Identification of novel mechanisms regulating the NAD^+-dependent deacetylase SIRT1

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Basel, den 02 Mai 2006

Prof. Dr. Hans-Jakob Wirz
(Dekan)

I declare that I wrote this thesis "Identification of novel mechanisms regulating the NAD^+-dependent deacetylase SIRT1" with the help indicated and only handed it in to the Faculty of Science of the University of Basel and to no other faculty and no other university.

Zurich, 13 April 2006

Dimitrios Anastasiou

Στους γονεις μου Κωστα & Ελενη

"Δημιουργωντας μια ποιηση πανω απο καθε καταστροφη"

Μανωλης Αναγνωστακης

Παρενθεσεις, Επιγνωση

ABSTRACT

Sirtuins comprise a highly conserved protein family which catalyse the deacetylation of proteins in an NAD^+-dependent manner. In *S. cerevisiae* and in *C. elegans* sirtuins have been shown to mediate the beneficial effects of caloric restriction on organismal longevity thus giving rise to the notion that may function as key regulators of the ageing process.

SIRT1 is the best characterised member of the mammalian sirtuin family which comprises seven homologues. It has been shown to associate with and deacetylate several proteins, predominantly transcriptional regulators such as p53, NFκB, MyoD and FOXO, thus functioning in processes as diverse as development, differentiation, senescence, survival, proliferation and metabolic regulation.

Understanding the dynamics of intracellular SIRT1 function has been hindered by the limitation of methods for accurately quantifying free intracellular [NAD^+] levels and thus SIRT1 activity. Furthermore, nothing is known about potential post-translational mechanisms involved in SIRT1 regulation.

During the course of this work, the task of identifying novel mechanisms that regulate SIRT1 function was undertaken. SIRT1 was found to be specifically phosphorylated in mitosis as well as in interphase. In addition, its subcellular localisation and turnover are sensitive to UV irradiation. Finally, various stresses induce caspase-mediated SIRT1 cleavage which has an impact on overall protein stability.

These data provide the first glimpse into the molecular regulatory mechanisms that dictate SIRT1 function. The implications of this work are discussed in the context of current knowledge as well as proposed novel functions of SIRT1.

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To start with, I would like to thank Prof. Wilhelm Krek for providing me the opportunity to collaborate with him for my first long-term investment into my scientific career, for the stimulating environment that has nurtured my inquiring mind and his insightful approaches to aspects of scientific discovery.

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Finally, I would like to exercise my right to provide my partner Pia and my parents Kosta and Eleni with my thanks in person. Anything else would only diminish the paramount role that they have played and continue to play in my life as well as the completion of the work presented here.

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List of frequently used acronyms and abbreviations

approx.	approximately
BER	base excision repair
BrdU	bromodeoxy-uridine
BSA	bovine serum albumin
CK2	casein kinase 2
DAPI	1,4,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DRB	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
DTT	dithiothreitol
DMSO	dimethyl sulfoxide
ES	embryonic stem (cells)
FCS	foetal calf serum
FISH	Fluorescence in-situ hybridisation
incl.	including
IP	immunoprecipitation
IR	ionising radiation
MAPs	microtubule associated proteins
MEFs	mouse embryonic fibroblasts
NER	nucleotide excision repair
MWCO	molecular weight cut-off
NHEJ	non-homologous end joining
$OD_{\#}$	optical density at # wavelength
O/N	overnight (typically 16 hours)
PBS	phosphate-buffered saline
PFA	paraformaldehyde
RNA	ribonucleic acid
ROS	reactive oxygen species
siRNA	small interfering RNA
TSA	trichostatin A
UV	ultraviolet

CHAPTER 1

ADAPTIVE CELLULAR RESPONSES TO ENVIRONMENTAL STIMULI

1.1 SIGNALLING PATHWAYS REGULATING ADAPTIVE RESPONSES TO NUTRIENT AVAILABILITY

1.1.1 Archetypal signaling strategies in bacteria and lower eucaryotes

Evolutionary considerations suggest that the ensemble of living organisms that constitute an environment's population stems from their ability to perpetuate under this environment's particular conditions. By definition, such populations are fit to thrive. Yet, living environments are dynamic rather than static and in combination with genetic variability contribute to the evolution of the species. Thus, a paramount feature of living organisms throughout the phyla is their ability to adapt to such environmental changes in order to increase their survival potential.

Unicellular organisms have evolved specific biochemical systems of variable complexity that allow them to respond to environmental changes such as fluctuating levels of nutrients.

Bacteria preferentially utilise glucose as their primary carbon source even in the presence of other sugars in their growth environment. Only following depletion of glucose can

other sugars such as lactose also be used for energy production, a phenomenon called diauxic growth. Jacob and Monod proposed the concept of the lac operon to explain this phenomenon which was subsequently confirmed and elaborated extensively (Lewis, 2005). In this model, the lac repressor can bind a *cis* acting element in the promoter region of genes encoding proteins that allow lactose production suppressing their expression under conditions of glucose abundance. When lactose is the primary carbon source, it binds to the repressor inducing a conformational change which reduces its affinity for the operator, leading to its dissociation from the promoter and allowing the expression of genes involved in the uptake and metabolism of lactose.

More advanced signaling cascades in bacteria adopt a simple two-component modular configuration comprising a sensor and an effector module. This is exemplified by the two-component signal transduction (or phosphorelay) system which is widely employed by bacteria and to a lesser extend by fungi and plants (Perraud *et al.*, 1999). In bacteria, two-component systems regulate basic cellular processes such as chemotaxis, osmoregulation, temperature sensing, metabolism and membrane transport (West and Stock, 2001).

The basic architecture of such systems is depicted in FIGURE 1-1. The sensor (HK) is a transmembrane protein (TM1 and TM2 are the membrane-spanning regions) which can dimerise through a dimerisation domain. The intracellular region of the protein contains a histidine kinase activity characterised by four conserved motifs (N, G1, F, G2). The effector component of the system (RR) contains a conserved regulatory domain and an effector domain. Environmental stimuli induce the histidine kinase activity of HK leading to its autophosphorylation (depicted with P in FIGURE 1-1). Following

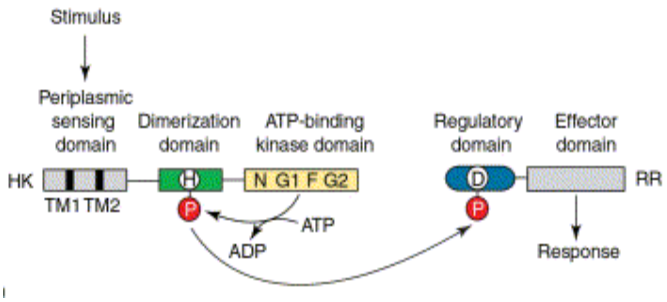


FIGURE 1-1. Schematic represenation of a basic two-component phosphotransfer system. A typical two-component phosphotransfer system consists of a dimeric transmembrane sensor HK and a cytoplasmic RR. A monomer of a representative HK is shown with transmembrane segments indicated by TM1 and TM2. Conserved sequence motifs N, G1, F and G2, are located in the ATP-binding domain. HKs catalyze ATP-dependent autophosphorylation of a specific conserved His residue (H). The activities of HKs are modulated by environmental signals. The phosphoryl group (P) is then transferred to a specific Asp residue (D) located within the conserved regulatory domain of an RR. Phosphorylation of the RR typically activates an associated (or downstream) effector domain, which ultimately elicits a specific cellular response.

Adapted from West and Stock, 2001

that, RR catalyses the transfer of the phosphate group to one of its own aspartic acid residues within the regulatory domain leading to the activation of the effector domain. More elaborate systems based on these principles including consecutive histidine/aspartic acid phosphorelay systems are also found (Perraud *et al.*, 1999).

Unicellular eucaryotic organisms such as the yeast *S. cerevisiae* exhibit increased complexity in the signaling cascades mediating adaptive responses, which reflect not only their architectural differences to procaryotes (e.g. in the case of regulated nucleocytoplasmic transport) but also their increased computational capacity in decision-making processes. A well-studied system, largely conserved also in higher eucaryotes is the mitogen-activated protein kinase (MAPK) signalling cascade in yeast.

The MAPK kinase signaling pathway regulates processes as diverse as mating behaviour and responses to osmotic stress and nutrient availability (FIGURE 1-2). Upon induction, a kinase cascade involving sequential phosphorylation/activation steps is initiated (FIGURE 1-3A). Surprisingly, multiple stimuli use a largely shared set of molecules to elicit diverse and specific responses raising the issue how specificity is attained in such systems.

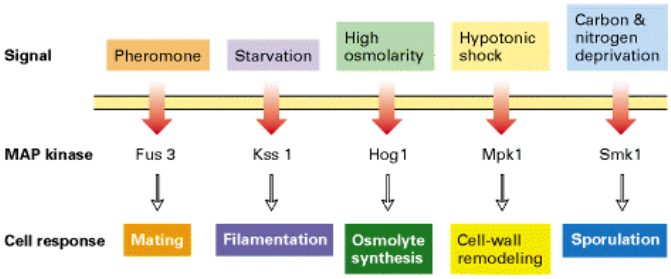


FIGURE 1-2. Multiple MAPK pathways regulate distinct cellular responses. Each pathway is triggered by a specific extracellular signal and leads to activation of a single MAP kinase, which mediates characteristic cellular responses.

Figure and legend adapted from Lodish *et al.*, 2000

A potential specificity mechanism involves the formation of protein complexes dedicated to a single pathway (FIGURE 1-3B). Complex formation is driven by scaffold proteins which can bring into proximity multiple components that are then allowed to phosphorylate each other but not proteins in a heterologous pathway (Dard and Peter, 2006). It has also been proposed that MAPK functions independent of their catalytic activity exist. In particular, the Fus3 and Kss1 MAPKs which mediate the mating and filamentous growth pathways respectively, bind with different affinities to a common scaffold protein Ste5. Thus Fus3 precludes the binding of Kss1 to Ste5 driving preferentially the mating pathway. More recently, it has been suggested that a more significant specificity factor is provided by the preferential substrate selectivity of Fus3 over

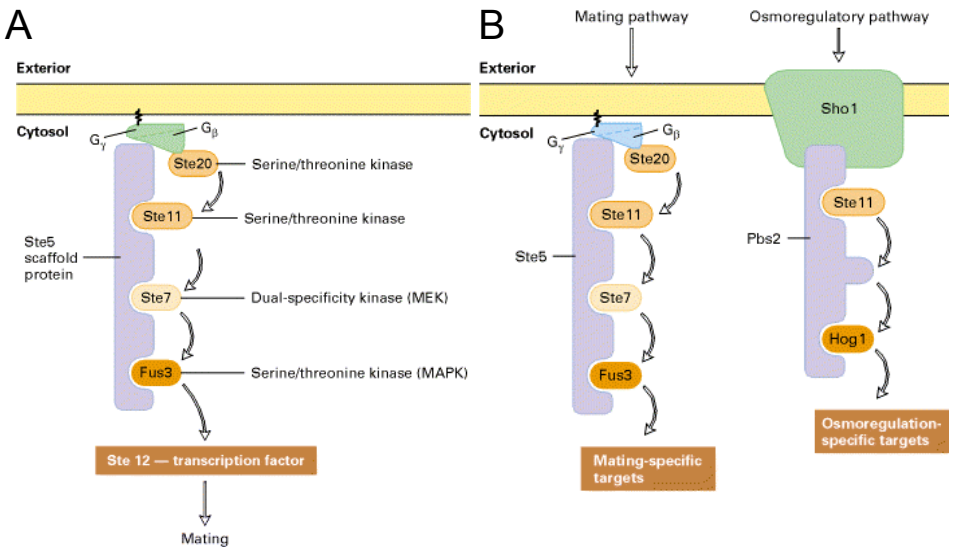


FIGURE 1-3. Mechanisms of specificity in yeast MAPK signaling. (A) MAPK cascade that transmits signals in the mating pathway in *S. cerevisiae*. The receptors for yeast **a** or a mating factors are both coupled to the same trimeric G protein. Ligand binding leads to activation of the G protein and dissociation of $G_{\alpha} \cdot GTP$ from the $G_{\beta\gamma}$ complex. In the yeast mating pathway, however, the physiological responses are induced by the dissociated $G_{\beta\gamma}$, which activates a protein kinase cascade. The final component, Fus3, is functionally equivalent to MAP kinase (MAPK) in higher eukaryotes. It phosphorylates transcription factors (e.g., Ste12) that control expression of proteins involved in mating-specific cellular responses. **(B)** Formation of pathway-specific complexes prevents "cross-talk" between pathways that contain a common component, such as Ste11 in these two pathways. These large complexes are assembled on the molecular scaffolds Ste5 and Pbs2. Unlike Ste5, which has no catalytic function, Pbs2 has MEK activity (analogous to Ste7 in the mating pathway). Once phosphorylated by Ste11, activated Pbs2 phosphorylates Hog1.

Figure and legend adapted from Lodish *et al.*, 2000

Kss1 towards Far1 rather than physical occlusion of Kss1 from the signaling scaffold (Breitkreutz and Tyers, 2002).

Thus, even in single-cell eucaryotes, elaborate networks are in action to sense and respond to environmental changes. Although the principles of adaptive responses delineated for unicellular organisms are broadly conserved in more advanced forms of life, multicellular organisms exhibit increased complexity in the form of functionally specialised organs and organ systems.

Reflecting this complexity, an additional level of co-ordination is required to sustain survival in response to environmental as well as intraorganismal changes. For this to be achieved elaborate endocrine systems are in action. Such a signaling system with central roles in animal physiology is mediated by the hormone insulin and the related insulin-like growth factors (IGFs), IGF1 and IGF2. Both at the intracellular as well as organismal level,

the sophistication of this system exemplifies the underlying basis of advanced biological systems design.

1.1.2 Major homeostatic pathways in higher eucaryotes

1.1.2.1 The insulin/IGF signaling system

The insulin/IGF sytem is involved in fundamental biological processes such as growth, proliferation, survival and metabolic regulation (White, 2003; Pollak *et al.*, 2004). For example, in response to feeding, insulin, which is produced in the pancreas, dictates the uptake and catabolism of glucose by peripheral tissues. IGF1 and IGF2 are produced primarily by the liver and have mitogenic capacity. IGF1 but not IGF2 production is dictated by pituitary gland-derived growth hormone underlying its function in regulating animal size (Kenyon, 2001). The insulin/IGF system has also an evolutionarily conserved function in determining organismal longevity which is tighly linked to its responsiveness to nutritional inputs (Kenyon, 2001).

The effects of insulin/IGF are mediated by binding to three receptors, the insulin receptor (IR), IGF1 receptor (IGF1R) and IGF2 receptor (IGF2R). A fourth family member exists named insulin receptor-related receptor (IRR) for which an endogenous ligand has not been identified (Kitamura *et al.*, 2003).

IR, IGF1R and IRR harbour ligand-activated tyrosine kinase activity in their intracellular domains which initiates downstream signaling cascades. Although several protein substrates of the insulin/IGF receptor tyrosine kinase activity have been identified, genetic ablation studies in mice suggest that the majority of insulin responses are mediated by insulin receptor substrates 1 or 2 (IRS1 or IRS2 respectively) (White, 2003). Thus, IRS1 is responsible for body growth control and peripheral insulin action, while IRS2 controls brain growth, body weight, glucose homeostasis and female fertility (White, 2003).

IRSs are scaffold proteins which upon their phosphorylation allow the docking of multiple kinases or other scaffold proteins that contain phospho-aminoacid binding domains. Upon binding to IRS proteins through its SH2 domains, the lipid kinase activity of phosphoinositide-3 kinase (PI3K) is induced and results in increased membrane phosphatidylinositol-3,4,5-triphosphate [PtdIns(3,4,5)P_3] levels (Vanhaesebroeck and Alessi,

2000). These lipids are preferentially recognised by the pleckstrin homology (PH) domains of protein kinase B (PKB, a.k.a. Akt) and phosphoinositide-dependent kinase (PDK). Following membrane recruitment, PDK phosphorylates PKB at S308 contributing to its activation.

A critical regulator of this pathway is the tumour suppressor protein PTEN (phosphatase and tensin homologue deleted on chromosome 10). PTEN is a lipid phosphatase which attenuates PKB activation by catalysing the reverse reaction to that of PI3K.

1.1.2.1.1 The PI3K-PKB signaling pathway

Regulation of cell survival

In the presence of growth factors, PKB activity promotes cell survival through a pleiotropic mode of action (FIGURE 1-4). Bad is a member of the Bcl-2 family of proteins which upon growth factor withdrawal, it translocates to mitochondria where, in collaboration with other pro-apoptotic members of the Bcl-2 protein family it elicits cytochrome-c release, the first step in the intrinsic cellular apoptotic pathway (Danial and Korsmeyer, 2004). PKB phosphorylates the pro-apoptotic protein Bad leading to its sequestration to the cytoplasm by 14-3-3 proteins thus preventing cell death (Datta *et al.*, 1999). PKB can also inhibit apoptosis downstream of cytochrome-c release by phosphorylating and inactivating caspase-9 (Datta *et al.*, 1999).

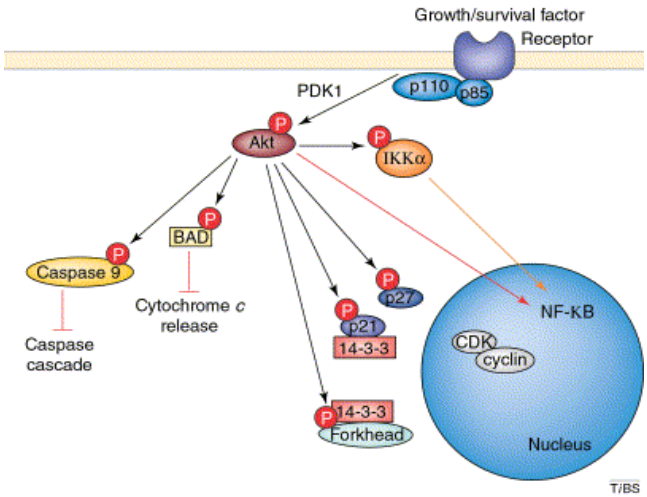


FIGURE 1-4. PKB-mediated pathways that regulate cell survival. See text for details.

Adapted from Mayo and Donner, 2002

Interestingly, the ability of PKB to prevent cytochrome-c release is coupled to glucose availability, requiring the phosphorylation of glucose by hexokinase, i.e. the first step in the glycolytic pathway (Gottlob *et al.*, 2001). Concomitant to this, PKB greatly enhances the mitochondrial localisation of hexokinase. Two mechanisms by which hexokinase localisation

to mitochondria prevents apoptosis have been proposed (Majewski *et al.*, 2004). Firstly, hexokinase precludes the recruitment of the pro-apoptotic protein Bax to mitochondria preventing cytochrome-c release. Secondly, it participates in the maintainance of mitochondrial integrity by regulating the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane. VDAC is involved in the exchange of metabolites such as adenine nucleotides and respiratory substrates across the outer mitochondrial membrane contributing to mitochondrial homeostasis. Upon glucose withdrawal, decreased hexokinase at mitochondria results in VDAC closure leading to mitochondrial outer membrane swelling and eventual rupture.

PKB also contributes to the activation of the anti-apoptotic pathway driven by the transcription factor NFκB. PKB phosphorylates and activates the inhibitor of κB kinase β (IKKβ). IKKβ in turn targets the inhibitor of κB (IκB) proteins for degradation allowing the activation of NFκB. NFκB target genes include the cellular inhibitor of apoptosis (cIAP) proteins that bind to and inactivate caspases (Datta *et al.*, 1999).

Forkhead or winged-helix transcription factors are also regulated by PKB activity. In the presence of growth factors, PKB phosphorylates FOXOs (forkhead box subclass O) and FOXA2 factors leading to their sequestration in the cytoplasm by 14-3-3 proteins (Plas and Thompson, 2005). Upon growth factor limitation, FOXOs can translocate to the nucleus where they bind cognate DNA sequences in target gene promoters modulating their expression. A FOXO target gene is Fas ligand (FasL) which upon binding to its cognate receptor induces apoptotic cell death in neuronal cells (Datta *et al.*, 1999). PKB-mediated phosphorylation prevents the pro-apoptotic function of FOXO through FasL expression.

Regulation of cell cycle

Another emerging function of PKB is in the regulation of the cell cycle (FIGURE 1-5). PKB attentuates the activity of the cyclin-dependent kinase (CDK) inhibitor p27 by at least two mechanisms. By directly phosphorylating p27, PKB induces its retention to the cytoplasm by 14-3-3 proteins preventing p27 from inhibiting nuclear CDK complexes (Shin *et al.*, 2002). Secondly, p27 is a transriptional target of FOXO transcription factors thus, in the presence of growth factors, nuclear exclusion of FOXO leads to reduced p27 transcription. Interestingly,

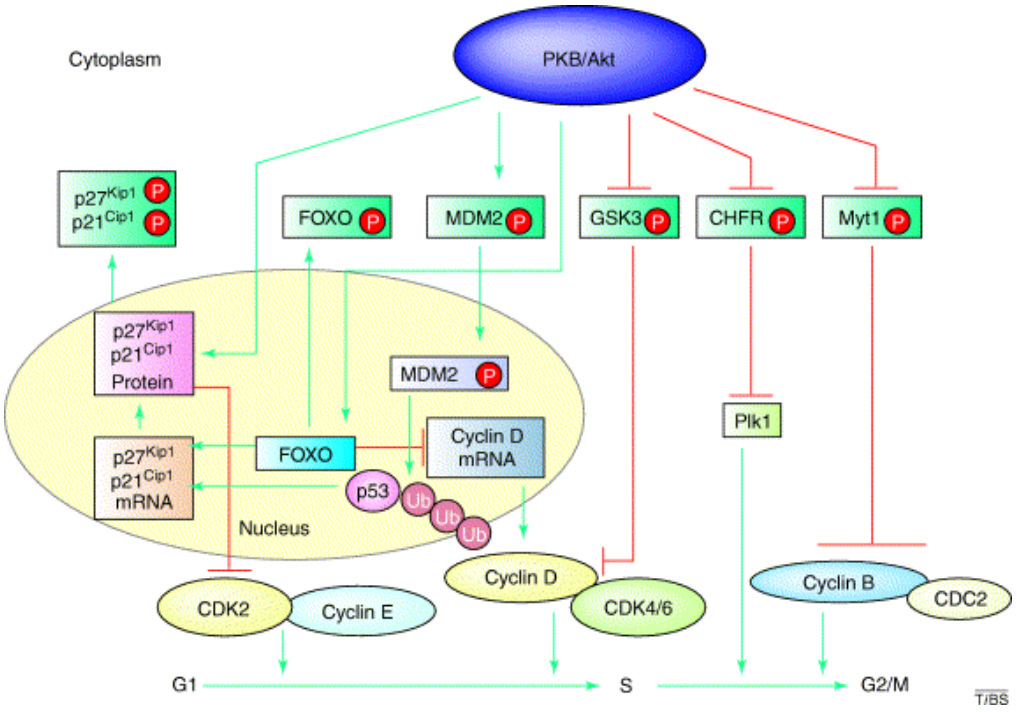


FIGURE 1-5. PKB-mediated pathways that regulate cell cycle progression. *See* text for details.

Adapted from Brazil *et al.* 2004

FOXO is also the target of another regulator of p27, the Skp2 ubiquitin ligase complex. Skp2 mediates entry into the S phase by ubiquitinating p27 targeting it for proteasome-mediated degradation (Sutterluty *et al.*, 1999). Similarly, PKB-mediated phosphorylation of FOXO targets it to proteasome-mediated proteolysis *via* Skp2-mediated ubiquitination (Huang *et al.*, 2005).

An alternative mode of G1/S regulation by FOXO was proposed by Ramaswamy *et al.* who showed that a FOXO1 species that cannot bind the p27 promoter but retains its ability to inhibit transcription of D-type cyclins is sufficient to induce cell cycle arrest (Ramaswamy *et al.*, 2002). Interestingly, PKB promotes cyclin D1 stability by inhibiting glycogen synthase kinase-3 (GSK-3) which under limiting growth factor conditions phosphorylates cyclin D1 and targets it for proteolysis (Diehl *et al.*, 1998).

Unlike the effects of PKB on FOXO and p27 where phosphorylation induces their nuclear exclusion, PKB phosphorylates and promotes the nuclear localisation of another E3 ubiquitin ligase component, mdm2 (mouse double minute 2). Nuclear mdm2 is thus able to target the transcription factor p53 for degradation, leading to reduced expression of its target genes including the CDK inhibitor p21 (Mayo and Donner, 2002). Thus PKB promotes S phase

entry by both inhibiting CDK inhibitors and by upregulating CDK activity through stabilisation of D-type cyclins.

Further evidence supports a role for PKB in the G2/M transition too as PKB activation either by *PTEN* inactivation or trangenic expression overrides DNA damage-induced G2/M arrest (Brazil *et al.*, 2004). A potential mechanism was provided by studies in the starfish oocyte. Myt1 is a Wee1-related kinase whose activity inhibits cyclinB/Cdk1 complexes thus attenuating cell cycle progression. PKB phosphorylates and inactivates Myt1, assisting the dephosphorylation and activation of Cdk1 and subsequent initiation of mitosis (Okumura *et al.*, 2002).

In addition, PKB-driven phosphorylation of CHFR (checkpoint protein with forkhead associated and ring finger domains) stabilises polo-like kinase 1 (Plk1) which is required for mitotic progression (Shtivelman, 2003) providing an additional mechanism of regulation of mitosis by PKB.

Regulation of metabolism

One of the first PKB substrates identified was GSK-3. GSK3 phosphorylation by PKB alleviates the GSK3-mediated inhibition of glycogen synthesis so that in the presense of insulin which signifies glucose abundance, glycogen synthesis is promoted (Cross *et al.*, 1995). Since then, the role of PKB in the regulation of metabolism has been expanded. PKB regulates the translocation of the glucose transporter GLUT4 to the plasma membrane to stimulate glucose uptake in response to insulin signaling but also to other receptor pathways such as EGF, IL-3 and TGF-β (Plas and Thompson, 2005). It is also possible that the enhancement of hexokinase recruitment to mitochondria by PKB (*see* above) increases the overall rate of glycolysis (Gottlob *et al.* 2001).

Furthermore, PKB can exert its effects on cellular metabolism through a class of target genes which are regulated by FOXO in a manner distinct from the aformentioned type D cyclins (Ramaswamy *et al.*, 2002). In this respect FOXOs activate the transcription of manganese superoxide dismutase (*MnSOD*) whose gene product encodes for an enzyme involved in the detoxification of reactive oxygen species. IGF-binding protein 1 (*IGF-BP1*) is also a downstream target of FOXO. IGF-BP proteins bind IGFs and regulate their plasma

availability since they attenuate their ability to activate their cognate receptors (Pollak *et al.*, 2004). Furthermore, FOXOs co-regulate the expression of metabolic genes in combination with nuclear receptors. FOXO in conjunction with peroxisome proliferator-activated receptor-γ (PPAR-γ) represses transciprtional activity of *IGF-BP1* and phosphoenolpyruvate carboxykinase (*PEPCK*) gene promoters. Conversely, in combination with PGC-1α (PPAR-γ co-activator 1α), FOXO induces the transcription of *PEPCK* and glucose-6-phosphatase (*G-6-Pase*) upon fasting in the liver thus contributing to the gluconeogenesis programme (Puigserver *et al.*, 2003). FOXO transcriptional responses are in addition fine-tuned by acetylation, more of which will be discussed in Chapter 2.

1.1.2.1.2 Endocrine functions of the IGF system and the regulation of longevity

Endocrine IGF signaling is central to organismal growth. IGF factors are produced in the liver a process controlled by growth hormone (GH). GH is produced by the pituitary gland in response to signals from the hypothalamus, mainly somatostatin and growth-hormone-releasing hormone (GHRH). The ability of GH to regulate IGF production though, is greatly influenced by dietary input. Under low food intake conditions, IGF production is suppressed (Thissen *et al.*, 1994).

It has been long known that organismal longevity can be extended by nutrient limitation, often referred to as caloric restriction, a phenomenon demonstrated in *S. cerevisiae, C. elegans*, *Drosophila* as well as mammals (Bordone and Guarente, 2005). Extensive genetic studies in these model systems have established that the beneficial effects of dietary limitation to life-span are mediated by the IGF signaling pathway which is well conserved in higher eucaryotic organisms (FIGURE 1-6) (Partridge and Gems, 2002).

The term 'replicative life-span' refers to the number of cell divisions a cell undergoes before cessation of cell division. In mammalian cells the discontinuation of division is also know as 'cellular senescence'. Chronological life-span refers to the amount of time that a cell is alive.

Mutations that abolish IGF signaling result in increased replicative life span (Kenyon, 2001). Interestingly, tissue-specific ablation of the insulin receptor in adipose tissue results in

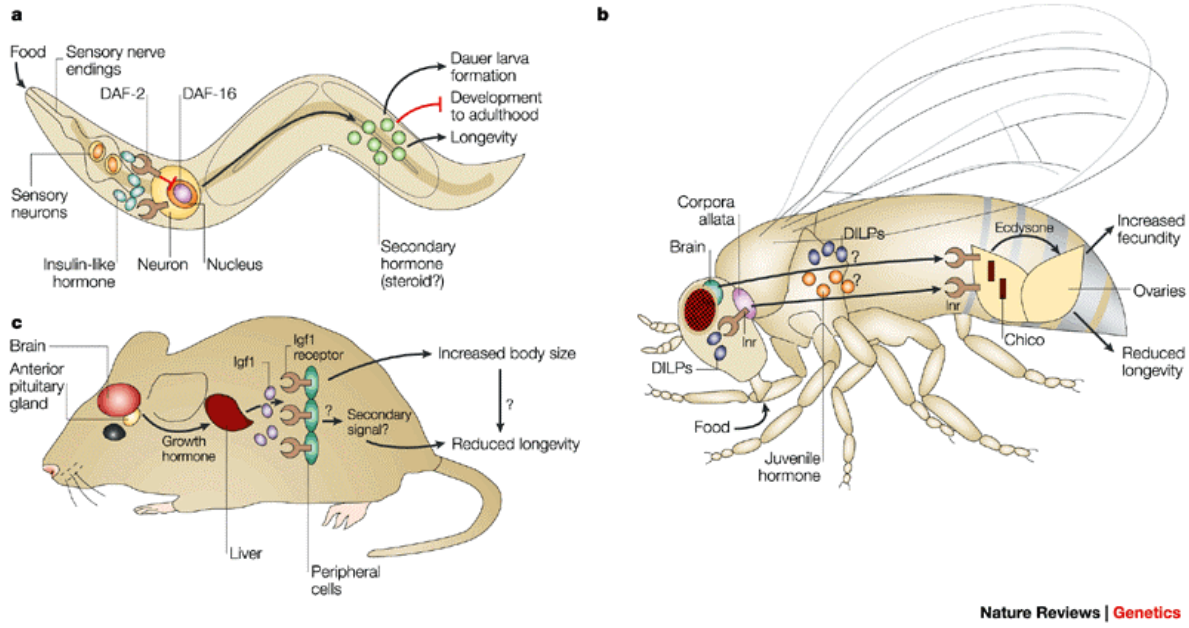


FIGURE 1-6. Neuroendocrine regulation of ageing. (a) Insulin/IGF signalling in a two-step hormone signalling system in *Caenorhabditis elegans*. In this model, food modulates the production of an insulin-like peptide hormone (INS) by chemosensory neurons. This acts on DAF-2, which is also expressed in the nervous system, to cause the production of a second hormone signal, which modulates development and ageing throughout the organism. Elements of this model are speculative, and the following remain to be determined: whether environmental stimuli regulate INS production and what these stimuli are; the role of DAF-16 in regulating secondary hormone production; and whether this hormone regulates longevity. **(b)** Two hypotheses for the role of insulin/IGF signalling in ageing in *Drosophila*. In both models, *Drosophila* insulin-like peptides (DILPs) are produced by the brain in response to environmental or internal nutritional stimuli. How the production of DILPs is regulated is unknown. In one version of this model, DILPs act directly on the ovaries, stimulating the production of the steroid hormone ecdysone; in the other, DILPs stimulate the production of the isoprenoid hormone juvenile hormone by the CORPORA ALLATA. **(c)** Insulin/IGF signalling in mice. This model proposes that Igf1, rather than insulin, acts as a modulator of ageing in mammals; this role of Igf1 in ageing remains to be shown directly. DAF, dauer larva formation abnormal; Igf1, insulin-like growth factor 1; Inr, insulin-like receptor.

Figure and legend adapted from Partridge and Gems, 2002

a ~18% increase in life-span in mice suggesting that specific metabolic effects are responsible for this effect (Bluher *et al.*, 2003).

The influence of IGF on life-span is tightly coupled to its ability to down-regulate forkhead transcription factors (Kenyon, 2005). In *C. elegans*, life-span extension due to mutations in the IGF pathway depend on the presence of DAF-16. In agreement to this, dFOXO overexpression in *Drosophila* results in lifespan extension. This function of FOXO factors is tighly coupled to a concomitant resistance to stress (Kenyon, 2001; Kenyon, 2005) which is also thought to operate in other mutant animals with extended life-span (Miggliacio *et al.*, 1999).

An explanation of this may lie with the genes regulated by forkhead factors. FOXO drives the expression of MnSOD, an enzyme involved in superoxide detoxification. *MnSOD* overexpression in *Drosophila* suffices to confer life-span extension (Kenyon, 2001). This evidence provides support of the "free radical theory" of ageing which states that the rate of ageing is related to the deleterious effects of free raicals upon the cell (Balaban *et al.*, 2005).

Although relatively little is known about the downstream effectors of free radicals it has been proposed that they cause an accumulation of mutations in the DNA leading to progressively aberrant cellular functions leading to cellular death and the decline of organ performance (Lombard *et al.*, 2005). Alternatively, there is evidence that signaling pathways that regulate cellular survival are regulated by reactive oxygen species (ROS). Jun N-terminal kinase (Jnk) is activated by phosphorylation which is counteracted by the action of phosphatases. The enzymatic activity of Jnk phosphatases is regulated by ROS in that high ROS levels oxidise a key residue in the phosphatase catalytic site leading to their inactivation (Kamata *et al.*, 2005). Thus Jnk kinases are allowed to elicit the cellular apoptotic programme which in turn may contribute to tissue decline (Balaban *et al.*, 2005).

Despite the lack of a classical IGF signaling pathway in yeast, homologues thereof have been also implicated in the regulation of cellular life-span. Mutations in *Sch9* a gene encoding for a homologue of PKB confer increased replicative life-span in *S. cerevisiae*. Interestingly, two recent studies identified yeast TOR1 as a negative effector of both replicative and chronological life-span in response to nutrient satiety consistent the interplay of this pathway with the IGF system in higher organisms (Kaeberlein *et al.*, 2005; Powers *et al.*, 2006). Thus, the conserved functions of IGF signaling in response to dietary factors appear to underlie the determination of cellular as well as organismal life-span.

1.1.2.2 The TOR signaling pathway

1.1.2.2.1 Signaling pathways regulating TOR activity

Under conditions of growth factor availability, cell proliferation is favoured. However, to ensure sustainable growth, cell division has to be co-ordinated with concomitant increases in

cell mass, primarily protein synthesis. A key signaling module in the regulation of cell growth is mediated by the target of rapamycin (TOR) kinase.

TOR activity is under the possitive influence of PKB (Figure 1-7). PKB phosphorylates and inactivates the GTPase-activating protein (GAP) tuberous sclerosis 2 (TSC2). TSC2 in complex with the putative chaperone TSC1 inactivates the small G protein Rheb (Plas and Thompson, 2005). Rheb is a positive regulator of the protein kinase activity of TOR.

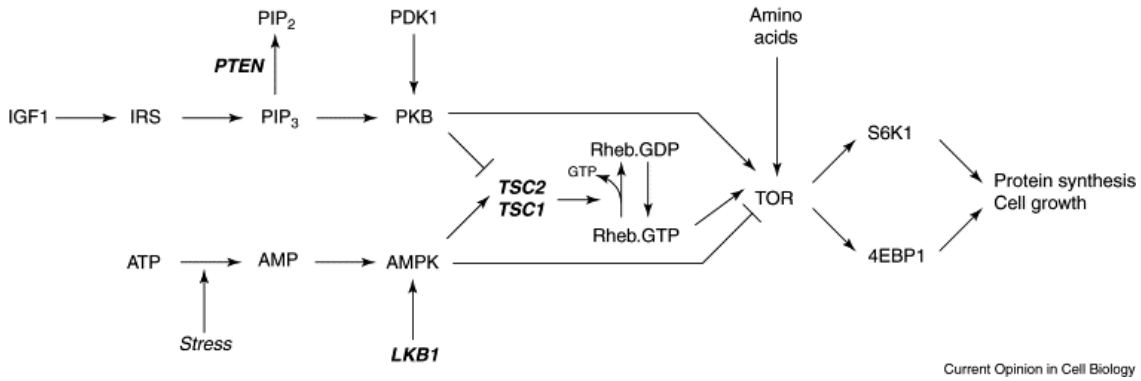


FIGURE 1-7. Signaling pathways that regulate TOR activity. See text for details.

Adapted from Hardie, 2005

Apart from the input growth factor signaling pathways TOR also responds to the energy status of the cell expressed as the AMP/ATP ratio (FIGURE 1-7) (Hardie, 2005). When glucose levels are low, the rate of glycolysis and ensuing oxidative phosphorylation are suppressed leading to a high AMP/ATP ratio which activates AMP-dependent protein kinase (AMPK). AMPK in turn phosphorylates TSC2 enhancing its GAP activity unltimately leading to TOR inactivation as described above. Upon energy deprivation, another kinase LKB1 also phosphorylates and activates AMPK parallel to AMP to ultimately inhibit TOR. TOR has also been proposed to directly sense the energy status of the cell due to an uncharacteristic high K_m for ATP rendering functional only under high energy conditions (Dennis *et al.*, 2001).

Nutrients, in particular aminoacids, have a positive impact on TOR function yet the molecular mechanisms involved remain controversial (Wullschleger *et al.*, 2006). It has been proposed that the effect of nutrients on TOR is mediated by the TSC1/2-Rheb axis, however this does not address the issue how aminoacids regulate TOR in yeast where TSC1/2 and Rheb homologues have not been identified. It is thus possible that aminoacids are directly sensed by TOR.

1.1.2.2.2 Functions of the TOR pathway

Two TOR complexes have been isolated in yeast and mammals named TORC1 and TORC2 (FIGURE 1-8) (Wullschleger *et al.*, 2006). Apart from TOR and LST8, a positive regulator of TOR kinase activity, the two complexes differ in their protein composition as well as function (FIGURE 1-9). TORC1 contains a protein termed raptor (for regulatory associated protein of mTOR) while TORC2 contains rictor (rapamycin-insensitive companion of mTOR, a.k.a. mAVO3).

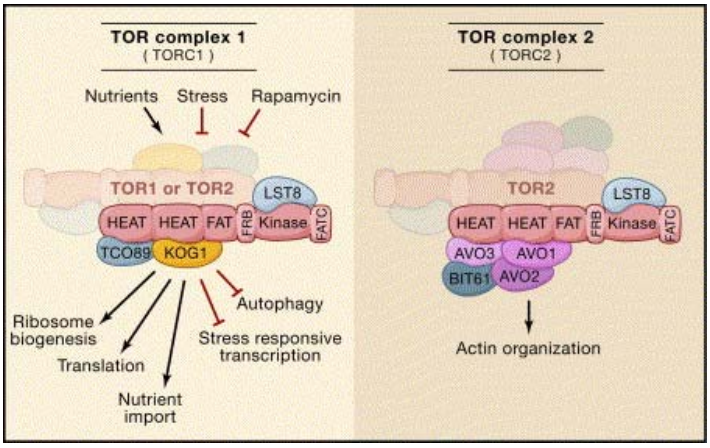


FIGURE 1-8. TOR complexes. Depicted are TOR-associated proteins (KOG1, TCO89, LST8, AVO1–3, and BIT61) and the domains found in TOR (HEAT, FAT, FRB, Kinase, and FATC). Both TORC1 and TORC2 are multimers, likely dimers. TORC1 mediates the rapamycin-sensitive signaling branch that couples growth cues to the accumulation of mass. Stimuli that positively regulate TORC1 and TORC1 outputs that promote the accumulation of mass are depicted with black arrows. Inputs that negatively regulate TORC1 and the stress- and starvation-induced processes that TORC1 regulates negatively are depicted with red bars. TORC2 signaling is rapamycin insensitive and is required for the organization of the actin cytoskeleton. Upstream regulators of TORC2 are not known.

Figure and legend adatped from Wullschleger *et al.,* 2006

TORC1 function is sensitive to rapamycin, a bacterial metabolite with potent anti-proliferative properties. This is not the case for TORC2. As numerous studies of TOR biology were based on rapamycin sensitivity of TOR-mediated pathways, little is known about TORC2 function. Recent data suggest that TORC2 regulates the actin cytoskeleton (Wullschleger *et al.*, 2006). Furthermore, TORC2 has been shown to phosphorylate PKB which in combination to the activity of PDK1 contributes to full PKB activation (Sarbassov *et al.*, 2005).

It is well accepted that TORC1 is a central regulator of cell growth. This is primarily through its ability to positively regulate ribosomal biogenesis and eventually cellular protein synthesis capacity which in turn is required for cell mass accumulation prior to cell division. TORC1 positively regulates the activity of ribosomal S6 kinase (S6K) a Ser/Thr kinase which phosphorylates the 40S ribosomal protein S6. It has been proposed that this phosphorylation event allows the ribosome to preferentially translate mRNAs containing a 5' tract of

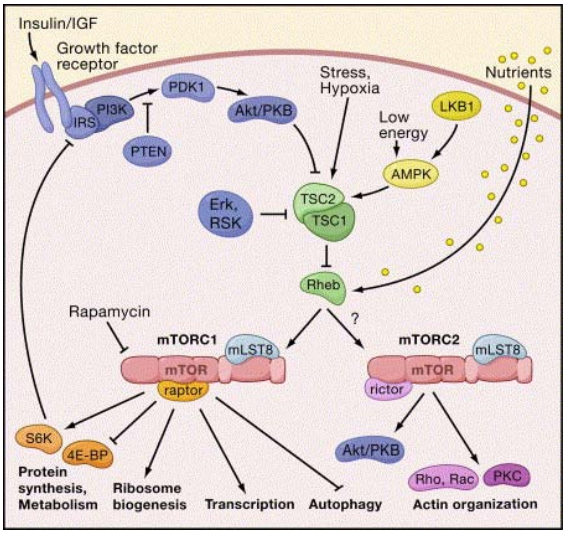


FIGURE 1-9. Model of the mTOR Signaling Network in Mammalian Cells. The mTOR signaling network consists of two major branches, each mediated by a specific mTOR complex (mTORC). Rapamycin-sensitive mTORC1 controls several pathways that collectively determine the mass (size) of the cell. Rapamycin-insensitive mTORC2 controls the actin cytoskeleton and thereby determines the shape of the cell. mTORC1 and possibly mTORC2 respond to growth factors (insulin/IGF), energy status of the cell, nutrients (amino acids), and stress. mTORC1 (and likely mTORC2) are multimeric, although are drawn as monomers. Arrows represent activation, whereas bars represent inhibition.

Figure and legend adatped from Wullschleger *et al.,* 2006

oligopyrimidine (5' TOP) which comprise 15-20% of total cellular mRNA and encode for components of the translational apparatus. However, recent data sugest that 5' TOP translation can take place in the absense of S6Ks or S6 phosphorylation leaving the issue of 5' TOP translation regulation by TORC1 open (Wullschleger *et al.*, 2006).

TORC1 also enhances cap-dependent translation by phosphorylating 4E-binding protein 1 (4E-BP1). 4E-BP1 can associate with the eucaryotic translation initiation factor 4E (eIF-4E) inhibiting its translation initiation function. Upon its phosphorylation by TORC1, 4E-BP1 dissociates from eIF-4E allowing it to associate with eIF-4G to stimulate translation initiation (Gingras *et al.*, 2001). Furthermore, TORC1 regulates the transcription of ribosomal protein genes by co-ordinate control of transcription factor activity (Wullschleger *et al.*, 2006).

Recently, a potential mediator of the effects of TORC1 on transcription was identified, termed URI (for unconventional Rpb5-interacting protein). URI mediates rapamycin-sensitive transcription programmes in yeast and mammals and can itself be phosphorylated in a rapamycin-sensitive manner (Gstaiger *et al.*, 2003). URI can associate with Rpb5, a common subunit of all mammalian RNA polymerases. Furthermore, URI also binds to a parafibromin-based complex whose orthologous counterpart in yeast is involved in transcriptional elongation and 3' processing. This complex also associates with RNAPolII in mammalian cells (Yart *et al.*, 2005). These data suggest that TORC1 may have broader functions in transcriptional regulation.

Finally, emerging evidence implies a role of TORC1 in metabolic regulation. TORC1 mediates the aminoacid-dependent transactivation capacity of PPARγ which mediates

adipogenesis. In addition, genetic ablation of S6K1 confers resistance to diet and age-induced obesity in mice (Um *et al.*, 2004). The underlying mechanism was attributed to the inhibitory phosphorylation of IRS1 by S6K under conditions of nutrient abundance which negatively regulates insulin signaling.

1.1.2.3 Molecular pathways sensing oxygen

Another important attribute of cells concerns their capacity to sense oxygen, an important factor for cellular functions such as oxidative phosphorylation. This is exemplified by solid tumours whose development is inhibited by agents that block their ability to elicit angiogenesis which otherwise provides the necessary supply of nutrients and oxygen to support survival (Reymond and Segrè, 2006; Carmeliet and Jain, 2000).

The key mediator the cellular response to hypoxia is a transcription factor called hypoxia-inducible factor (HIF). HIF acts in gene promoters as a heterodimer between an α and a β subunit the latter also being known as ARNT (for aryl hydrocarbon receptor nuclear translocator) both of which are members of the basic helix-loop-helix Per/Arnt/Sim (PAS) family of transcription factors (Kaelin, 2005). HIFβ is a stable protein in contrast to HIFα which, under conditions of normal oxygen tension, is modified by hydroxylation at proline residues by a class of enzymes knowns as prolyl hydroxylases. This modification tags HIFα for recognition by the E3 ubiquitin ligase von Hippel-Lindau (VHL) which ubiquitinates it and targets it for proteasomal degradation (Kaelin, 2005). At the same time hydroxylation at an arginine residue in the HIFα transactivation domain blocks its binding to the co-activator p300 thus blunting any residual transcriptional activity (FIGURE 1-10).

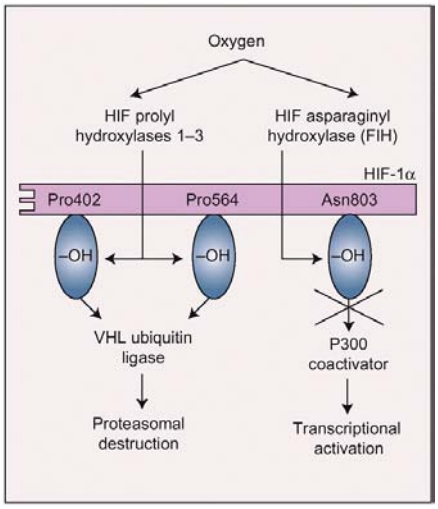


FIGURE 1-10. Dual regulation of HIF- subunits by prolyl and asparaginyl hydroxylation. Hydroxylation sites are indicated for the human HIF-1 polypeptide.

Figure and legend adapted from Pugh and Ratcliffe, 2003

The prolyl hydroxylase activity of the HIF modifying enzymes exhibits an intrinsic dependence on oxygen with a K_m suitable to serve as sensor of oxygen availability (Kaelin, 2005). Thus under hypoxic conditions, HIFα is not

hydroxylated, cannot be recognised by VHL and thus is stabilised. Once in the nucleus, HIF drives the expression of target genes involved in physiological as well as metabolic responses that allow cells to adapt to low oxygen conditions (*see* also discussion in Chapter 5). Towards this end, HIF target genes include angiogenesis-promoting factors such as VEGF (vascular endothelial growth factor) as well as other growth factors (e.g. TGF-β) and genes involved in glycolysis (Semenza, 2002).

Interestingly, recent reports provide evidence both in *Drosophila* and mammalian cells that hypoxia inhibits the TOR pathway *via* the TSC1/2 complex (Liu *et al.*, 2006; Brugarolas *et al.*, 2004; Reiling *et al.*, 2004). There is however some controversy whether this phenomenon is dependent on HIF. Brugarolas *et al.* reported that hypoxia inhibits TOR through the HIF target gene *RTP801*/*REDD1* (for regulated in development and DNA damage responses) and that this effect is independent of AMPK activity (Brugarolas *et al.*, 2004). In contrast, Liu *et al.* showed that *ARNT*-deficient fibroblasts retain their ability to inhibit TOR under hypoxic conditions leading them to propose that hypoxia-induced changes in cellular energy status activates the AMPK pathway which also contributes to TOR inactivation (Liu *et al.*, 2006).

Irrespective of the exact molecular details, these data provide a first glimpse into the mechanisms involved in the growth inhibitory effects of hypoxia. Furthermore, they demonstrate the existense of intimate connections between basic homeostatic pathways that ensure the co-ordinate control of cellular activities in response to environmental factors.

The molecular circuitries described above provide a picture of the complexity underlying the first level of adaptive cellular responses to environmental stimuli. Many of the effects elicited by these pathways require the expression of new genes as demonstrated for HIF and forkhead factors. The regulation of gene expression is a second level at which multiple regulatory inputs convert to implement transcriptional programmes that support cellular functions. Thus the following section will review the mechanisms involved in the regulation of gene expression.

1.2 REGULATION OF CHROMATIN STRUCTURE AND GENE EXPRESSION

1.2.1 Regulation of chromatin structure

Eucaryotic genomes comprise thousands of genes encoded in the DNA in a way that allows gene expression suited to support specific cellular needs to be achieved with remarkable accuracy.

A critical factor in this feat is the packaging of the DNA into ordered structures called nucleosomes which are arranged in a "bead-on-a-string" configuration comprising the chromatin fiber. Nucleosomes consist of a strectch of DNA wrapped around a proteinaceous core of core histones (H2A, H2B, H3, and H4) arranged as an octamer and stabilised by linker histones (H1, H1°, and H5). Nucleosomes are spatially positioned to form a 30-nm chromatin fiber which in turn can further compact to increasingly thicker structures to form chromosomes which is the configuration of DNA during cell division (FIGURE 1-11) (Falsenfeld and Groudine, 2003).

In interphase cells, distinct regions of chromatin can be observed cytologically known as euchromatin and heterochromatin. Heterochromatin is thought to be tightly condensed and thus inaccessible to DNA binding factors unlike euchromatin which adopts a more relaxed conformation (Grewal and Moazed, 2003).

Chromatin structure can be altered by replacement of the core histones with specialized histone variants, ATP-dependent nucleosome remodeling enzymes, or by covalent modification of histones within the nucleosome.

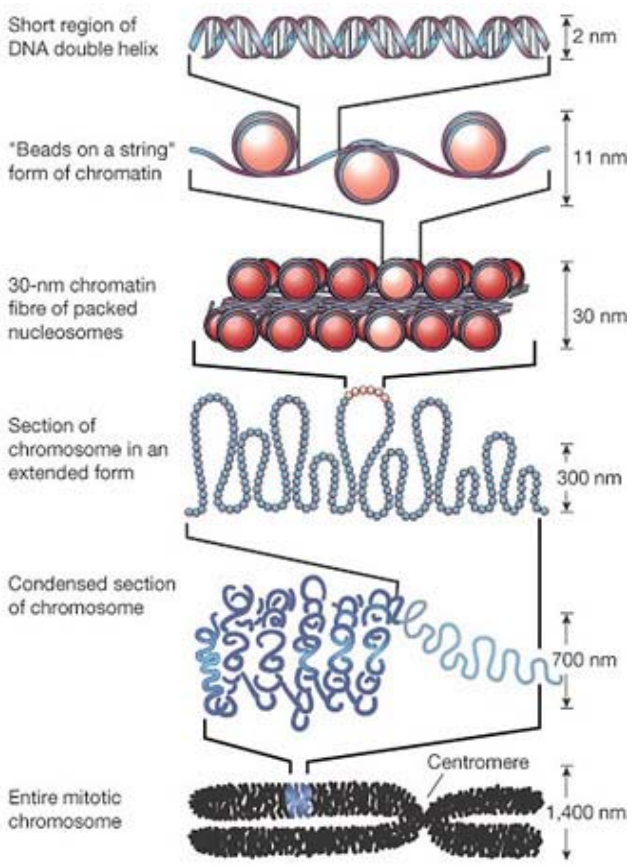


FIGURE 1-11. Hierarchical organisation of nuclear DNA structure in eucaryotes. *See* text for details

Adapted from Falsenfeld and Groudine, 2003

1.2.1.1 Histone variants

Variants for all except histone H4 have been identified and are thought to have occurred through gene duplication (Gilbert *et al.*, 2005). Histone variants are located in distinct chromatin regions where they are proposed to participate in the formation of specialised chromatin structures. CENP-A is an H3 variant found in centromeric chromatin which is characterised by increased compaction. H2A.Z may be able to influence chromatin structure since H2A.Z-containing nucleosomal arrays are less condensed and thus may facilitate transcription. Converesely, another H2 variant, macroH2A has been associated with X-chromosome inactivation and may interfere with gene transscription (Gilbert *et al.*, 2005).

1.2.1.2 ATP-dependent nucleosome remodeling

Nucleosomes perform a dual function as structural components of chromatin and as regulators of gene expression. Nucleosome position is precisely determined so that key transcription factor binding sites are exposed while maintaining proper DNA packaging. During processes that require active nucleosome repositioning, such as replication and transcription, nucleosomes can be mobilised on the chromatin fiber or the histone-DNA contacts within individual nucleosomes can be discrupted by ATP-dependent chromatin remodeling complexes which use the energy derived from ATP hydrolysis to perform their task (Smith and Peterson, 2005).

A central function in ATP-dependent chromatin remodeling complexes is performed by a helicase-like protein of the SWI/SNF (switch genes/sucrose non-fermentors) family. This class of helicases has also been subdivided into three subfamilies based on primary sequence homology as well as the individual charateristics of the corresponding remodeling complexes: the SWI2/SNF2, Mi-2/CHD and ISWI families (Smith and Peterson, 2005).

The SWI2/SNF2 complexes have been implicated in the regulation of gene transcription in yeast but also mammalian organisms where they participate in differentiation, early development and cytokine-mediated gene expression. the catalytic components contain bromodomains which mediate interaction with acetylated histone tails. Mutations of complex components have also been associated with tumour progression, in particular lung and

gastric cancers. Aside from their role in transcriptional regulation, SWI2/SNF2 complexes have also been implicated in global chromatin structure control during mitosis when chromosomes undergo major structural changes.

The ISWI (imitation SWI)-based complexes contain ATPases which are characterised by a different histone-binding domain than SWI/SNF complexes, namely the SANT domain. Although they also participate in transcriptional regulation, they have also been implicated in global nucleosome assembly and positioning. This is likely to be coupled to replication as ISWI components co-localise with replication foci in mammalian cells. Furthermore, ISWI complexes are thought to be involved in transciptional repression as well as the formation of silenced regions on chromatin (Smith and Peterson, 2005).

The ATPases of the third class of remodeling complexes, the Mi-2 family, contain yet another histone binding domain, the chromodomain. Many of Mi-2 complexes are thought to participate in transcriptional repression by virtue of their association with histone deacetylases (HDACs, *see* below).

1.2.1.3 Covalent histone modifications and the histone code concept

The ability of histones to nucleate the assembly of nucleosomes can be attributed to their overall basic charge which attracts the negatively-charged phosphate backbone of the DNA. In addition, histones play important roles in the regulation of chromatin structure and associated functions as acceptors of posttranslational modifications in their N-termini (FIGURE 1-12). At least five such modifications have been shown to occur in histones: acetylation,

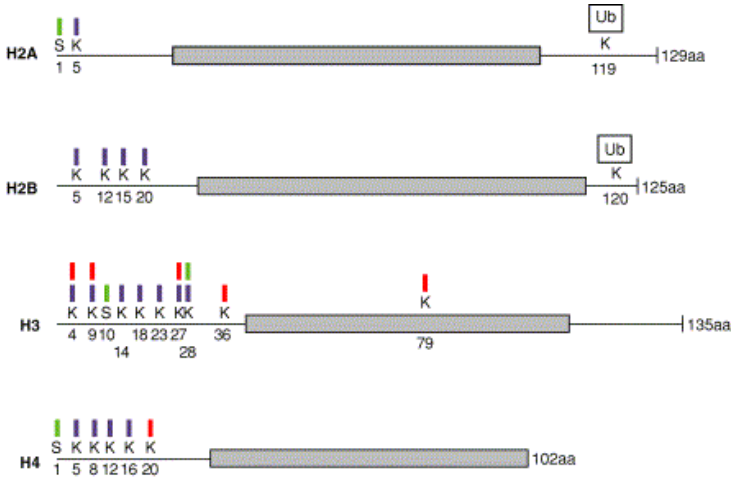


FIGURE 1-12. Histone modifications. Each histone protein consists of the N- and C-terminal tails and a central globular domain (gray box). The N- and C-terminal tails of core histones can be chemically modified by methylation (red bar), acetylation (blue bar), phosphorylation (green bar), or ubiquitination (Ub) at several residues along the length of the protein.

Figure and legend adapted from Gilbert *et al.*, 2005

methylation, phosphorylation, ADP-ribosylation and ubiquitination. At least the first three of these are thought to exert their function primarily by disrupting histone/DNA contacts in the nucleosome thus altering chromatin structure directly, while there is evidence that modified histone tails serve as platforms for other DNA regulatory complexes *via* the recruitment of proteins (Jenuwein and Allis, 2001).

1.2.1.3.1 Histone acetylation

Early experiments provided evidence that histone acetylation correlates with areas of high DNase sensitivity and transcriptional activity suggesting that these chromatin regions exhibit a lower degree of compaction (Roth *et al.*, 2001). Indeed, the acetyl group serves as a moiety partially neutralising the basic charge of histones thus weakening interactions with the surrounding DNA although biophysical evidence has not provided any evidence for gross structural changes in chromatin fiber structure (Gilbert *et al.* 2005).

At the global level, euchromatin which is associated with transcriptionally competent regions of the genome, contains high levels of acetylated histones whereas heterochromatin is characterised by histone hypoacetylation (Grewal and Moazed, 2003). Heterochromatin is concentrated around functional chromosomal regions such as centromeres and telomeres and participates in genomic stability by maintaining the structure of these regions intact.

Histone acetylation can occur at specific lysine residues which are highly conserved throughout the species. It is regulated by enzymes called acetyltransferases which transfer acetyl groups from acetyl-CoA to histones and is removed by deacetylases. Either of these classes of enymes are recruited to specific sites of the genome by sequence-specific transcription factors to regulate gene expression. Acetylated lysines are recognised by dedicated protein interaction domains called bromodomains.

Three families of histone acetyltransferases have been described (FIGURE 1-13): GNAT for (Gcn5-related N-acetyltransferases), MYST (named after its founding members MOZ, Ybf2/Sas3, Sas2, and Tip60) and p300/CBP (for CREB binding protein). The best-characterised among them and of interest for this thesis is the p300/CBP family.

p300 and CBP were independently identified as binding partners of the E1A adenoviral oncoprotein and cAMP response element binding factor (CREB). They contain

HAT family	HAT enzyme	Organisms known	Complex	Specificity	Function
GNAT	Gcn5	Yeast to humans	SAGA, ADA ADA2	H3, H2B	Coactivator
	PCAF	Human, mice	PCAF	H3, H4	Coactivator
	Hat1	Yeast	HatB	H4 [K5, K12], H2B	Histone deposition, silencing
	Elp3	Yeast to humans	Elongator	H3, H4	Transcriptional elongation
	Hpa2	Yeast		H3, H4	Unknown
	ATF-2	Humans, mice		H2B, H4	Sequence-specific transcription factor
MYST	Sas2	Yeast		H4 [K16]	Silencing
	Sas3	Yeast	NuA3	H3, H4, H2A	Silencing
	MORF	Humans		H4>H3	Unknown
	TIP60	Humans	TIP60	H4>>H3, H2A	HIV Tat interaction, DNA repair, Apoptosis
	Esa1	Humans	NuA4	H4, H2A	Cell cycle progression
	MOF	Drosophila	MSL	H4 [K16]	X- chromosome hyperactivation dosage compensation
	HBO1	Humans	HBO1	H3, H4	DNA replication
	MOZ	Humans	AML1	H3, H4> H2A	Transcription activation, others?
P300/CBP	p300	Multicelular organisms		H2A, H2B, H3, H4	Coactivator
	CBP	Multicelular organisms		H2A, H2B, H3, H4	Coactivator
Hormone Receptor Coactivators	ACTR	Humans, mice		H3>H4	Hormone Receptor coactivators
	SRC-1	Humans, mice		H3>H4	Hormone Receptor coactivators
	TIF2	Humans, mice			Hormone Receptor coactivators
TAFII250	TAFII250	Yeast to humans	TFIID	H3>H4,H2A	TBP-associated factor/ Cell cycle progression
TFIIIC	TFIII220	Humans	TFIIIC	H3, H4 >H2A	RNA polymerase III –mediated transcription
	TFIII110	Humans			
	TFIII90	Humans			
Nut1	Nut1	Humans	Mediator	H3>>H4	RNA polymerase II –mediated transcription

FIGURE 1-13. Overview of histone acetyltransferases (HATs).

Adapted from Vaquero *et al.*, 2003

multiple domains which mediate their interaction with transcription factors and recruit them to specific sites on the genome (Goodman and Smolik, 2000). Interestingly, the majority of proteins identified to-date bind in close proximity to second of the two zinc fingers of the histone acetyltransferase domain (HAT, or simply AT). Genetic studies in the mouse along with data derived from patients support a role of these proteins in tumour suppression. Mice heterozygous for *CBP* develop a range of hematopoietic malignansies due to bone marrow abnormalities (Kung *et al.*, 2000). It is of note, however, that apart from histones, p300/CBP were found to acetylate non-histone proteins such as other transcription factors and thus regulate their activities (*see* below).

Histone acetylation is reversed by histone deacetylases or HDACs. Three classes of deacetylases have been described phylogenetically (FIGURE 1-15): class I which comprises HDAC1, 2, 3 and 8, class II HDAC4, 5, 6, 7, 9 and 10 and class III SIRT1-7 (Eckwall, 2005; de Ruijter *et al.*, 2003). Despite their name, it is now clear that deacetylation of proteins other than histones can be catalysed by HDACs, as is the case for HDAC6 which is associated with microtubules and deacetylates tubulin (Hubbert *et al.*, 2002).

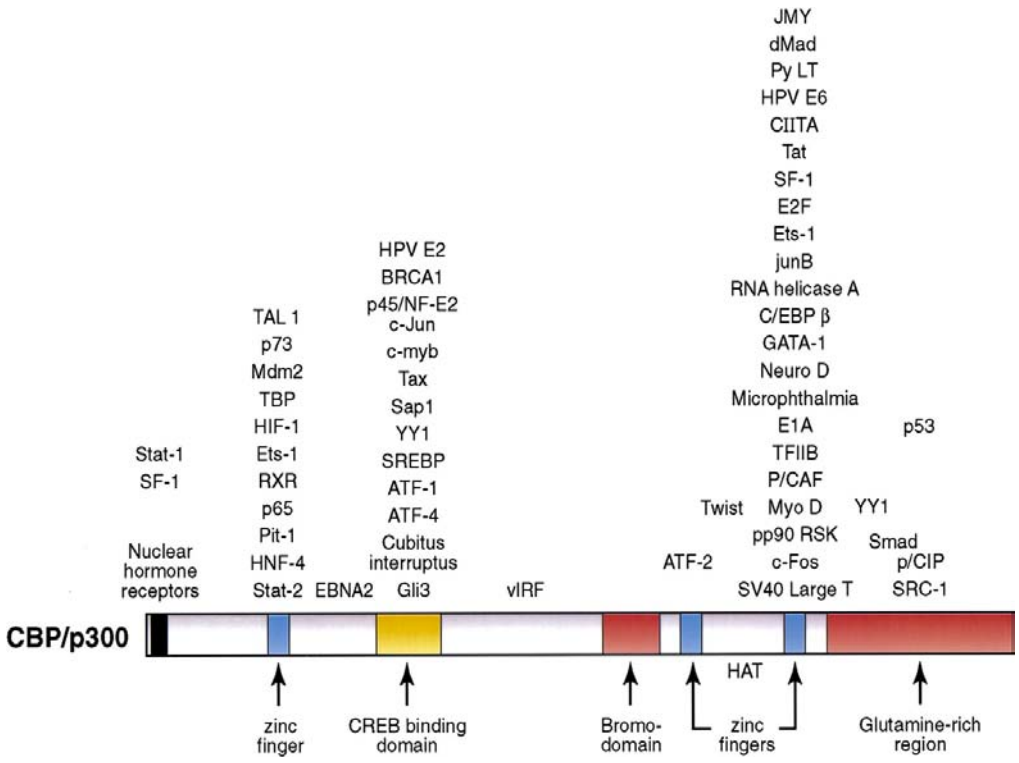


FIGURE 1-14. Domain organisation and known binding partners of the co-activators CBP/p300.

Adapted from Goodman and Smolik, 2000

Class I HDACs are thought to be ubiquitously expressed whereas Class II HDACs are restricted to specific tissues and thus are proposed to be involved in differentiation. Similar to acetyltransferases, HDACs act in the context of multicellular complexes which target them to specific genomic sites where they participate in the regulation of gene transcription. Furthermore, as recombinant HDACs alone do not exhibit robust deacetylase activity, it is likely that such complexes contain additional co-factors required for the deacetylase reaction (de Ruijter *et al.*, 2003).

Three protein complexes have been characterized that contain both HDAC1 and HDAC2: Sin3, NuRD (nucleosome remodelling and deacetylating) and Co-REST. HDAC3 is found as part of SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) and N-CoR (nuclear receptor co-repressor). HDACs can act in conjunction with each other although the exact purpose of this co-operation is not clearly understood.

Class III deacetylases are the focus of this thesis and will be discussed in detail in Chapter 2.

HDAC group	HDACs members	TSA sensitivity	NAD⁺ dependence	Localization	Function
Class I (Rpd3)	HDAC1, HDAC2, HDAC3, HDAC8	Yes	No	Nuclear and ubiquitous localization.	Involved in a variety of functions, such as transcriptional repression and cell differentiation.
Class II (Hda1)	HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10	Yes	No	Tissue-specific expression.	Transcriptional repression, microtubule regulation
Class III (yeast Sir2)	Sir2, HST1-4 Homologues in different organisms from yeast to humans. (Arabidopsis, *C. elegans*, *D. Melanogaster*, etc…) e.g. Human Sirt1-7	No	Yes	Some are nuclear, others cytoplasmic and mitochondrial.	Involvement in silencing. Connection with aging. Function in development, gene repression and DNA repair.

FIGURE1-15. Overview of protein deacetylase families.

Adapted from Vaquero *et al.*, 2003

1.2.1.3.2 Histone methylation

In addition to acetylation, histones can be modified by methyl groups by histone methyltransferases (HMTs) (FIGURE 1-16). Unlike acetylation, up to three methyl groups can be attached to either a lysine or arginine side-chain (Gilbert *et al.*, 2005). Lysine methyltransferases belong to the SET domain-containing family of proteins while arginine methyltransferases to the PRMT1. Methylation of specific residues marks the transcriptional competence of whole chromosomal regions such as the inactive X chromosome as well as individual genes. Thus, H3-K9, H3-K27, H4-K20 methylation correlates with silent chromatin, whereas H3-K4, H3-K36, H3-K79 mark transcriptionally active chromatin although this general rule is subject to exceptions (Sims *et al.*, 2003, Vaquero *et al.*, 2003). Methylated lysines are recognised by dedicated protein interaction domains called chromodomains.

Until recently, it was thought that unlike acetylation histone methylation is a stable modification that could only be reversed by histone exchange (Gilbert *et al.*, 2005). Tsukada *et al.*, reported the identification of an enzyme that catalyses the removal of methyl-groups from histones (Tsukada *et al.*, 2006). The demethylase in question, called JHDM1 (for JmjC domain-containing histone demethylase 1), preferentially demethylates methyl-K36 of histone H3 and is conserved in yeast and humans. Thus, it is very likely that additional demethylases exist with distinct sequence specificities.

Interestingly, DNA itself is also methylated and this modification correlates with transcriptional repression too. In some occassions there is a correlation between specific histone methylation events such as at H3K9 and DNA methylation. A recent report by Viré *et al.* provided a molecular explanation for this by showing that the Polycomb Group (PcG) EZH2 methyltransferase which methylated H3-K27, directly interacts with DNA methyltransferases (DNMTs) to recruit them to specific genomic sites where DNA is subsequently methylated (Viré *et al.*, 2006).

HMT group	HMT	Specificity	Function
Arginine	PRMT1	H4-R3 (non-histone proteins)	Transcriptional activation (signal transduction, etc)
	PRMT2	Unknown	Coactivator of estrogen receptor
	PRMT3	Unknown	Cytoplasmic (mitosis?)
	PRMT4/ CARM1	H3-R2, -R17, -R26. (Also at the C-terminal)	Transcriptional coactivator
	PRMT5	H2A, H4 (non-histone proteins)	Transcriptional repressor and spliceosome formation
Lysine SET domain	Suv39H1, Suv39H2	H3-K9	Heterochromatin formation, silencing
	G9a	H3-K9, H3-K27	Early embryogenesis role, transcriptional repression
	ASH1	H3-K4, -K9, H4-K20	Establishment of epigenetic, active transcription patterns
	Set1	H3-K4	Silencing
	Set2	H3-K36	Silencing, transcription
	Set7	H4-K20	Development, silent chromatin. Involved in aging
	Set9	H3-K4	Transcriptional activation
	ESC-E(z)	H3-K27	Polycomb-mediated silencing
	SETDB1	H3-K9	Silencing-mediated by the corepressor KAP-1
Dot	Dot1	H3-K79	Silencing by precluding Sir binding to bulk chromatin

FIGURE 1-16. Overview of protein methyltransferase families.

Adapted from Vaquero *et al.*, 2003

1.2.1.3.3 Histone phosphorylation

All histones, including H1, have been shown to be modified by phosphorylation (FIGURE 1-17). Among them the best characterised is histone H3 phosphorylation which occurs at residues S10 and S28. H3-S10 phosphorylation appears early in G2 in pericentromeric heterochromatin and spreads throughout mitotic chromosomes. In relation to this, H3-S10 phosphorylation has been implicated in mitotic chromosome condensation, yet this appears not to be an absolute requirement in all species as in yeast mutations of phosphorylated H3 tail residues do not cause any mitotic defects (Vaquero *et al.*, 2003).

Rapid H3 phosphorylation is also observed upon stimulation with mitogens. The significance of this is not clear but it may reflect the concomitant induction of proliferation (Cheung *et al.*, 2000). C-terminal phosphorylation of a histone H2A variant, H2A.X is observed

rapidly following DNA damage caused by ionising radiation as well as in response to caspase-mediated apoptotic signaling. Similarly, H2B is also phosphorylated during apoptosis. Thus, it has been postulated that H2A.X and H2B phosphorylation correlate with the occurrence of DNA double strand breaks, yet little is known about the kinase pathways involved (Cheung *et al.*, 2000).

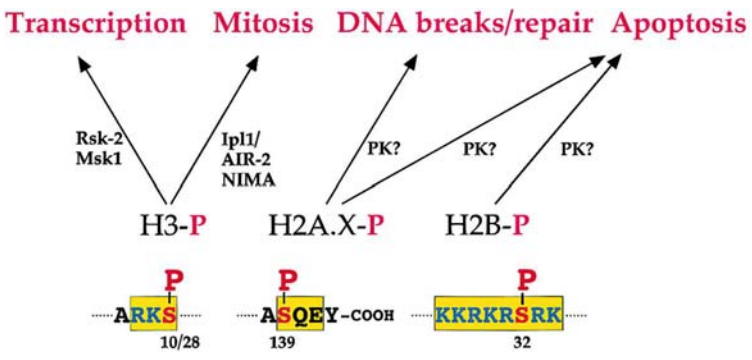


FIGURE 1-17. Cellular processes are associated with histone phosphorylation. Phosphorylation of H3, H2A.X, H2B has respectively been associated with mitogen stimulation (and thus potentially transcription), chromosome condensation (Mitosis), DNA damage, and apoptosis. The putative enzymes responsible for these phosphorylation events and the sites of phosphorylation, where known, are as indicated, and PK? refers to unknown protein kinases. The amino acid sequences surrounding those phosphorylation sites are indicated at the bottom, and the yellow boxes typically indicate stretches of basic amino acid residues flanking the phosphorylated serines.

Figure and legend adapted from Cheung *et al.*, 2000

1.2.1.3.4 Histone ubiquitination

Histone ubiquitination is a less understood modification. Unlike methylation, acetylation and phosphorylation, ubiquitination occurs in the C-terminus of histones. It was initially linked to transcriptional activation as monoubiquitinated histones where found in actively transcribed chromatin in *Tetrahymena* (Vaquero *et al.*, 2003). In agreement to this the transcriptional co-activator $TAF_{II}250$ has been reported to have a histone monoubiquitination activity. Furthermore, an interplay between histone ubiquitination and histone methylation is implied by the observation that lack of H3-K123 ubiquitination prevents methylation of H3 at K4 (Vaquero *et al.*, 2003).

1.2.1.3.5 Histone ADP-ribosylation

Histone ADP-ribosylation occurs in glutamic acids in a polyglutamate stretch or single arginines (Vaquero *et al.*, 2003). Although mono-ADP-ribosylation can also occur, poly-ADP-ribosylation is relevant to histone function and can comprise more than 100 ADP-ribosyl moieties with extensive branching (Jacobson and Jacobson, 1999). Poly-ADP-ribosylation is

highly dynamic being catalysed by poly-ADP-ribosyltransferases (PARPs) and removed by poly-ADP-ribose glycohydrolases (PARGs).

PARP1 is required to recruit the DNA repair machinery in response to DNA damage it is though possible that PARP1 has a stimulatory effect on gene transcription by modifying not only histones but also transcription factors. Conversely, PARP1 has also been shown to inhibit RNAPolII transcriptional elongation (Vaquero *et al.*, 2003 and references therein). PARP activity requires NAD^+ and one of its enzymatic products is nicotinamide both of which are features of the enzymatic reaction catalysed by sirtuin deacetylases (*see* Chapter 2). The possible significance of this will be discussed in Chapter 5.

1.2.1.3.6 Epigenetics and the 'histone code' hypothesis

As is evident from the above, histone modifications serve as recognition sites for specific protein domains to recruit additional chromatin-modifying complexes that regulate gene expression. Thus, the modification status of histone tails in addition to sequence elements encoded on the DNA can dictate chromatin structure and thus transcriptional activity. The recognition of this led credence to the notion of a "histone code" which postulates that covalent chromatin modifications are carriers of information that contribute to gene expression (Jenuwein and Allis, 2001).

The histone code along with DNA methylation are so-called epigenetic events as they are not encoded in the genome, yet they correlate with specific phenotypes (Pennisi, 2001). Although epigenetic regulation underlies basic biological phenomena such as inheritence and differentiation (Pennisi, 2001), the field of epigenetics has only recently started to provide detailed molecular mechanisms primarily fuelled by the discovery of the enzymes involved in histone and DNA modification, the identification of specific protein interaction domains recognising such modifications and the elucidation of the interlinks between them.

Given this knownledge, it is of great interest to identify links between specific epigenetic phenomena and associated phenotypes as it becomes apparent that information other than DNA nucleotide sequence is contained within chromatin.

1.2.2 Transcriptional regulation

Extracellular stimuli can evoke specific transcriptional responses by eliciting changes in chromatin structure. Multiple mechanisms of specificity that ensure appropriate pathway activation are in place, however a major role in this process is played by the sequence-specific binding of transcription factors to DNA elements that can affect the activity of the basal transcriptional machinery. These factors and their assembly into functional protein complexes can be controled either by specific small molecule ligands such as lipids, cholesterol and xenobiotics, or by posttranslational modifications often smilar to the ones occuring in histones. This section will provide an overview of the current knowledge underlying transcriptional regulation necessary for the understanding of the concepts discussed in later chapters of this thesis.

1.2.2.1 Basal transcription

mRNA synthesis from a gene-encoding DNA sequence is catalysed by RNA Polymerases (RNAPols). Three distinct RNAPols exist in eucaryotic cells which were first distinguished from their variable sensitivity to amanitin, a mushroom poison. They are designated RNAPolI, RNAPolII and RNAPolIII.

RNAPolI catalyses the transcription of precursor rRNA (pre-rRNA), which is processed into 28S, 5.8S, and 18S rRNAs. RNAPolIII transcribes the genes encoding tRNAs, 5S rRNA as well as other small, stable RNAs including U6 RNA involved in RNA splicing and the 7S RNA of the signal-recognition particle involved in the transport of proteins into the endoplasmic reticulum (Lodish *et al.*, 2000). RNAPolII transcribes all protein-encoding genes as well as four small nulcear RNAs involved in RNA splicing.

DNA sequences located at the beginning (5') of the gene coding region recruit RNAPols to initiate gene transcription (FIGURE 1-18). Such sequences constitute the core promoter element which is able to sustain transcriptional initiation by RNAPols.

Initiation of RNAPolII-catalysed transcription starts by the recognition of the TATA box element by the TATA-binding protein (TBP) of TFIID. TBP binding causes a dramatic conformational change on the DNA which is thought to contribute to the downstream

transcriptional activation events (Orphanides *et al.*, 1996). RNAPolII transcription initiation can also occur in the absence of TATA elements where other sequences such as the initiator (Inr) or the downstream core promoter element (DPE) take up its role (Butler and Kadonaga, 2002).

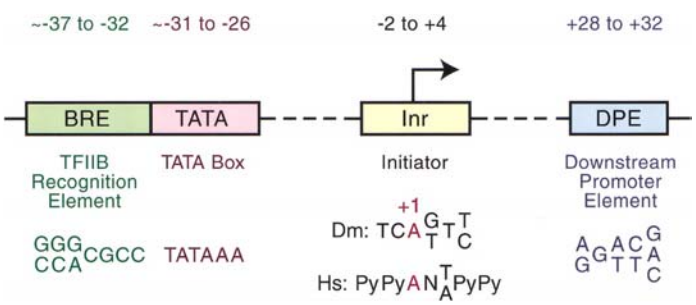


FIGURE 1-18. Core promoter elements. Some core promoter motifs that can participate in transcription by RNA polymerase II are depicted. Each of these elements is found in only a subset of core promoters. Any specific core promoter may contain some, all, or none of these motifs. The BRE is an upstream extension of a subset of TATA boxes. The DPE requires an Inr, and is located precisely at +28 to +32 relative to the A_{+1} nucleotide in the Inr. The DPE consensus was determined with *Drosophila* transcription factors and core promoters. The Inr consensus sequence is shown for both *Drosophila* (Dm) and humans (Hs).

Figure and legends adapted from Butler and Kadonaga, 2002

TFIID nucleates the ordered recruitment of a multisubunit complex which comprises activites that lead to promoter 'melting', i.e. nucleosome disruption and opening of the DNA double helix, rendering it competent as a transcription template. Following that, transcriptional elongation from TATA-driven promoters requires the phosphorylation of the C-terminal domain (CTD) of RNAPolII which is catalysed by CDK7.

rDNA genes contain distinct promoter elements that recruit RNAPolI. These elements are recognised by upstreat binding factor (UBF) which nucleates the formation of the RNAPolI holoenzyme (Grummt, 2003).

Three types of RNAPolIII promoters have been described based on the sequence elements encompassed by each (Schramm and Hernandez, 2002). In contrast to RNAPolI and RNAPolII core promoters which are located upstream or at the very 5' of the mRNA-encoding region, RNAPolIII promoters encoding for tRNA and 5S rRNA are intragenic while these encoding for snRNAs contain a TATA box and a proximal sequence element (PSE).

In addition to promoter elements which act in-*cis* DNA sequences which act in-*trans* play important roles in transcriptional regulation. These elements are called enhancers and in contrast to promoters whose spatial positioning with respect to the transcribed region is important for their function, enhancers can promote gene transcription in a position- and orientation-independent manner.

Events following promoter recruitment of RNAPolI and RNAPolIII are similar in nature to those described for RNAPolII although the individual protein components as well as regulatory mechanisms involved are clearly distinct. For the rest of this section, the focus will be on RNAPolII-related mechanisms.

1.2.2.2 Transcription factors and their regulation

1.2.2.2.1 Mechanisms of transcription factor action

Transcription factors are modular proteins which comprise a DNA-binding domain, a protein interaction domain that mediated homo- or hetero-dimeriation and an effector domain which mediates transcriptional activation or repression (Merika and Thanos, 2001). Such a modular structural configuration in combination with differential arrangements of enhancer and promoter gene elements allows transcription factors to assemble into variable protein complexes to implement distinct gene expression programmes.

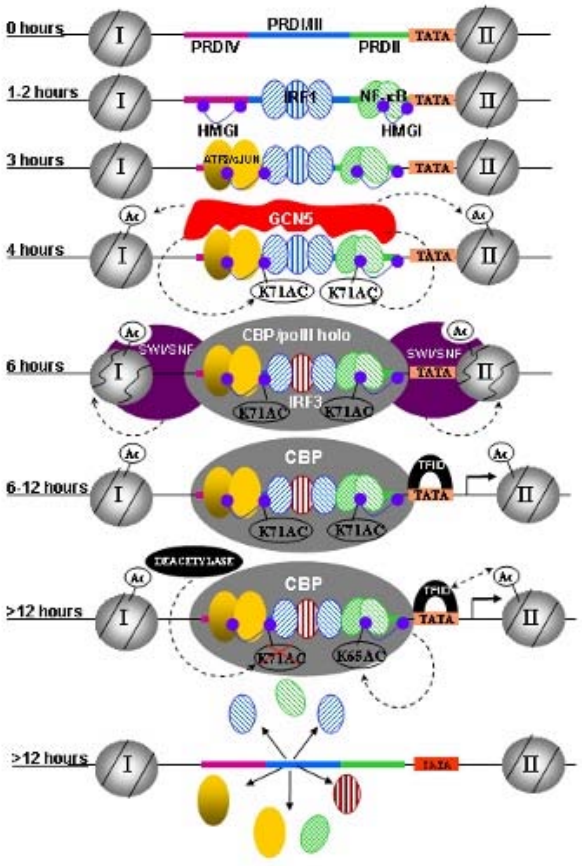


FIGURE 1-19. Transcriptional regulation at the IFN-β gene promoter. *See* text for details.

Adapted from http://www.fleming.gr/en/investigators/Thanos/index.html

The molecular machinery activated in response to viral infection and regulates transcription of the interferon-β gene has been extensively studied and will be used here as an exemplar of inducible gene expression mechanisms. Activation of IFN-β gene transcription in response to viral infection is mediated by at least three transcription factors NF-κB (nuclear factor κB), members of the

IRF (interferon regulatory factor) family, and the ATF-2 (activating transcription factor-2)/c-Jun heterodimer (Merika and Thanos, 2001).

These factors (activators) act in concert upon viral entry to form a complex known as the 'enhanceosome' to specifically activate IFN-β gene transcription (FIGURE 1-19). Assembly of these factors on the IFN-β gene promoter is dictated by enhancer elements which are found in a specific spatial positioning pattern. In a different context any of these factors can mediate other processes such as response to cytokines, UV irradiation or the proliferative response to mitogens. At the same time none of them alone can elicit the response to viral infection (Merika and Thanos, 2001). Enhanceosome assembly is greatly facilitated by a high mobility group protein HMG I(Y) which binds to four sites in the enhancer sequence and induces a conformational change on the DNA that lowers the free energy of activator binding (Merika and Thanos, 2001). At the same time HMG I(Y) contributes to the stability of the enhanceosome.

Upon its assembly, the enhanceosome, recruits the co-activator GCN5 which acetylates a nucleosome masking the promoter TATA element. At the same time GCN5 also acetylates HMG I(Y) at K71 contributing to enhanceosome stability. TATA box exposure allows the assembly of the RNAPolII pre-initiation complex along with the acetyltransferase CBP which in turn recruits the SWI/SNF remodelling complex and acetylates promoter histones, a prerequisite for SWI/SNF-mediated nucleosomal remodelling (Agalioti *et al.*, 2000). This cascade culminates with the assembly of the RNAPolII holoenzyme and transcriptional initiation.

1.2.2.2.2 Post-translational regulation of transcription factors

Apart from histone modifications, transcription factor modifications are also well-established events in the regulation of transcription. These include acetylation, phosphorylation, ubiquitination, sumoylation and proteolytic cleavage to mention but some and have variable effects in the ability of transcription factors to modulate gene expression. Many of the modifying enzymes described for histones also target transcription factors. Such modifications provide a way of modulating transcription factor activity by upstream signaling cascades as described in section 1.2. The wealth of studies on the regulation of the tumour suppressor p53 provide a characteristic example of this.

p53 is a transcription factor that binds to specific DNA elements as a tetramer. It is a highly unstable protein due to its constant ubiquitination by the E3 ligase Mdm2 (a.k.a. Hdm2) which targets it for proteasomal degradation. Mdm2 also binds an N-terminal transactivation domain thus inhibiting downstream transcriptional induction. Upon exposure of cells to genotoxic stresses, p53 ubiquitination is suppressed allowing its accumulation in the nucleus where it activates or represses more than 150 target genes to control cell cycle and survival (Bode and Dong, 2004).

Multiple pathways are involved in p53 stabilisation. Acetylation targets among other sites the lysine residues ubiquitinated by Mdm2 contributing to p53 stabilisation. Furthermore, p53 acetylation enhances its ability to bind to DNA and consequently its transcription activation capacity (Luo *et al.*, 2004). Phosphorylation by multiple kinases can also affect p53 targeting by Mdm2, increase its transcriptional activity and modulate its subcellular localisation by affecting NLS recognition (Bode and Dong, 2004). Furthermore, phosphorylation of p53 renders it a substrate for the peptidyl-prolyl isomerase Pin1 which induces a conformational change that enhances its transactivation capacity (Zheng *et al.*, 2002; Zacchi *et al.*, 2002). Recently, methylation of p53 by a SET domain methyltransferase was also reported to modulate protein stability (Chuikov *et al.*, 2004).

Acetylation can also have more subtle effects on transcription factor activity. Upon stimulation the NFκB transcription factor is released from the inhibitory protein IκB to translocate to the nucleus and activate transcription (Chen and Greene, 2004). Similarly to p53 acetylation has been shown to enhance binding to DNA. At the same time though, acetylation reduces its affinity for IκB albeit by targeting different lysine residues. Conversely, HDAC3-mediated deacetylation contributes to the attenuation of the NFκB response by enhancing NFκB interaction with IκB, a complex that is actively exported to the cytoplasm (Chen *et al.*, 2001).

1.2.2.2.3 Ligand-mediated modulation of transcription factor activity

Similarly to procaryotic cells, some eucaryotic transcription factors harbour domains that can be directly bound to by specific ligands. Several small molecules including aminoacids (proline, arginine), intermediate metabolites (benzoic scid, orotic acid) and

sugars are known to regulate transcriptional switches with the primary goal to modulate metabolic output depending on the cellular needs as relayed by these molecules (Sellick and Reece, 2005).

The family of nuclear receptors comprise transcription factors most of which are regulated by ligand binding (Weatherman *et al.*, 1999). Nuclear receptor ligands include among others hormones such as estrogen and growth hormone, metabolites such as bile acids, lipids and cholesterol and xenobiotics such as drugs. The natural ligands for many nuclear receptors are unknown thus such receptors are described as 'orphan'.

Ligand effects are mediated through a C-terminal ligand binding domain (LBD) and an N-terminal DNA binding domain (DBD) linked by a flexible linker region which is proposed to allow not only conformational changes but also contribute to the recruitment of co-activators and co-repressors.

Co-activator and co-repressor recruitment sites are overlapping in the LBD. Thus it is postulated that ligand binding induces a conformational change that allows the exchange of co-repressors for co-activator complexes to induce gene expression (Nagy and Schwabe, 2004). Recently, two F-box/WD-40 containing proteins, transducin β-like (TBL1) and the related TBLR1, were identified as critical components for this exchange mechanism. Upon ligand binding TBL1 and TBLR1 recruit the 19S proteasome to induce the clearance of co-repressor complexes allowing the binding of co-activators to the liganded receptors (Perissi *et al.*, 2004).

1.2.2.2.4 Transcriptional regulatory networks

The complexity of the mechanisms in place to determine gene transcription by multiple regulatory events allows an enormous degree of flexibility in the implementation of specific gene expression programmes suited to environmental cues. During development for example, multipotent stem cells derived from a single zygote form the starting cell pool which will generate hundreds of different cell types to form tissues, organs and together an organism.

Recent technical advances are starting to provide an integraded view of how transcription factor networks drive the spatio-temporaly precise execution of complex biological processes (Blais and Dynlacht, 2005). During early development, the transcription

factors OCT4, SOX2 and NANOG are important for the maintainance of the pluripotent character of stem cells in that loss-of-function results in aberrant differentiation. Genome-wide analysis of target promoter occupancy revealed an elaborate gene regulatory network dictated by OCT4, SOX2 and NANOG which among other features drives the expression of homeobox transcription factors important for development (Boyer *et al.*, 2005). This work implied the existence of autoregulatory and feedforward loops which enable the maintainance of the stem cell's pluripotent character while enabling it to repond to developmental cues in order to give rise to appropriate cell types (Boyer *et al.*, 2005).

1.2.2.3 Additional mechanisms of gene regulation

Finally, in addition to the above mechanisms regulating gene expression other factors may also contribute to it.

Chromosomes are arranged into so-called chromosome territories in the interphase nucleus in that they occupy distinct nuclear regions. For example the rDNA-containing acrocentric chromosomes 13, 14, 15, 21 and 22 associate preferentially with nucleoli. Human chromosomes 18 and 19, although of comparable size they differ in gene density with chromosome 18 being gene-poor (~4.3 genes per Mbp) and chromosome 19 gene-rich (22.7 genes per Mbp). Chromosome 18 is located in the nuclear periphery whereas chromosome 19 is positioned more internally (Gilbert *et al.*, 2005). Furthermore, chromosome 19 spreads in an area larger than that of chromosome 18 suggesting that the chromatin in the former adopts a less condensed conformation than that in the latter.

It is thought that interchromatin domains (ICDs) which are chromatin-free regions, contain a high density of transcriptional apparatus components to which genes located in euchromatin have access (Gilbert *et al.*, 2005). Moreover, foci of transcription have been observed in nuclei (Francastel *et al.*, 2000). However, little is known about the way nuclear architecture can affect gene expression in reponse to specific stimuli or whether it simply provides an organisational framework for proper execution of the cell's transcriptional activities.

Finally, post-transcriptional regulatory mechanisms exist to determine gene expression. A mechanism of post-transcriptional regulation previously identified in plants and

C. elegans has been recently shown to apply in mammalian cells. It is known as RNA interference (RNAi) and it acts by the formation of precisely processed small RNA molecules that guide molecular complexes such as the RISC (RNA-induced silencing complex) or RITS (RNA-induced initiation of transcriptional gene silencing) to recognise complementary sequences on target mRNAs (Tomari and Zamore, 2005). This drives the degradation of the target mRNA and thus reduction of the respective gene product synthesis. There is evidence that engogenous small RNA molecules known as microRNAs (miRNAs) are involved in development and disease thus further expanding the cellular toolbox or transcription regulatory mechanisms (Wienholds and Plasterk, 2005).

1.3 Conclusion

This chapter provided an overview of the signaling mechanisms involved in the transduction of environmental cues to the nucleus and described mechanisms available therein that allow the implementation of transcriptional programmes determined by the nature of the upstream signals. The importance of elucidating such basic cellular processes is emphasised by the increasing appreciation that deviation from these finely designed pathways underlies the cause of many disease states such as cancer and diabetes.

Prompted by our interest in undertanding how nutrient availability and cellular energy status modulate gene trasncription, we initiated the investigation of the sirtuin family of deacetylases. This family comprises proteins with reported histone deacetylase activity which, unlike HDACs, depends on NAD^+. Thus, sirtuins are prime candidates to function as nodes that couple cellular redox status and transcriptional regulation.

CHAPTER 2

THE SIRTUIN FAMILY OF PROTEIN DEACETYLASES

2. INTRODUCTION TO THE SIRTUIN FAMILY

2.1 Discovery of sirtuins and determination of their enzymatic activity

The sirtuin family of enzymes comprises protein members from across all three phyla. They are characterised by a highly conserved core domain which carries a catalytic activity and in is flanked by N- and C-terminal regions of variable length which are particularly prevalent in the more recent additions of the evolutionary time-scale.

Early studies implicated the yeast sirtuin Sir2 as part of the genetic network that regulates the position-dependent expression of genes which determine mating type in *S. cerevisiae*, a phenomenon also known as gene silencing (Ivy *et al.*, 1986; Kimmerly and Rine, 1987; Rine and Herskowitz, 1987). An indication as to the actual activity of Sir2 was given by the finding that silenced chromatin correlated with concomitant hypoeacetylation of histones and that Sir2 overexpression resulted in significant histone deacetylation *in vivo* (Braunstein *et al.*, 1993). This implied that Sir2p harbors a deacetylase activity. However, initial attempts to demonstrate such enzymatic action *in vitro* were unsuccesful.

In search of a catalytic activity it was noted that the *CobB* gene in *Salmonella typhimurium*, a bacterial sirtuin member, could compensate for loss-of-function mutations in another gene, *CobT* (Tsang and Escalante-Semerena, 1998). *CobT* encodes for a 5,6-

dimethylbenzimidazole phosphoribosyltransferase that transfers the 5-phospho-α-D-ribosyl moiety from nicotinate mononucleotide onto 5,6-dimethylbenzimidazole (FIGURE 2-1). This produces α-ribazole-5'-phosphate a precursor for the in cobalamin biosynthesis pathway. Given its ability to compensate for loss of CobT, it was proposed that CobB and, by association, sirtuins, could catalyse a similar, pyridine nucleotide transferase-like reaction which yet had to be distinct given the absence of the cobalamin biosynthesis pathway in higher eucaryotes.

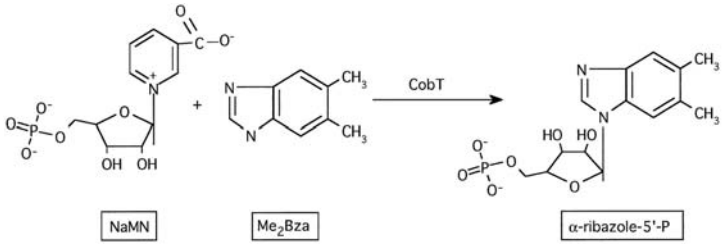


FIGURE 2-1. The reaction catalysed by CobT. *See* text for details.

Adapted from Trzebiatowski *et al.*, 1997

Using computer-based homology searches, Frye provided the first comprehensive description of the sirtuin family, discovering the first five human orthologues and provided experimental evidence that some bacterial, yeast and human sirtuin members harbor an ADP-ribosyltransferase activity (Frye, 1999). Interestingly, this activity seemed to depend on nicotinamide adenine dinucleotide (NAD^+). Subsequent studies provided support for these conclusions and showed that Sir2 requires this enzymatic activity to perform its chromatin silencing function (Tanny *et al.*, 1999).

Given the evidence for an NAD^+-dependence of the enzymatic activity Imai *et al.* investigated whether NAD^+ regulates the effect of Sir2 on histone H3 and H4 acetylated peptides (Imai *et al.*, 2000). They could demonstrate that the vast majority of the reaction product was deacetylated rather than ADP-ribosylated peptides. This reaction absolutely required NAD^+ and none of NADH, NADPH or $NADP^+$ could substitute for it. This provided the first evidence that Sir2 is an NAD^+-dependent histone deacetylase. This exceptional property for chromatin-modifying enzymes implied that sirtuins may provide a link between nutrient availability sensed as the intracellular NAD^+ levels and transcriptional regulation. Further studies also confirmed the same enzymatic activity for other sirtuins in yeast,

Drosophila and mammals. An exception to this consensus is mammalian SIRT6 which was recently shown to be an ADP-ribosyltransferase (Liszt *et al.*, 2005).

Phylogenetic studies of the sirtuin concerved core domain (Frye, 2000) have identified four classes of proteins according to their evolutionary relationship (FIGURE 2-2) with a variable presence across the phyla. Furthermore, some gram-positive bacteria and *Thermotoga maritima* exhibit sequence similarities between class II and class III sirtuins and thus are classified separately as class U. A multiple sequence alignment of representative members of sirtuins from across the phyla is shown in FIGURE 2-3.

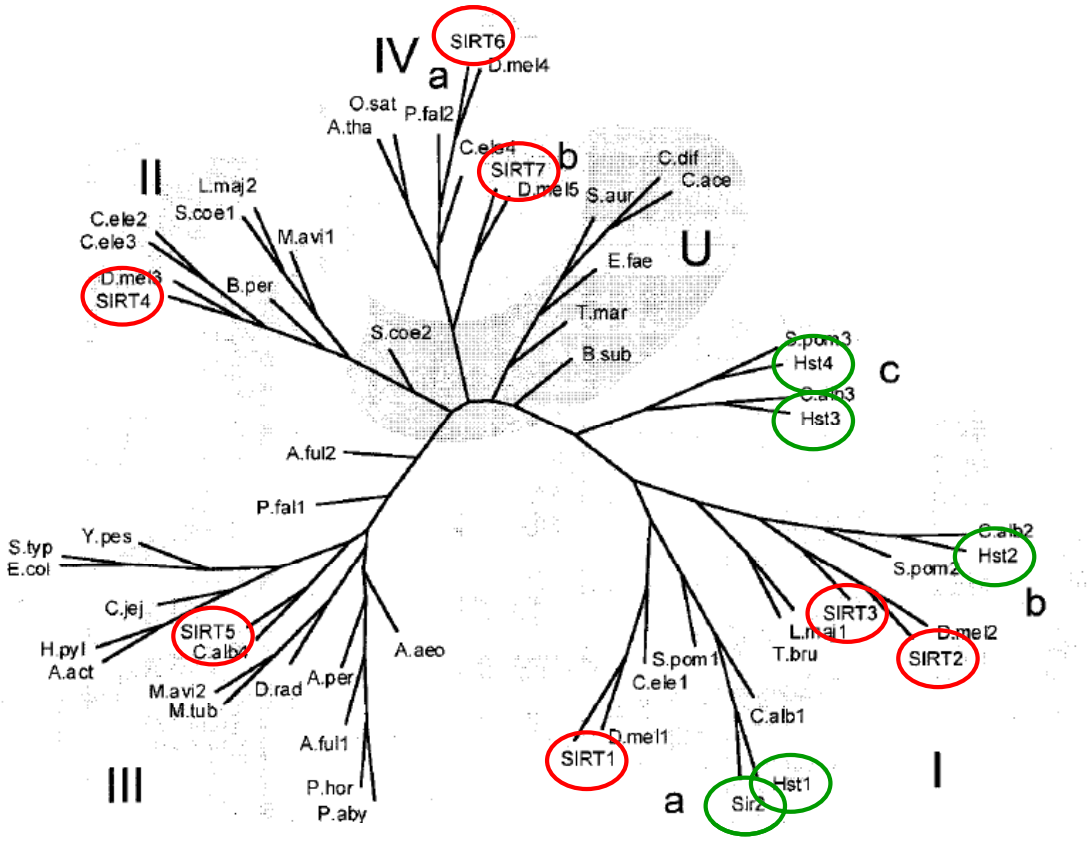


FIGURE 2-2. Phylogenetic analysis of sirtuins. Human sirtuin homologues are circled in red while their yeast counterparts are indicated in green.

Adapted from Frye, 2000

2.2 Structural and enzymatic properties of sirtuins

As delineated in the previous section, two enzymatic activities have been associated with sirtuins, that of ADP-ribosyltransferase and that of NAD^+-dependent deacetylase. The

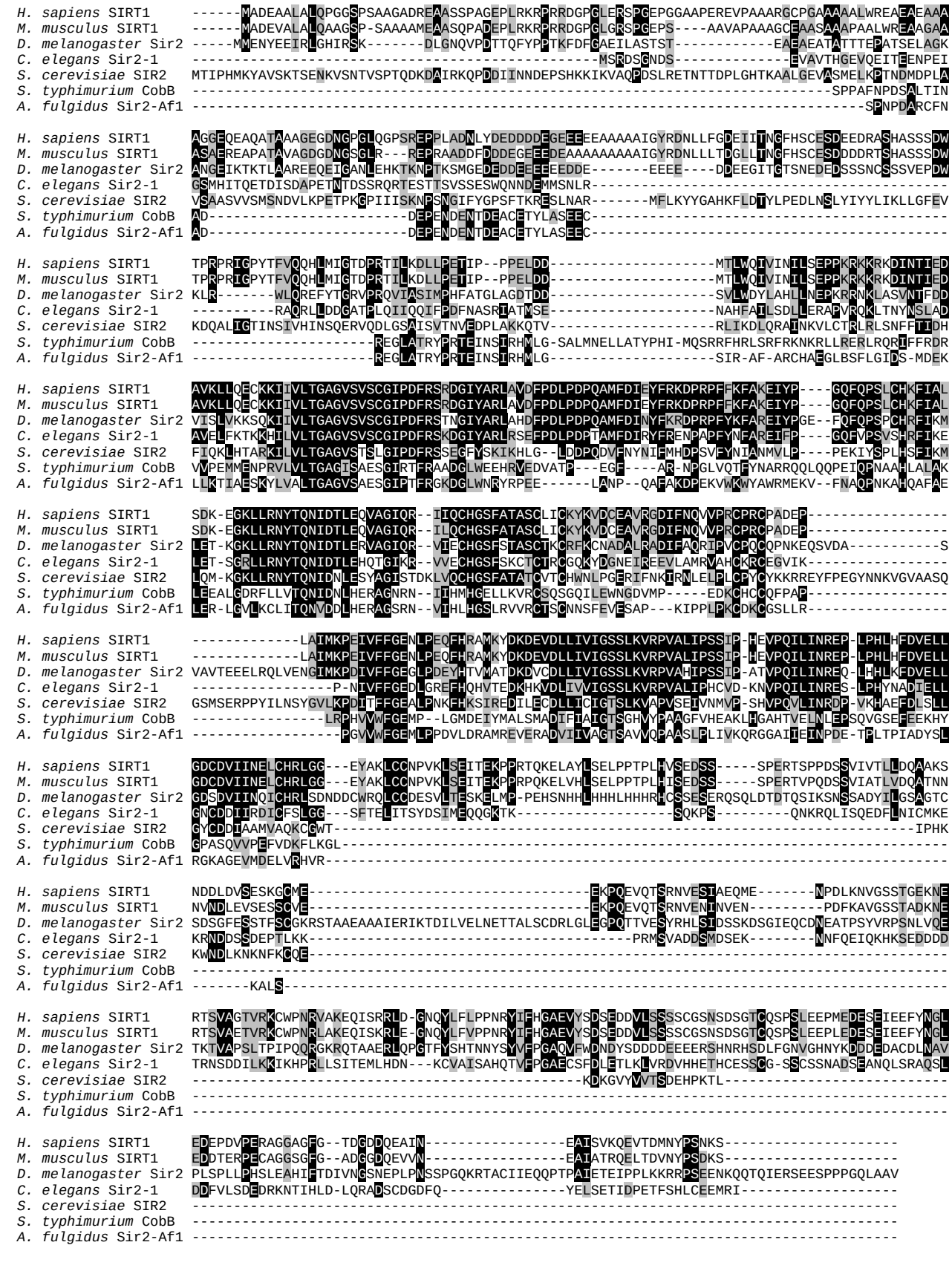


FIGURE 2-3. Multiple sequence alignment of sirtuin members from different species. Accession numbers of the sequences used are repsectively from top to bottom: Q96EB6, Q923E4, O96505, Q21921, P06700, P0A2F3, O28597.

NAD^+-dependence of deacetylase activity was an unprecedented paradigm in the field of histone-modifying enzymes, the original proposed role of sirtuins, and consequently has attracted considerable effort to elucidate the enzymatic mechanism of the catalysed reaction. This effort was fuelled initially by biochemcal studies primarily based on quantitative measurements of sirtuin enzymatic products and, upon their availability, on structural data.

The crystal structures of several sirtuins from across the phyla and in complex with different substrate forms have been determined. TABLE 2-1 summarises these crystalographic studies.

TABLE 2-1. Available crystal structures of sirtuin family members

Protein	Organism	Co-crystalised with:	Resolution	Reference
SIRT2 (aa 34-356)	*H. sapiens*	-	1.7 Å	Finnin *et al.*, 2001
Sir2-Af1	*Archaeoglobus fulgidus*	NAD^+	2.1 Å, 2.4 Å	Min *et al.*, 2001
Sir2-Af2	*Archaeoglobus fulgidus*	18-aa Ac-K382 p53 peptide	2.0 Å	Avalos *et al.*, 2002
Sir2-Af1	*Archaeoglobus fulgidus*	NAD^+	1.47 Å	Chang *et al.*, 2002
Hst2	*S. cerevisiae*	-	2.5 Å	Zhao *et al.*, 2003a
Hst2 (1-294)	*S. cerevisiae*	NAD^+	2.7 Å	Zhao *et al.* , 2003b
Hst2 (1-294)	*S. cerevisiae*	2'-O-acetyl-ADP-ribose & Ac-H4 peptide	1.5 Å	Zhao *et al.*, 2003b Zhao *et al.*, 2004a
Hst2 (1-294)	*S. cerevisiae*	carba-NAD^+ & Ac-H4 peptide	1.75 Å	Zhao *et al.*, 2004a
CobB	*E. coli*	AcK16-H4 peptide	1.96 Å	Zhao *et al.*, 2004b
Sir2-Af2	*Archaeoglobus fulgidus*	NAD^+	2.3 Å	Avalos *et al.*, 2004
Sir2-Af2	*Archaeoglobus fulgidus*	ADP-ribose	2.3 Å	Avalos *et al.*, 2004
Sir2-Af2	*Archaeoglobus fulgidus*	Nicotinamide	2.4 Å	Avalos *et al.*, 2005
Sir2-Tm	*Thermotoga maritima*	Nicotinamide	1.4 Å	Avalos *et al.*, 2005

These studies show the existence of a catalytic core that adopts an elongated shape containing two domains connected by four cross-over loops (FIGURE 2-4). The smaller domain consists of a three-stranded antiparallel β-sheet, two α-helices and a large portion of the long loop connecting the two domains. This domain binds a structural zinc ion through two pairs of cycteines which are highly conserved among sirtuins. This zinc-binding module has the same topology as a RING finger motif which is known to mediate protein-protein interactions although it lacks the secong zinc stom of the RING finger (Finnin *et al.*, 2001). The larger domain adopts a reverse Rossmann fold structure characteristic of NAD^+- and $NADP^+$-binding enzymes. Six parallel strands form a central β-sheet flanked by four α-helices one one side of the β-sheet and two α-helices on the other. In between the two domains there is a large groove that contains the NAD^+ binding site and comprises residues with high degree of conservation across all classes which form the catalytic domain of the enzyme.

Three distinct regions are found in the NAD^+ binding pocket (FIGURE 2-5) (Min *et al.*, 2001; Finnin *et al.*, 2001). Site A that binds the adenine-ribose moiety of NAD^+, site B where the nicotinamide-ribose moiety is bound and site C burried deep inside the NAD^+-binding pocket and contains residues that affect enzymatic activity despite the fact that it makes no direct contact with NAD^+.

The structure of Sir2-Af2 bound to

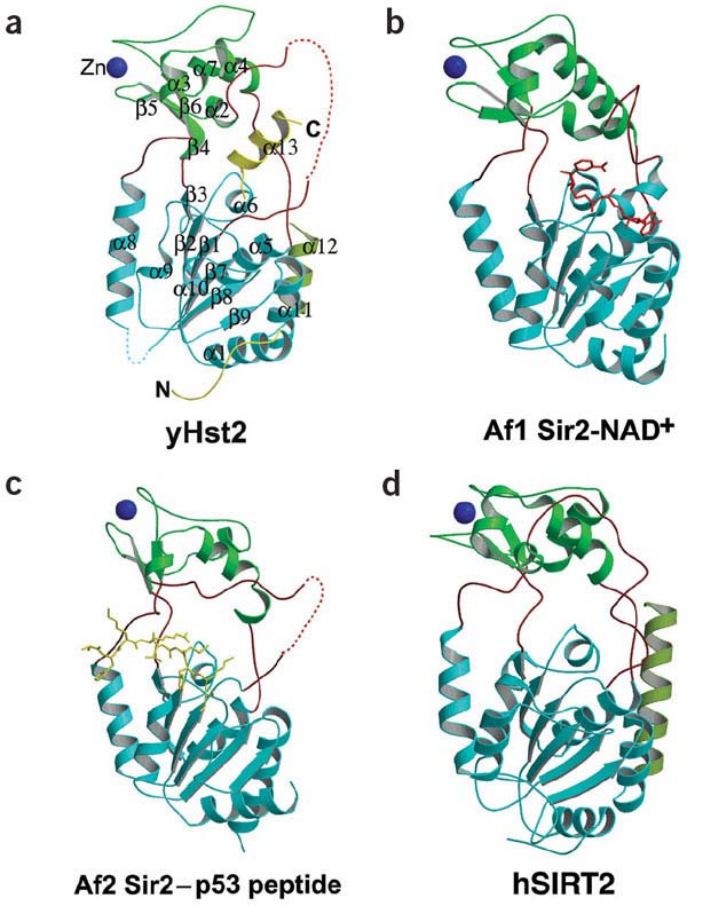


FIGURE 2-4. Representative crystallographic structures of sirtuins. (a) Structure of yHst2. The catalytic core is color-coded to distinguish the large conserved domain (cyan), structurally more variable small domain (green), the loops connecting the small and large domains (brown) and the bound zinc ion (blue). Features present uniquely in Hst2 are yellow (N-terminal loop and α13 helix) and light green. **(b)** Structure of the Af1 Sir2−NAD^+ complex with the NAD^+ (red). **(c)** Structure of the Af2 Sir2−p53 peptide complex with the p53 peptide (yellow). **(d)** Structure of human SIRT2.

Figure and legend adapted from Zhao *et al.*, 2003

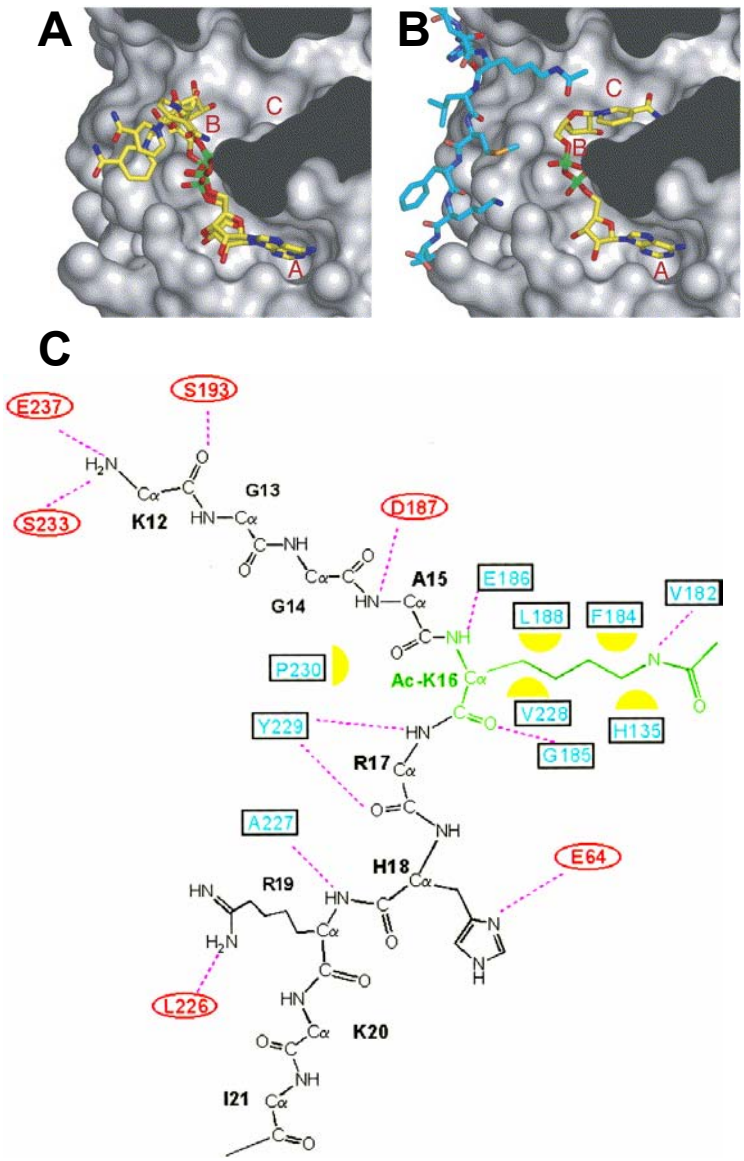


FIGURE 2-5. The sirtuin NAD$^+$-binding pocket. (A) In the absence of substrate peptide, NAD$^+$ can bind in the A and B pockets of sirtuins in alternative, nonproductive conformations. **(B)** In the presence of substrate peptide (modeled in blue), NAD$^+$ binds in a precise productive conformation that buries its nicotinamide moiety in the highly conserved C pocket of sirtuins. (A) and (B) are based on the crystal structure of the archaeal sirtuin Sir2-Af2. **(C)** Schematic representation of the observed interactions between yeast Hst2 and histone H4 acetyl-K16 peptide.

Adapted from Avalos *et al.*, 2005 [(A) and (B)]; Marmorstein, 2004 (C)

acetylated p53 peptide (FIGURE 2-4b) and of Hst2p bound to acetylated histone H4 peptide (FIGURE 2-6) showed that substrate binding occurs in the large pocket between the Rossmann fold and the zinc binding module with the acetylated lysine inserted in a hydrophobic tunnel placing acetyl group adjacent the NAD$^+$-binding site (FIGURES 2-5B,C & 2-6).

In the absence of substrate peptide, the nicotinamide ring of NAD$^+$ appears unstructured. When acetylated

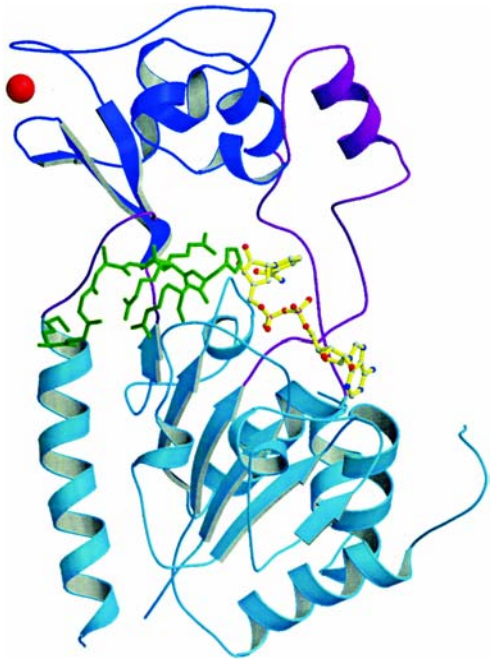


FIGURE 2-6. Crystal structure of Hst2 in complex with acetyl-K16 histone H4 peptide and carba-NAD$^+$.

Adapted from Marmorstein, 2004

peptide substrate or mimics thereof are present (Avalos *et al.*, 2004), NAD^+ undergoes a conformational change that places the nicotinamide ring in the highly concerved C pocket to induce a productive conformation (FIGURE 2-5).

The crystallographic analyses along with biochemical studies have led to a proposed mechanism of NAD^+-dependent deacetylation (FIGURE 2-7). Binding of the acetylated substrate to the catalytic pocket facilitates the binding of NAD^+ in a productive conformation (Avalos *et al.*, 2005; Borra *et al.*, 2004; Marmorstein, 2004). The reaction starts with the nucleophilic attack of the 1' carbon of the ribose ring leading to the cleavage of nicotinamide from NAD^+ and formation of an active *O*-alkyl amidate (FIGURE 2-7 step i). The rate of this reaction is similar to that of the reverse (step ii), thus to proceed in the next step nicotinamide has to be either quickly released or adopt a conformation that prevents its condensation with *O*-akyl amidate. Nicotinamide can exist in two interchangable conformations, an entrapped or a reactive state. In a reactive state, the pyridine ring is in position to react with the C1' of the *O*-acyl amidate and reform NAD^+, in a reaction known as nicotinamide exchange, leading to a non-productive catalysis (FIGURE 2-7 step I and FIGURE 2-9). In the entrapped state, the pyridine ring establishes contacts in the distal site of the C pocket away from the *O*-acyl-amidate allowing the reaction to proceed. The *O*-acyl-amidate too can adopt two conformations, a contracted and an extended one, which also influences the

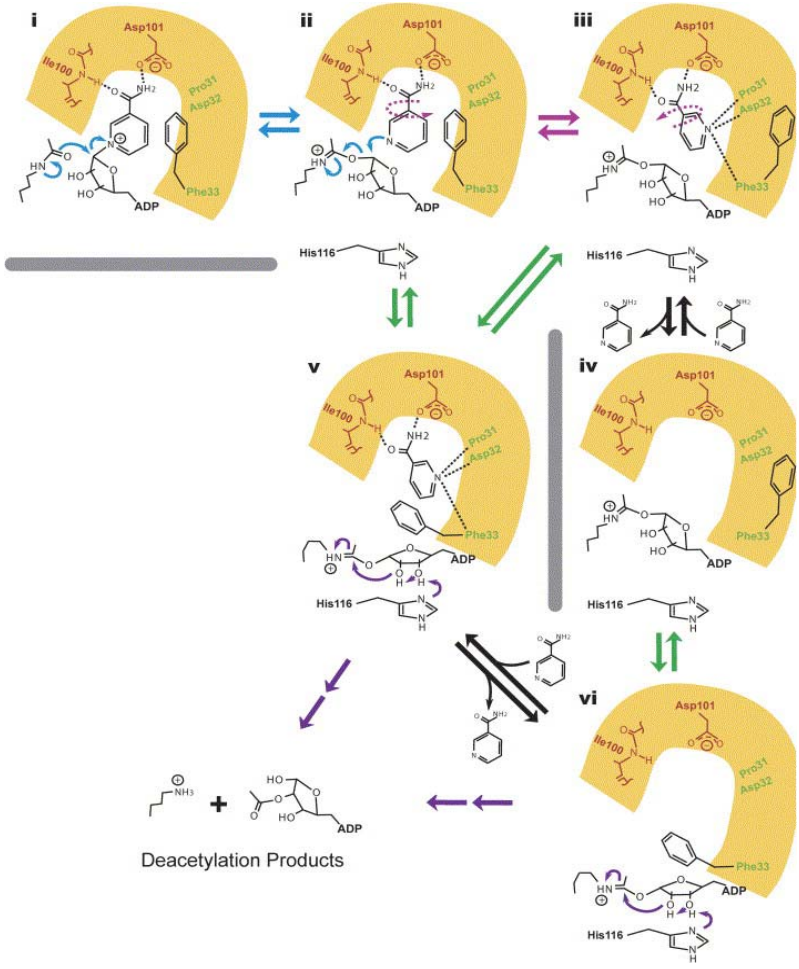


FIGURE 2-7. Mechanism of sirtuin-catalysed deacetylation. *See* text for details.

Adapted from Avalos *et al.*, 2005

outcome of the reaction due to the different relative orientations of the intermediate and a catalytic histidine residue (Avalos *et al.*, 2005). The contracted form favours deacetylation. The ribose ring of the nicotinamide-proximal site of NAD^+ (henceforth: nicotinamide ribose) is then rotated to a position that allows the catalytic histidine to deprotonate the 3' hydroxy group of the nicotinamide ribose eliciting a cascade that leads to the formation of a cyclic acyldioxalane involving the 1' and 2' oxygens of the ribose ring. A water molecule stably positioned by hydrogen-bonding with an asparagine residue then performs the nucleophilic attack of the cyclic acyldioxalane resulting in the production of deacetylated lysine of the protein substrate and a mixture of 2'-*O*-acetyl-ADP-ribose and 3'-*O*-acetyl-ADP-ribose (Jackson *et al.*, 2002). Thus, unlike other HDACs whose enzymatic activity leads to the release of acetate, sirtuins produce two distinct products, nicotinamide and 2',3'-*O*-acetyl-ADP-ribose (FIGURE 2-8).

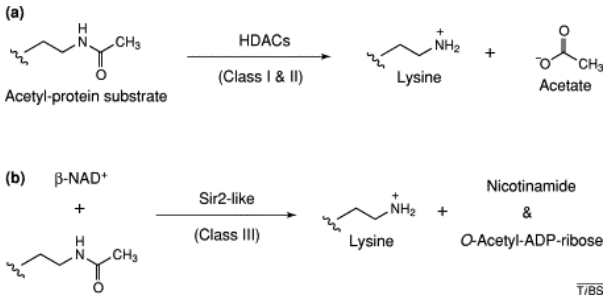


FIGURE 2-8. General reactions catalyzed by known histone and/or protein deacetylases. **(a)** Class I and II histone deacetylases (HDACs) catalyze the hydrolysis of acetyl-lysine side-chains to generate deacetylated lysine and acetate as products. **(b)** Class III, silent information regulator 2 (Sir2)-like deacetylases (e.g. yeast Sir2) require the coenzyme NAD^+ and produce deacetylated lysine, nicotinamide and *O*-acetyl-ADP-ribose as enzymatic products.

Figure and legend adapted from Denu, 2003

This mechanism also provides an explanation as to the inhibitory properties of nicotinamide upon sirtuins. The flexibility of the intradomain loop that contributes to the structure of the C pocket may allow the release of nicotinamide through partial disassembly of the C pocket. If nicotinamide binds to the C pocket while the *O*-acyl-amidate intermediate is there, a base-exchange reaction takes place leading to the re-formation of NAD^+ and preventing the deacetylation reaction (FIGURE 2-9).

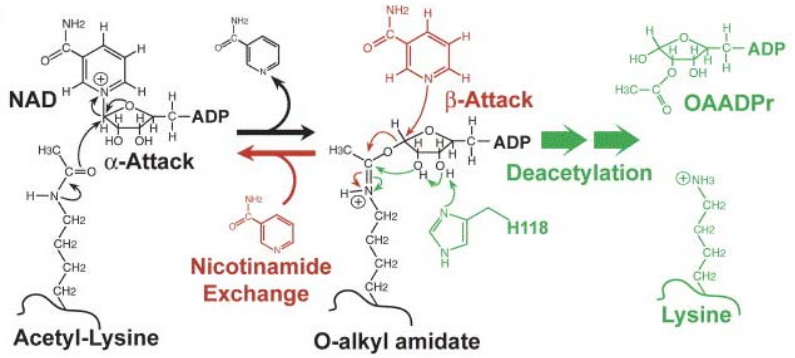


FIGURE 2-9. The nicotinamide exchange reaction. *See* text for details.

Figure and legend adapted from Avalos *et al.*, 2005

2.2.1 Structural insights into the regulation of Hst2p

The crystal structure of Hst2p revealed additional features which provided interesting insights into potential regulatory mechanisms of its enzymatic activity (Zhao *et al.*, 2003a). In particular, the α13 α-helix at the C-terminal extension of the protein folds back to the main body of the protein forming contact with residues within the cleft between the to lobes of the core domain (FIGURE 2-4a). Superimposition of the Hst2p structure with that of Sir2-Af1 revealed that the α13 helix interacts with highly conserved residues that mediate interactions with NAD^+. In addition, the β1-α2 loop forms contacts with NAD^+ in the Sir2-Af1-NAD^+ structure but cannot be modeled in the absense of NAD^+ (Finnin *et al.*, 2001; Avalos *et al.*, 2002; Zhao *et al.*, 2003) most probably due to high atomic temperature factors indicating that this region is highly mobile. These observations imply that by folding into the NAD^+ binding site of the catalytic pocket, the C-terminus of Hst2p precludes NAD^+ binding and thus acts as an autoinhibitory domain. Validation to this theory was provided by the fact that an Hst2p species lacking the α13 helix exhibits a lower K_m value for NAD^+ than the full-length form.

Another insight into the regulation of Hst2p came from the arrangement of Hst2p in the crystal lattice used for the determination of its structure. It indicated a symmetry-related trimeric configuration with the extended N-terminal loop of Hst2p forming contacts with the active site of a neighbouring Hst2p molecule in the lattice (FIGURE 2-10). In particular the N-terminal methionine of Hst2p superimposes well with the acetyl-lysine residue seen in the Sir2-Af2-Ac-p53 peptide structure. This implies that residues within the N-terminal region may influence the catalytic function of the enzyme by interfering with substrate binding. Sendimentation equilibrium experiments confirmed that the N-terminal region mediates trimerisation and that absence of 7 N-terminal amino acids results in enhanced enzymatic activity towards acetylated

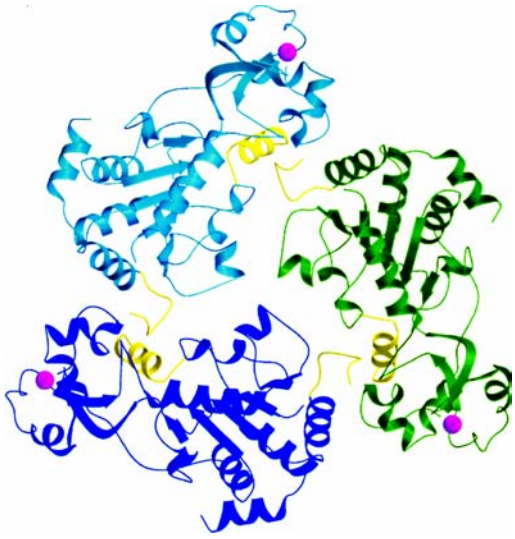


FIGURE 2-10. Structure of the Hst2 trimer. The three subunits of the trimer are shown in blue, aqua and green. The N- and C-terminal extensions that occupy the acetyl-K and NAD^+ binding sites are highlighted in yellow.

Adapted from Marmorstein, 2004

substrates.

The proposed autoinhibitory function of the C-terminus of Hst2p is very reminiscent of the Src kinase regulatory mode where C-terminal extension when phosphorylated on a specific tyrosine residue can bind the SH2 domain of the enzyme and impose an autoinhibitory closed conformation (Harrison, 2003). Thus, as Hst2p shares N- and C-terminal extensions with other members of sirtuins, including mammalian SIRT1, it is possible that the observed structural features reflect true regulatory mechanisms.

2.2.2 Fate of the sirtuin deacetylation products

Apart from the deacetylated lysine, nicotinamide and 2',3'-*O*-ADP-ribose are produced as a result of sirtuin-mediated deacetylation (FIGURE 2-8). The function of these molecules has not been firmly established yet, however some insights have been provided by recent work in the field.

2.2.2.1 Nicotinamide and NAD^+ biosynthesis pathways

Biochemical studies along with the structural data presented above provided good evidence that the involvement of two naturally occuring metabolites in the catalysis, NAD^+ and nicotinamide, may provide a potential mechanism of regulation of sirtuin activity. For this, we shall review the basic pathways of NAD^+ biosynthesis in the cell which will be of importance to understand the underlying principles and significance of the work addressing the function of sirtuins discussed below.

In both procaryotic and eucaryotic cells there are two pathways that participate in NAD^+ production, the *de novo* pathway and the salvage pathway (FIGURE 2-11). The converging molecule of the two pathways is nicotinic acid mononucleotide (NaMN).

In the *de novo* pathway NaMN derives from the degradation of tryptophan in a six-step reaction where the last step is catalysed by quinolinate phosphoribosyl transferase (BNA6/QPT1). In animals this reaction occurs mainly in the liver and kidney. Inadequate dietary supplementation of tryptophan leads to a condition known as pellagra characterised by diarrhea, dermatitis and dementia.

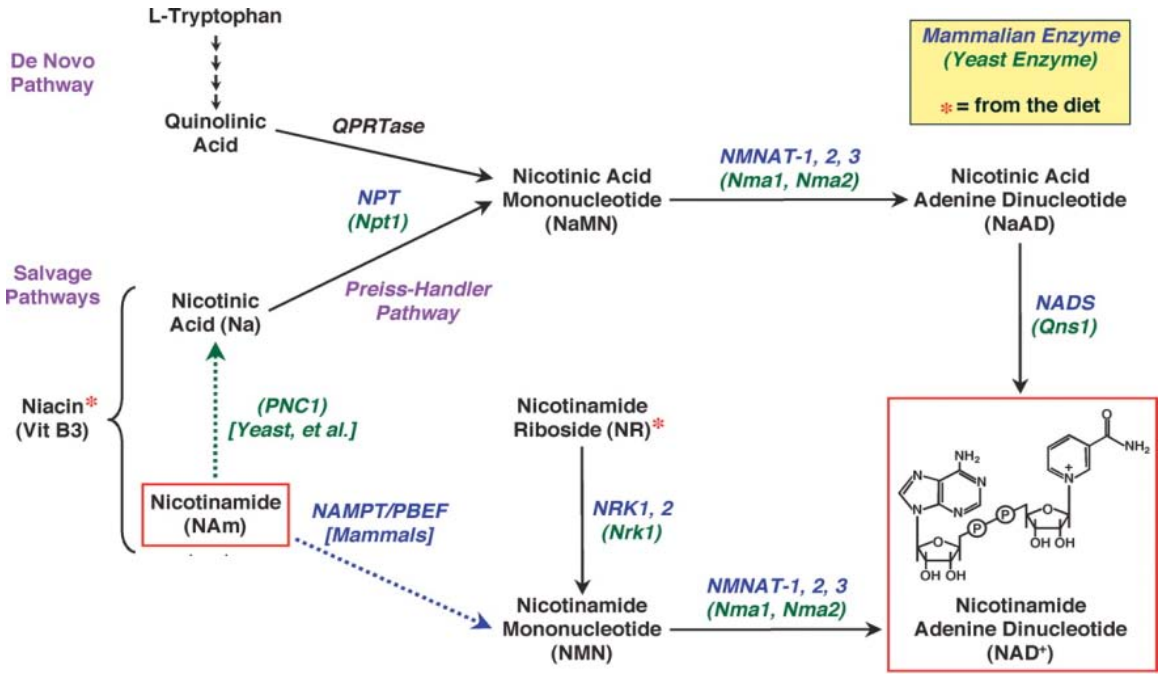


FIGURE 2-11. NAD$^+$ biosynthetic pathways. The biosynthesis of NAD$^+$ occurs through both salvage and de novo pathways. The salvage pathways begin with either nicotinamide or nicotinic acid, collectively referred to as niacin or vitamin B3. One salvage pathway leading from nicotinic acid (Na) to NAD$^+$, known as the Preiss-Handler pathway, goes through two intermediates, nicotinic acid mononucleotide (NaMN) and nicotinic acid adenine dinucleotide (NaAD). A parallel salvage pathway leading from nicotinamide (NAm) to NAD$^+$ goes through one intermediate, nicotinamide mononucleotide (NMN). The *de novo* pathway leads from tryptophan to quinolinate, which connects to the Preiss-Handler salvage pathway through NaMN. Recently, nicotinamide riboside (NR) was also shown to be a precursor for NAD$^+$ synthesis, connecting to the NAm salvage pathway through NMN (Bieganowski and Brenner, 2004).

Figure and legend adapted from Kim et al., 2005.

In the salvage pathway, in a reaction catalysed by nicotinate phosphoribosyl transferase (NPT1), NaMN is synthesised from nicotinic acid (a.k.a. nicotinate or niacin) and 5-phosphoribosyl-α-pyrophosphate (PRPP) which is derived from α-D-ribose-5-phosphate of the pentose phosphate pathway. Nicotinic acid can be derived extracellularly by import through nicotinic acid permease (TNA1). Alternatively it comes from the hydrolysis of nicotinamide (Nam) catalysed by a nicotinamidase encoded by the *PNC1* gene.

NaMN derived from either pathway is converted to desamido-NAD (NaAD) by nicotinate mononucleotide adenylyl transferase encoded by the *NMA1* and/or *NMA2* genes. Finally, an NAD$^+$ synthase encoded by *QNS1* converts NaAD to NAD$^+$. Nicotinamide derived from NAD$^+$ through the action of NAD$^+$ utilising enzymes can then be hydrolysed to give NA completing the salvage pathway circle. In mammals exists a pathway that can use Nam directly for NAD$^+$ synthesis bypassing its conversion to Na. This involves the conversion of

Nam into nicotinamide mononucleotide (NamMN) which is then converted to NAD^+ by the action of the same enzyme that catalyses NAD^+ formation from NaMN (Voet and Voet, **Biochemistry**, Wiley 1995, p825).

NAD^+ is a key molecule in many metabolic, primarily catabolic, processes such as glycolysis and the tricarboxylic acid (TCA) cycle, where it participates as a proton acceptor leading to its reduction to NADH. It is also important in DNA metabolism since enzymes like DNA ligase and poly(ADP) ribose polymerase (PARP) depend on NAD for their activity.

The ratio of NAD^+ to NADH is important for the redox state of the cell and has been shown to flactuate in response to metabolic changes. Accordingly, it can regulate the activity of enzymes such as glyceraldehyde 3-phosphate dehydrogenase in glycolysis and pyruvate dehydrogenase which converts pyruvate to acetyl-CoA that is used in the TCA cycle. Thus, homeostatic mechanisms exist that ensure the constant re-oxidation of NADH to NAD^+ e.g. through the respiratory chain. Nevertheless, limitations in the accurate experimental determination of the intracellular [NAD^+]/[NADH] ratio have fueled an extensive ongoing debate as to the exact quantitation of this ratio and how it fluctuates (Lin and Guarente, 2003).

The relevance of the interaction between NAD^+ metabolic pathways and the function of sirtuins is further exemplified by the recent evidence in the work of Pan *et al.* which revealed a network of synthetic lethal interactions between NPT1 and components of the replication machinery (Pan *et al.*, 2006). In the same study, Hst3 shared the majority of these interactions although it did not exhibit synthetic lethality with Npt1 itself suggesting that they participate in a novel NAD^+-dependent pathway regulating DNA replication in yeast.

Revollo *et al.* found that in mammals the rate-limiting enzyme in NAD^+ biosynthesis is nicotinamide phosphoribosyltransferase (NamPT) (FIGURE 2-11) rather than nicotinamide/nicotinic acid mononucleotide adenylyl transferase (Nmnat) (Revollo *et al.*, 2004). Accordingly, overexpression of NamPT increased cellular NAD^+ levels and this correlated with transcriptional activation of mouse SIRT1 on a heterologous reporter. Furthermore, the transcription profiles induced by overexpression of either NamPT or SIRT1 in MEFs significantly overlapped, indicating that regulation of intracellular NAD^+ by the mammalian salvage pathway also had a potential impact on SIRT1.

Araki *et al.* observed that increased NAD^+ levels due to overxpression of Namnt1 provided neuroprotection depending on SIRT1 (Araki *et al.*, 2004). This work will be discussed in more detail in section 2.3.5.1.7.

Some other aspects of interest in relation to this issue will be presented in the discussion of his thesis (Chapter 5).

2.2.2.2 The function and fate of 2',3'-O-ADP-ribose

Borra *et al.* hypothesised that being a distinctive protein deacetylation reaction product, 2',3'-*O*-acetyl-ADP-ribose may serve either as a second messenger in a manner reminiscent of cAMP, or as a substrate for other proteins. They tested their hypothesis by microinjecting purified *O*-acetyl-ADP-ribose into starfish (*A. miniata*) immature oocytes a system previously used to probe the biological function of proteins compounds. At low mM concentrations, *O*-acetyl-ADP-ribose was able to block oocyte maturation (Borra *et al.*, 2002). In starfish immature oocyte cell-free extracts *O*-acetyl-ADP-ribose is rapidly metabolised suggesting the existence of enzymes that mediate its turnover. Thus it is possible that either *O*-acetyl-ADP-ribose or a metabolite thereof is responsible for the observed effects. The oocyte maturation blockade is recapitulated by injection of recombinant yeast sirtuin Hst2p in a manner dependent on its enzymatic activity. Microinjection of either Hst2p or *O*-acetyl-ADP-ribose into one cell of a two-cell stage embryo also caused a delay in cell cycle progression of the injected cell.

In a subsequent study, Rafty *et al.* identified a class of hydrolases termed Nudix that could robustly catalyse the cleavage of *O*-acetyl-ADP-ribose into AMP and acetylated ribose-5'-phosphate (Rafty *et al.*, 2002). However, in cellular extracts *O*-acetyl-ADP-ribose was subject to modifications inconsistent with Nudix activities an associated with novel unidentified enzymes. Rafty *et al.* predicted the existence of cytoplasmic esterases that hydrolysed *O*-acetyl-ADP-ribose to acetate and ADP-ribose and nuclear *O*-acetyl-ADP-ribose-dependent acetyltransferases.

In a study with exciting implications, Kustatscher *et al.* discovered that the macro domain of histone macroH2A1.1 (mH2A1.1) can bind *O*-acetyl-ADP-ribose through a distinct binding pocket with μM affinity (Kustatscher *et al.*, 2005). mH2A1 is a histone variant found

associated with heterochromatic foci in senescent and quiescent cells. Interestingly, the gene encoding for mH2A1 can give rise to two alternatively spliced variants with distinct spatiotemporal distribution during development. Among these isoforms, only one is able to bind *O*-acetyl-ADP-ribose suggestive of a potential regulatory role for the alternative splicing of this histone and adding another link between NAD^+ and DNA metabolism.

These experiments suggested that the enzymatic products of sirtuins and in particular *O*-acetyl-ADP-ribose may have novel unexplored functions aside the deacetylation of proteins conferred by the enzymes themselves.

2.2.3 Specificity of sirtuins

As for all catalysed reactions, an issue of interest is whether the enzymes involved exhibit substrate specificity and if yes how this is attained. Ultimately, provided enough information as to the associated biological functions, enzymatic specificity may provide important clues for the effective therapeutic targetting of the enzyme in question.

Early studies of sirtuin deacetylation activity showed that the yeast Sir2p may target histones for deacetylation based on the fact that overexpression of Sir2p in yeast leads to histone hypoacetylation. Imai *et al.* provided evidence that Sir2 has preference for residues Ac-K9 and Ac-K14 in histone H3 as well as residue Ac-K16 in histone H4 *in vitro* in agreement to previous data indicating a requirement for these residues for silencing (Imai *et al.*, 2000). Some evidence to support this is also provided by recent studies in mammalian cells that show SIRT1 may deacetylate both histones H3 and H4 with preference to Ac-K9 and Ac-K16 respectively as well as histone H1 Ac-K26 *in vivo* (Vaquero *et al.*, 2004). However, the high degree of phylogenic conservation of sirtuins and the fact that bacteria do not have canonical histones raised the possibility that additional sirtuin deacetylation targets exist, a hypothesis proved to be correct in both prokaryotic and eukaryotic organisms. Despite the emergence of new substrates a consensus deacetylation target sequence failed to emerge.

In order to investigate the issue of substrate specificity for the human sitruin SIRT1, Blander *et al.* performed an unbiased study using oriented peptide libraries (Blander *et al.*, 2005). This approach has been succesfully used before for the identification of protein domain consensus binding sites as well as kinase target sequences. This screen involved the

incubation of a degenerate peptide library containing acetylated lysines with recombinant SIRT1 and the selective purification of deacetylated peptides. This is achieved by reacting the ε-amino group of deacetylated lysine with a photocleavable linker which is tagged with biotin. After removal of the linker, the purified peptides bearing sites specifically targeted by SIRT1 were analysed by Edman degradation. This allowed the determination of the frequency at which specific aminoacids occur in the regions flanking the deacetylated lysine. This approach clearly showed that SIRT1 shows no sequence specificity *in vitro* (Blander *et al.*, 2005). In a subsequent study, Khan *et al.* using yeast Hst2p showed that this sirtuin shows preference for acetylates lysines situated within unstrustured protein regions (Khan *et al.*, 2005).

These approaches imply that sirtuins, unless promiscuous, attain specificity *in vivo* by mechanisms other than recognition of substrate primary sequence. Crystal structures of sirtuins in complex with peptide substrates only are available and show only weak contacts between sirtuin residues and the region surrounding the target lysine. It is not for sure that this applies in the context of the entire protein substrate but if true, it would support the notion that sirtuins may deacetylate their targets specifically by virtue of their localisation, binding partners that act like scaffold proteins or both.

2.2.4 Small molecule modulators of sirtuins

Metabolites of the NAD^+ biosynthetic pathway such as nicotinamide, NADH and NAD^+ itself have been useful tools in preliminary pharmacological studies of sirtuins due to their availability and low cost. However, their prominent role in basic cellular metabolic processes as well as our limited knowledge of mammalian sirtuin biology prompted the search of both activators and inhibitors that could provide sirtuin-specific targeting.

A study by Howitz *et al.* identified a class of compounds called polyphenols as sirtuin activating compounds (STACs). Among them, resveratrol, a molecule found in red wine, exhibited the most potent effects on sirtuins by lowering the K_m of the enzyme for acetylated substrate (Howitz *et al.*, 2003). Given the previously documented effects of resveratrol on neuroprotection, cardioprotection and chemoprotection (Pervaiz, 2003), the study of Howitz *et al.* raised the exhiting possibility that the molecular target of resveratrol is a sirtuin. In a follow-up study, Wood *et al.* could show that resveratrol and other STACs could extend life-

span in *Drosophila* as well as *C. elegans* in a manner dependent on Sir2 and non-additive to caloric restriction (Wood *et al.*, 2004).

However, subsequent studies by Kaeberlein *et al.* and Borra *et al.* challenged these observations (Kaeberlein *et al.* 2005, Borra *et al.* 2005). Kaeberlein *et al.* could find no evidence that resveratrol increased life-span in three different yeast strains, while both studies provided evidence that the observed effect of resveratrol depended on the particular fluorophore attached to the peptide used to assay sirtuin activity. Thus, resveratrol seems to be a substrate-specific activator of sirtuins and whether it truly regulates sirtuins *in vivo* remains to be further investigated especially following recent evidence that enhanced NAD^+ biosynthesis and SIRT1 activity promote neuroprotection in mice (Araki *et al.*, 2004).

Forward chemical genetic approaches have also yielded novel compounds that inhibit sirtuin activity. Forward chemical genetic screens involve assaying of libraries of small organic molecules for their ability to inhibit or enhance a particular phenotype. Grozinger *et al.* assayed a library of 1,600 compounds for their ability to allow expression of a reporter gene from the telomeres that would lead to cell death (Grozinger *et al.*, 2001). Their work yielded three inhibitors, A3, M15 and sirtinol, the latter being the most potent exhibith IC_{50} values of 68 and 38 μM for yeast Sir2p and hSIRT2 respectively.

Bedalov *et al.* used an ameliorated version of this screen in that inhibition of telomeric silencing rescued cell growth, having the advantage that they could eliminate false hits due to cytotoxic compounds (Bedalov *et al.*, 2001). Their study identified 11 out of 6,000 compounds screened to inhibit telomeric silencing. Among these, only 1, splitomicin was subsequently shown in a secondary screen to be capable of inhibiting silencing also at rDNA and mating type loci. Splitomicin exhibits an IC_{50} of 60 μM for yeast Sir2p *in vitro*. Mutagenesis analysis revealed that splitomicin may target the acetylated lysine binding pocket of the enzyme. In a later study, Hirao *et al.* identified dehydrosplitomicin and compound 26, two splitomicin analogues as specific inhibitors of the yeast sirtuins Hst1 and Sir2 respectively (Hirao *et al.*, 2003).

Recently, Solomon *et al.* identified a novel compound in a high-throughput screen using bacterially expressed SIRT1 (Solomon *et al.*, 2006). The compound, named EX-527 inhibits SIRT1 potently with an IC_{50} of 38 nM and is highly specific since its IC_{50} values for

SIRT2 and SIRT3 are in the μM range. This compound was succesfully used for intracellular inhibition of SIRT1 as described in section 2.3.5.1.4.

It is likely that such compounds will be of great use for the study of sirtuin biology, especially when activators/inhibitors targeting other sirtuin homologues are developed (*see* discussion in Chapter 5). The emergence of powerful novel technologies such as RNA interference and gene targeting by homologous recombination allow efficient genetic manipulation of protein expression in mammalian cells. However, RNAi is limited by the protein turnover rates so that a gradual rather than accute protein depletion takes place. Furthermore, the presence of several homologues in a protein family, at times allows the functional compensation of the depleted protein. Small molecule compounds that can accutely interfere with enzymatic activity can offer important tools to overcome such difficulties provided that their specificity is reasonably establised. However, the translation of such compounds into therapeutics will only depend on our understanding of a protein's biological function.

2.3 FUNCTIONAL STUDIES OF SIRTUINS

2.3.1 Sirtuin functions in prokaryotic organisms

2.3.1.1 Bacterial sirtuins

The ability of *Salmonella typhimurium* cobB to compensate cobT mutations in the cobalamin biosynthesis pathway provided the first hints into the biochemical activity of sirtuins (Tsang and Escalante-Semerena, 1998). Interestingly, strains of *Salmonella enterica* that lack the *cobB* gene are unable to utilise propioniate and low acetate concentration as carbon sources because in such strains the acetyl-CoA synthase (ACS) enzymes required to convert these substances into acyl-CoA derivatives are inactive (Starai *et al.*, 2002). ACS from *cobB*$^-$ but not *cobB*$^+$ strains was highly acetylated implying that its enzymatic activity is regulated by acetylation (Starai *et al.*, 2002).

ACS mediates acetyl-CoA production in a two-step mechanism. First, acetate is adenylated to yield acetyl-AMP and subsequently ACS catalyses the formation of a thioester bond to produce acetyl-CoA (Starai *et al.*, 2004). Acetylation of ACS at K609 by the acetyltransferase Pat (protein acetyltransferase) specifically inhibits the ATP-dependent adenylation of acetate but leaves the thioester bond forming activity unaffected (Starai *et al.*, 2004; Starai *et al.*, 2002). Incubation of inactive ACS with NAD^+ and cobB resulted in a dramatic increase of ACS activity suggesting that the deacetylase activity harboured by cobB acts as an activator of ACS (Starai *et al.*, 2002).

Interestingly, ACS K609 is located within a sequence which is highly conserved among AMP-forming enzymes including propionyl-CoA synthase and luciferase (Starai *et al.*, 2002). Despite lack of experimental evidence, it is tempting to speculate for a broader role of acetylation in regulating similar reactions and thus an expanded role of sirtuins in these processes.

2.3.1.2 Archaeal sirtuins

Similar to bacteria, some but not all Archaea do not have histone-like proteins to organise their genome. Instead, this role is performed by small 7-10 kDa proteins (Bernander, 2003). One such protein known as Sso10b and later renamed Alba (acetylation lowers binding affinity) which binds to DNA as a dimer, was identified as a interaction partner of the sirtuin orthologue from *Sulfolobus solfataricus* ssSir2.

Alba was shown to be acetylated at K16 by an archaeal orthologue of Pat acetyltransferase (Marsh *et al.*, 2005) which results in lower affinity for DNA (Bell *et al.*, 2002). This was further supported by the crystal structure of Alba and molecular modeling that revealed a role of K16 in the binding of the protein to DNA (Wardleworth *et al.* 2002; Zhao *et al.*, 2003). ssSir2-mediated deacetylation induces the ability of Alba to mediate silencing in an *in vitro* reconstituted transcription system, an attribute that correlates with the ability of Alba to bind DNA (Bell *et al.*, 2002; Marsh *et al.*, 2005).

These data indicated that archaeal sirtuins may regulate gene transcription by modulating the ability of proteins to bind DNA. Furthermore, as the chromosomal DNA of *Sulfolobus* is differently organised in exponentially growing *versus* stationary phase cells, the regulation of Alba acetylation by ssSir2 may have a broader role in genome organisation (Bernander, 2003).

2.3.2 Sirtuin functions in *S. cerevisiae*

2.3.2.1 Regulation of chromatin silencing in *S. cerevisiae* by sirtuins

2.3.2.1.1 Silencing at mating type loci

Two types of haploid cells are known in the yeast *Saccharomyces cerevisiae*, denoted **a** and α. These cell types are determined by the selective expression of the mating type locus (*MAT*) alleles *MAT***a** and *MAT*α which encode transcriptional regulators that dictate the expression of gene sets specific for each type.

Certain yeast strains known as homothallic are able to interconvert between the two cell types. These strains harbor an active *HO* gene encoding a site-specific endonuclease

that cleaves the mating locus. Such interconversion also requires two more genes namely *HMR* and *HML* which in most yeast strains encode cryptic copies of the entire *MAT***a** and *MAT*α loci respectively (and in other strains, mostly natural isolates, the inverse configuration). These loci act as sequence templates in the interconversion between the *MAT***a** and *MAT*α alleles: upon cleavage of the *MAT* locus by the HO endonuclease, they provide the template for copying the *MAT* allele that replaces the original allele in the *MAT* locus.

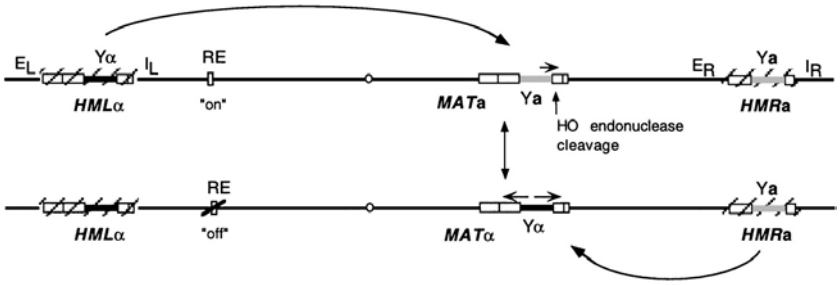


FIGURE 2-12. Mating type loci on S. cerevisiae chromosome III. *See* text for details.

Adapted from Haber, 1998

The *HMR* and *HML* loci are found in the proximity of the telomeres in the same chromosome as the *MAT* locus and remain silenced due to their location within the genome that adopts a heterochromatic structure. Heterochromatin formation at the *HMR* and *HML* loci is dictated by the flanking DNA sequences (E_L, I_L, E_R, I_R, FIGURE 2-12) which are responsible for the recruitement of *trans*-acting factors required for silencing. These sequences have properties of autonomously replicating sequences (ARS) when placed in plasmids but only HMR-E and HMR-I are shown to be *bona fide* origins of replication in the chromosome.

Genetic screens to identify determinants of silencing in the *HML* and *HMR* loci led to the cloning of several genes whose mutations resulted in activation of silent mating gene expression: *RAP1*, origin recognition complex (*ORC1*) and *ABF1* and four genes termed ***SIR1-4*** for Silence Information Regulator.

The *HMR-E* silencer is a 140-bp DNA sequence that harbours binding sites for the gene products of *RAP1*, *ORC1* and *ABF1*, Rap1p, Orc1p and Abf1p respectively. Interestingly, none of these proteins is specific to silencer regions. Rap1p and Abf1p are found in many promoter regions where they stimulate transcription while ORC1 is found in origins of replication throughout the genome. However there is evidence that the silencing functions of these proteins may by separable from the others. For example the N-terminus of Orc1p is not required for replication but it is required for silencing (Bell *et al.*, 1995). Mutations in any two of the three binding sites of these proteins suffices to abolish silencing while a single

copy of any of these binding sites is insufficient to establish silenced chromatin. Conversely, the Sir proteins are devoid of direct DNA binding ability. Thus it is thought that the combined binding of Rap1p, Orc1p and Abf1p to the silencer creates a platform for the recruitment of Sir proteins by means of protein-protein interactions.

Isolation of native Sir complexes from *S. cerevisiae* showed that while Sir2p and Sir4p form a soluble complex, Sir3p fails to co-purify in stoichiometric amounts. Furthermore, Sir3p and Sir4p bind preferentially to the hypoacetylated tails of histone H3 and histone H4 *in vitro* and Sir mutations that disrupt these interactions abolish silencing (Hecht *et al.*,1995). Mutational analyses also showed that the regions of histones H3 and H4 required for Sir3p and Sir4p binding are also required for silencing. Importantly, genomic regions occupied by Sir complexes overlap with the location of hypoacetylated histones suggesting a tight connection between these two phenomena.

The initiating step in the establishment of Sir-dependent chromatin silencing is the binding of Sir1p to the silencer. Sir1p binds directly to Orc1p and is confined to the silencer region without spreading adjacently. Tethering of Sir1p to silencers by a heterologous DNA binding domain can support the establishment of silenced chromatin in the absence of ORC or Rap1p. Both Sir3p and Sir4p can bind to Rap1 and the Sir3p-Rap1p interaction can also occur *in vitro* in the absense of other yeast proteins (Moretti *et al.*, 1994). Sir4p is recruited to the silencer through its interaction with Sir1p/Rap1p independently of Sir2p and Sir3p. Thus Sir2p is most likely recruited to the silencer in complex with Sir4p. Sir3p is engaged in the complex through its interactions with Sir4p and Rap1p.

Once located to the silencer the Sir complex spreads towards the gene to be silenced. Sir2p and Sir3p are essential for this. The histone deacetylase activity of Sir2p which is dispensable for the recruitment of the complex to the silencer is required for this function. Upon its recruitment to the silencer Sir2p is brought in the proximity of acetylated histones H3 and H4 in the nearby nucleosome which it deacetylates creating high affinity sites for more Sir3p and Sir4p. In turn, this allows the sequential binding of more Sir2 complexes allowing their spreading along with the consequent deacetylation of histones. Thus chromatin loaded with Sir complexes adopts a compact structure which renders it silent.

2.3.2.1.2 Chromatin silencing at telomeres

Sir complexes also participate in another site of silencing in yeast at telomeres. This phenomenon, reminiscent of position-effect variegation is known as telomere position effect (TPE) and refers to the silencing of genes in the proximity of telomeres. Telomeric silencing shares many of the features of silencing at mating-type loci yet it differs in that it is independent of Sir1p and that recruitment of the Sir complex in yeast telomeric repeats is mediated by both Rap1p and a protein known as Hdf1 or yKu70 (Guarente, 1999).

Luo *et al.* found that Sir4p can bind to Rap1p independent of other Sir proteins or the Ku complex in the telomeric region proximal to the chromosome end (Luo *et al.*, 2002). The reverse is true as the distance from chromosome ends increases suggesting that as in the case of mating type loci, the Rap1p-Sir4p complex participates in the seeding of the silenced chromatin at the telomeres. A schematic model of the proposed mechanism for the establishment of silencing at yeast telomeres is shown in Figure 2-13.

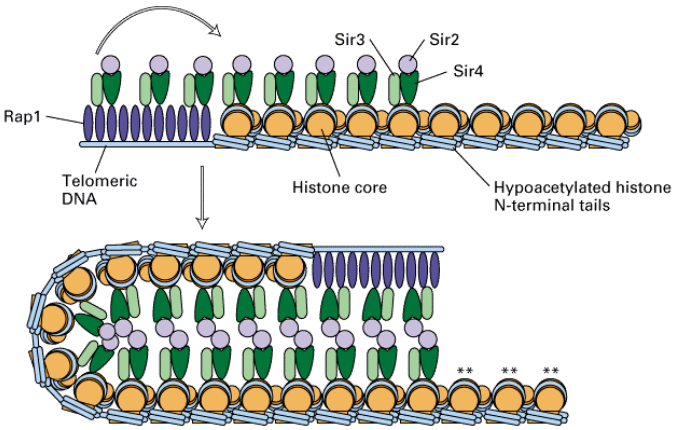


FIGURE 2-13. Schematic model of Sir-mediated silencing mechanism at yeast telomeres. Multiple copies of Rap1 bind to a simple repeated sequence at each telomere region, which lacks nucleosomes (top). This nucleates the assembly of a multiprotein complex (bottom) through protein-protein interactions between Rap1, Sir2, Sir3, Sir4, and the hypoacetylated amino-terminal tails of histones H3 and H4 of nearby nucleosomes. Asterisks represent hyperacetylated histone amino-terminal tails. The heterochromatin structure encompasses ~4 kb of DNA neighboring the Rap1-binding sites, irrespective of its sequence. The actual structure of the higher-order heterochromatin is not yet understood.

Adapted from Grunstein, 1997

Hediger *et al.* used live imaging of yeast telomeres to unveil another function of the Sir proteins at telomeres. They found that Ku and Sir proteins share a redudant role in telomere anchoring to the nuclear periphery (Hediger *et al.*, 2002). Telomere anchoring is thought to be important for the establishment of epigenetic states. Both Ku and Sir support telomere anchoring in the absence of eachother, but while Ku's contribution spans the entire cell cycle, that of Sirs is limited to the S-phase: In the absence of Ku, telomeres are less efficiently

tethered to the nuclear periphery during G1. This work provided evidence for a mechanism that explains the juxtaposition of silenced chromatin in distinct sub-nuclear compartments.

The Ku complex is phylogenetically conserved from bacteria to humans (Daley *et al.*, 2005). It comprises two subunits, termed Ku70 and Ku80. Its best known functions are in non-homologous end joining (NHEJ), V(D)J recombination and DNA damage repair where it participates in the recognition of double-strand DNA breaks and serves to recruit the appropriate protein complexes such as DNA ligase that fulfill the corresponding functions (Boulton and Jackson, 1998).

The work of Tsukamoto *et al.* identified Sir4 as a binding partner of yKu70 in a yeast two-hybrid screen. They could show that in accordance to the previously established roles of the Sir proteins that Sir2p, Sir3p and Sir4p but not Sir1 participate in the illegitimate recombination, end-joining and double-strand break repair pathways (Tsukamoto *et al.*, 1997). Mutations in the corresponding genes confer higher sensitivity to genotoxic stresses such as ionising irradiation. Interestingly, upon DNA damage, both Sir and Ku proteins re-localise from subtelomeric foci to sites of DNA damage albeit with different kinetics providing support for the notion that subtelomeric chromatin serves as a reservoir for proteins that are relocated to specific nuclear sites upon physiological stimuli (Martin *et al.*, 1999).

With regards to the role of the Sir complex in DNA damage repair, it has been suggested that its recruitment at sites of DNA damage induces a heterochromatin-like structure which may prevent the transcriptional and replication machineries from accessing the site until it has been repaired. Alternatively, the localised compact state of chromatin may facilitate the re-joining of the damaged DNA (Critchlow and Jackson, 1998).

2.3.2.1.3 Chromatin silencing at the rDNA locus

Another site where silencing holds important roles in the yeast genome is the loci that encode ribosomal RNAs (rDNA). The rDNA locus has a repetitive nature in that it comprises a 9.1 kb sequence that is tandemly repeated 100-200 times. Each rDNA repeat encodes the 35S RNA which is transcribed by RNAPolI and serves as the precursor for the 25S, 18S and 5.8S rRNAs and the RNAPolIII-transcribed 5S rRNA. These genes are separated by non-transcribed spacer regions. Sir2p was shown to be more highly associated with these spacer

regions than with the 35S coding region and this gave rise to the as yet unproven notion that a silencer sequence similar to the one found in mating type loci is also present therein. Several RNA PolII-transcribed genes when introduced within the rDNA repeats are silenced (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997).

Unlike mating type and telomeric loci, *Sir3 and Sir4* are not required for silensing at the rDNA loci. Rather an entirely separate Sir2p-containing complex called RENT (for regulator of nucleolar silencing and telophase) is required. The RENT complex was identifed by two groups using distinct starting points for their investigation. Seeking to understand the mechanism of rDNA silensing, Straight *et al.* compared gel patterns of proteins which interact with GST-Sir2 *vs.* GST-Sir4 C-terminal fragment fusions to identify Sir4p components that are specific to Sir2p. They found a protein with an apparent mass of 175 kDa which they termed Net1 by virtue of its previously given names (*NUS1*-nucleolar silencing protein, *ESC5*-establishes silent chromatin and *TUB2*-telophase arrest bypassed) (Straight *et al.*, 1999).

Shou *et al.* also identified Net1p in a genetic screen for proteins that allow a yeast strain bearing a deletion of the *cdc15* gene and arrests in telophase to survive (Shou *et al.*, 1999). *Cdc15* encodes a protein kinase which is part of a signalling pathway known as the mitotic exit network (MEN). The MEN is required for proper exit from mitosis primarily through its ability to inactivate mitotic cyclin-dependent kinases. Its function is to induce and maintain the release of the phosphatase Cdc14p from the nucleolus to the cytoplasm at anaphase and beyond during which Cdc14p dephosphorylates and thus inactivates mitotic kinases allowing cells to enter the next G1. To understand the mechanism underlying the genetic interaction between Cdc14p and Net1p, Shou *et al.* used immunoaffinity purification to identify binding partners of Net1. Among the proteins identified were Cdc14p and Sir2p (Shou *et al.*, 1999). Visintin *et al.* also indepedently identified Net1p as a Cdc14p partner in a yeast two-hybrid screen (Visintin *et al.*, 1999).

Net1 was shown to be required for normal growth and rDNA silencing. In agreement to this, chromatin immunoprecipitation experiments showed that Net1 is associated with rDNA loci independent of Sir2p. Conversely, Sir2p binding to rDNA requires the presence of Net1. Thus Net1 seems to be the protein that recruits Sir2p to rDNA consistent with the absence of Sir4 from these loci. At the same time Cdc14p also binds to the rDNA loci in a Net1-dependent manner (Straight *et al.*, 1999). The binding of Sir2p, Net1p and Cdc14p to rDNA

coincides with their localisation to nucleoli throughout the cell cycle until the anaphase/telophase transition where both Sir2p and Cdc14p are released to the cytoplasm. The release of Cdc14p from the nucleoli coincides with its activation because when bound to Net1p its phosphatase activity is inhibited.

In strict terms, the observations above do not prove the existence of a ternary complex comprising Sir2p, Net1p and Cdc14p. Indeed, Shou *et al.* even demonstrated that loss of rDNA silencing does not bypass lethality caused by a mutation in *tem1* whose product is an activator of the MEN. This implies two separable functions for Net1p/Cdc14p and Net1p/Sir2p complexes. Sequential co-immunoprecipitation experiments, mutational analysis on Net1p in an attempt to dissect these two functions in rDNA silencing and mitotic exit as well as genetic analysis of the interaction between MEN and Sir2 would be required to firmly establish the interconnectivities between these pathways.

2.3.2.2 Regulation of meiotic checkpoint function and recombination

Aside its function in rDNA silencing as part of the RENT complex, the localisation of Sir2p in the nucleolus also functions in securing faithful meiotic progression (San-Segundo and Roeder, 1999). Proper synapsis of homologous chromosomes during meiosis is guarenteed by checkpoint systems. Mutations in meiotic checkpoint genes such as *zip1* cause cells to arrest in the pachytene phase of meiosis I (Sym *et al.*, 1993). Pch2p is a protein identified in a genetic screen for mutants that can bypass the pachytene checkpoint elicited by *zip1* mutation. *zip1-pch2* double mutants sporulate and undergo mitotic recombination but the haploid progeny show poor viability due to chromosome missegregation.

The proper function of Pch2p requires its localisation to the nucleolus, an event which is dependent on Sir2p. In the absence of Sir2p, Pch2p is localised to the nucleoplasm and these cells exhibit high rates of recombination in the rDNA locus similar to cells defective for Pch2p alone. This may arise due to the increased presence of the meiotic recombination protein Hop1p in the nucleolus.

The exact role of Sir2p in meiotic checkpoint function is not understood but two pieces of experimental evidence suggest that it may contribute through its role in the establishment of the structural characteristics of silenced chromatin (San-Segundo and Roeder, 1999). Firstly, in

a strain carrying deletion of the rDNA array with the essential rDNA genes provided as an extrachromosomal plasmid, Pch2p still localises to nucleoli-like structures but is defective in its meitotic checkpoint function. In this strain, mutation in *sir3* induces the relocalisation of Pch2p and Sir2p to the telomeres and partially restores checkpoint function. A second experiment showed that in the presence of intact rDNA, overexpression of Sir4p induces the relocalisation of Pch2p to telomeres but only partially abolishes meiotic checkpoint function and this is depedent on *sir2*. This evidence sustains the notion that the telomeric locus provides some element similar to the rDNA arrays that supports the meiotic checkpoint function of Pch2p.

Interestingly, mutations in another pachytene checkpoint protein Dot1 (a.k.a. Pch1) disrupt Pch2 and Sir2 localisation from the nucleoli (San-Segundo and Roeder, 2000). Dot1 is a distinct H3 histone methyltransferase which lacks the charactersitic SET domain of other lysine methyltransferases and can trimethylate H3-K79 (Vaquero *et al.*, 2003). This implies a possible interconnection between histone methylation and Sir-mediated silencing which has not been experimentally addressed further yet.

2.3.2.3 Regulation of DNA replication

Given the association of Sir2p with ORC, it is not surprising that recently a novel role for Sir2p in the control of replication has emerged (Pappas *et al.*, 2004). Replication of DNA takes place during the S-phase of the cell cycle. However, assembly of pre-replication complexes (pre-RC) at origins of replication occurs as early as at the anaphase of the previous cell cycle. Cdc6p orchestrates pre-RC assembly by initiating recruitment of its components such as the MCM (minichromosome maintainance) helicase to ORC at the origins of replication. The temperature-sensitive *cdc6-4* mutant is lethal due to its inability to assemble pre-RCs.

A genetic screen for rescue of this phenotype identified mutations in *sir2* and to a lesser degree *sir3* and *sir4* as potential candidates. Loss of *sir2* was shown to supress the lethality of other replication initiation mutants, notably *orc-5* and *mcm2*, but not that of genes acting at steps subsequent to pre-RC assembly such as the kinase and DNA polymerase α-encoding genes *cdc7* and *cdc17* respectively. Subsequently, chromatin immunoprecipitation

experiments showed that *Sir2p* deletion restored the loading of MCM2 helicase to origins albeit to a different extend depending on the ARS tested.

The exact mechanism by which Sir2p inhibits replication is unclear. Interestingly, deletion of two other deacetylases, Hda1p and Rpd3p does not phenocopy the *sir2* deletion suggesting that the observed effects on pre-RC assembly are specific to Sir2p. It has been demonstrated that targeted increase of acetylation near ARS induces earlier activation of the affected originsshowing increased recruitment of Cdc45p, a temporal marker for the initiation of DNA synthesis (Vogelauer *et al.*, 2002). Thus sir2 may inhibit replication by deacetylating histones at ARS, deacetylate a replication complex component or even regulate the transcription of a gene required for replication.

2.3.2.4 Sir2 and the regulation of life-span in *S. cerevisiae*

2.3.2.4.1 Molecular mechanisms that determine life-span in *S. cerevisiae*

Yeast cells multiply by the budding of a daughter cell from the mother cell. Cell division is asymmetrical in that daughter cells are smaller and consist mainly of newly synthesised molecules including cell wall components and proteins. Ageing in yeast is associated with disinct phenotypes, namely increase in size, sterility and cell surface deterioration (Sinclair *et al.*, 1998). The "ageing" of the mother cell can be followed microscopically by removing the daughter cell away each generation. Also, because budding leaves a scar on the surface of the mother cell, counting of the bud scars can be used to define a yeasst cell's age. Thus, it was shown that each yeast cell divides by and large a fixed number of times, approximately 20 (Sinclair *et al.*, 1998) indicating that there exists a genetic component underlying life-span determination.

An important work that shed light in the process of ageing in yeast was provided by Kennedy *et al.* (Kennedy *et al.*, 1994) who found that yeast daughter cell derived from old mothers exhibited limited life-span. This suggested that a factor may be present in the mother cells that accumulates after each cell division and can be transmitted in a stochastic manner to the daughter cells at increased propensities as the cell ages. Interestingly, subsequent generations of these daughter cells did not inherit the decreased life-span

phenotype suggesting that it did not derive from genomic mutations (Kennedy *et al.*, 1994). Another major discovery came from a genetic screen by Kennedy *et al.* based on the fact that genes conferring stress resistance also associated with increased longevity (Kennedy *et al.*, 1995). Among other genes they isolated *SIR4-42* a mutant of the Sir4 gene that conferred a 45% increase in life-span. The mutation comprised a truncation at the C-terminus that prevented the assembly of the Sir complex at the mating type and telomeric loci because it is required for the interaction of Sir4p with Rap1p. However, deletion of *Sir4*, while abolishing mating type locus and telomeric silencing, it also decreases life-span suggesting that the *SIR4-42* mutant acted as dominant for some functions. It was subsequenlty shown that in *SIR4-42* strains Sir3p and Sir4p re-localise to nucleoli while this phenomenon is also observed in old yeast cells (Kennedy *et al.*, 1997). This observation provided an explanation as to why old yeast cells are sterile: relocalisation of the Sir complex alleviates silencing in the mating type loci allowing the expression of both a- and α-specific genes. These data further suggested a tight link between gene silencing and ageing.

Kaeberlein *et al.* (Kaeberlein *et al.*, 1999) discovered that *Sir3* and *Sir4* deletions decreased life-span due to elimination of silencing at the mating type loci which indirectly caused increased levels of rDNA recombination. High rDNA recombination is detrimental to the yeast cell because it leads to the excision of rDNA locus fragments that form extrachromosomal rDNA circles (ERCs) (Sinclair and Guarente, 1997). ERCs contain ARS which drive ERC replication in each cell cycle (Figure 2-14). Thus after each division, mother cells accumulate ERCs which are thought to titrate away essential components for the replication of the genome eventually leading to the

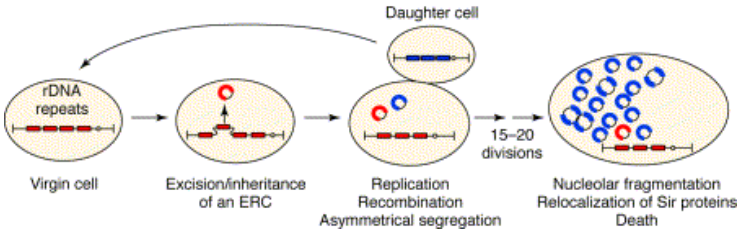


FIGURE 2-14. The ERC model of yeast aging. In young yeast cells, the rDNA locus comprises 100–200 tandem copies of a 9.1 kb repeat (red rectangles) and is located within a compact nucleolus. Homologous recombination between rDNA repeats results in the formation of an extrachromosomal rDNA circular molecule, or ERC (red circle). ERCs can replicate via the ARS elements in each repeat (ERCs derived from the first ERC are shown in blue). Asymmetrical segregation of ERCs results in their exponential accumulation in mother cells. Daughters rarely inherit ERCs and regenerate their rDNA array, perhaps by gene conversion. The rate of ERC accumulation is such that after 15 divisions following ERC excision, the total DNA content of ERCs may equal that of the total yeast genome. Cell death occurs by titration of either vital replication or transcription components.

Figure and legend adapted from Sinclair, 1998

inability of the cell to divide further. The localisation of Sir2p to the nucleolus at sites of rDNA loci, suppresses the recombination that gives rise to ERCs, allowing yeast cells to divide more times. Indeed, a second copy of *Sir2* suffices to confer an increase of up to 30% in the replicative life-span of yeast (Kaeberlein *et al.*, 1999).

Interestingly, Aguilaniu *et al.* provided a novel mechanism by which Sir2p may increase life span. Their starting point was the fact that protein carbonylation, an irreversible effect of oxidative damage, accumulates with replicative age (Aguilaniu *et al.*, 2003). Furthermore, carbonylated proteins are preferentially segregated in the mother cells during cytokinesis. However, mother cells bearing a *Sir2* deletion failed to retain carbonylated proteins suggesting that Sir2p protects progeny cells from oxidative damage by dictating the retention of oxidatively damaged cellular components at the mother cell.

2.3.2.4.2 Regulation of life-span by caloric restriction

Given that limitation of food uptake in mammals and rodents had been shown to result in increased longevity, Lin *et al.* tested whether caloric restriction in yeast could also extend its replicative life-span. Limiting the glucose content in the growth medium from 2.0% to 0.5% resulted in an increase of average life-span from 21.2 to 26.2 generations (Lin *et al.*, 2000). Glucose activates the cAMP/PKA pathway and mutations in its components (e.g. the GDP/GTP exchange factor Cdc25) or in enzymes that are involved in glucose metabolism such as hexokinase also exhibit increased life-spans (FIGURE 2-15). Thus caloric restriction also seems to positively influence replicative life-span in yeast.

Interestingly, caloric restriction does not increase the life-span of yeast cells carrying mutations in the *Sir2* gene. Similarly, mutation of the *Npt1* gene which is involved in the salvage pathway of NAD^+ biosynthesis also abolishes the beneficial effects of caloric restriction. In agreement to the known function of Sir2p in limiting rDNA recombination, cells carrying mutation in the *cdc25* gene to mimic caloric restriction show decreased levels of recombination of a reporter plasmid concomitant to reduced ERC levels. Subsequent studies also demostrated that calorically restricted yeast exhibits enhanced silencing (Lin *et al.*, 2002).

From these results it was inferred that the enzymatic activity of Sir2p is required for the extension of life-span due to limitation of nutrients. This however raised the question as to how caloric restriction regulates Sir2p activity.

2.3.2.4.3 Proposed mechanisms of Sir2p-mediated life-span extension by caloric restriction in *S. cerevisiae*

The utilisation of glucose as a carbon source in yeast depends on its abundance in the growth environment. When glucose is unlimited, pyruvate is directed to the fermentation pathway which yields only 2 ATP molecules. When glucose is scarce, pyruvate is shunted to the tricarboxylic acid cycle (TCA) and respiration to yield 28 molecules of ATP per glucose molecule.

Lin *et al.* showed that in reduced glucose growth conditions respiration is increased (Lin *et al.*, 2002). To determine whether respiration mediates the effects of caloric restriction on longevity they deleted the gene encoding cytochrome c1 (*CYT1*) to impede the function of the mitochondrial respiratory chain. Such cells were not responsive to caloric restriction. Hap4p is a transcription factor responsible for the switch from fermentation to respiration through activation of many respiratory chain genes.

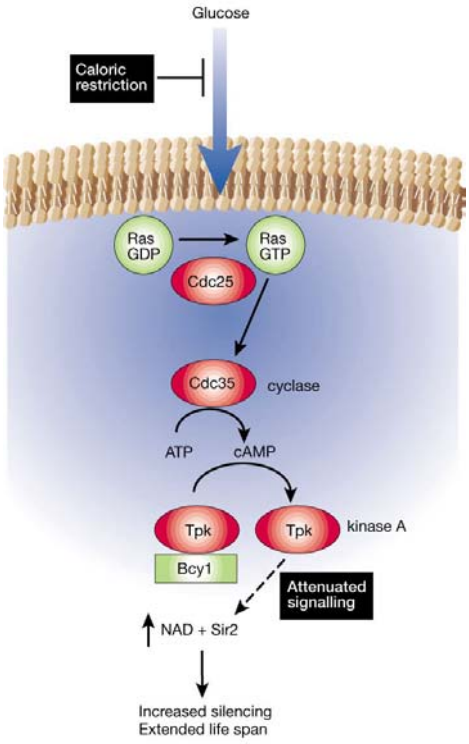


FIGURE 2-15. Glucose sensing signaling pathway in *S. cerevisiae*. The carbon source glucose stimulates a signal transduction pathway including a Ras GTP-binding protein, a GTP/GDP exchange factor, an adenylate cyclase, and cAMP-dependent protein kinase A (PKA). Without cyclic AMP, PKA is in a complex with the inhibitor protein Bcy1. Signalling is attenuated either by lowering the glucose levels in the media (caloric restriction) or by mutating genes that encode components of the pathway (for example, Cdc25, Cdc35 or tyrosine protein kinase (Trk), shown in red). This reduction in signalling leads to an increase in silencing by Sir2 and its NAD cofactor and an extended life span.

Adapted from Guarente and Kenyon, 2000

Yeast cells overexpressing Hap4p show increased respiration expressed as increased oxygen consumption, have approx. 35% increased life-span while exhibiting enhanced rDNA silencing in a manner depending on Sir2p. These effects were dependent on an intact respiratory chain and were

not additive to those elicited by low glucose growth conditions. Furthermore, it is possible that Hap4p overxpression resulted in increased resistance to ROS, This was however disproven by the fact that these cells exhibited similar sensitivities to oxidising agents and mRNA levels of antioxidant enzymes. Based on these observations, Lin *et al.* proposed that the effects of caloric restriction on Sir2 may be either due to the reduced carbon flow through glycolysis because of the elevated ATP levels and/or due to increased NAD^+/NADH ratio because of increased rates of NADH oxidation. Nevertheless both proposals remained experimentally unproven.

Anderson *et al.* addressed this issue by creating an NAD^+-reporter strain. They exploited the fact that NadR protein from *Salmonella typhimurium* binds to a DNA sequence called the NAD box in a manner that depends on NAD^+ concentration. Their strain expresses a fusion of NadR fragment and the transcriptional activation domain of GAL4 so that the fusion activates transcription of a gene required for survival under the control of UAS in an NAD^+-dependent way (Anderson *et al.*, 2003a). Thus fluctuations of intracellular NAD^+ levels directly correlate with viability. Using this system they found that upon caloric restriction their reporter line grew slower because of low NAD^+ because the phenotype could be rescued with acetaldehyde which induces NADH oxidation and presumably increases the NAD^+ pool. Furthermore, using highly-sensitive ^{13}C NMR spectroscopy they could show that the intracellular pool of NAD^+ is not greatly altered by caloric restriction. *In vitro* deacetylation assays also showed that neither Sir2p nor SIRT1 activity are altered by NADH when concentrations within the physiological range of NAD^+/NADH were used. Finally, depletion of NADH by treatment with acetaldehyde did not alter silencing of rDNA reporters suggesting that fluctuation of NADH levels are unlikely to affect Sir2 activity.

In a subsequent study, Anderson *et al.* investigated whether the natural sirtuin inhibitor nicotinamide can alter cellular Sir2p activity. They could show that increased levels of Npt1, an enzyme in the NAD^+ salvage pathway, suffices to induce life-span extension associated with increased silencing and rDNA stability (Anderson *et al.*, 2003b). At the same time steady-state NAD^+ levels were unaltered indicating that the observed effects were not due to increased NAD^+. Because the above were true for most of the enzymes involved in the NAD^+ salvage pathway, they concluded that increased flux through this pathway suffices to elicit life-span extension. In a follow-up study they investigated whether nicotinamide affects Sir2p

activity. Nicotinamide is not only an intermediate in the NAD^+ salvage pathway but also one of the products of the sirtuin-catalysed deacetylation reaction. Nicotinamide was found to be a non-competitive inhibitor of both Sir2p and SIRT1 and decreased the life-span of wild type yeast cells concomitant to inducing recombination of rDNA arrays and inhibiting silencing.

To address whether nicotinamide is important in determining life-span increase upon caloric restriction, Anderson *et al.* showed that caloric restriction could not extend the life-span of yeast lacking *Pnc1* (Anderson *et al.*, 2003b). Interestingly, caloric restriction as well as other stress conditions that are known to increase life-span such as heat shock, osmotic stress and amino-acid starvation are all potent inducers of Pnc1p protein levels. Increased Pnc1p activity could increase Sir2p activity by stimulating NAD^+ synthesis. This is unlikely to be the case since supplementation of nicotinic acid, the product of the reaction catalysed by Pnc1p did not increase gene silencing at rDNA. Furthermore, deletion of Npt1p, a protein that feeds nicotinic acid to the NAD^+ salvage pathway downstream of Pnc1p, did not affect rDNA silencing when intracellular NAD^+ levels were restored suggesting that Pnc1p can increase Sir2p activity in the absence of an intact NAD^+ salvage pathway. Finally, Anderson *et al.* could show that reduction of nicotinamide levels by driving its excretion had a positive impact on rDNA silencing as well as life-span.

Lin *et al.* then argued that the effects of caloric restriction are actually exerted through modulation of the levels of NADH (Lin *et al.*, 2004). They could show that caloric restriction decreases NADH levels while it leaves NAD^+ levels unaffected. This was dependent on an intact mitochondrial respiratory chain and could also be observed in cells with genetically increased respiration *via* the overexpression of the transcription factor Hap4p. Furthermore, NADH levels decreased upon caloric restriction indepedently of the presence of Sir2p. *In vitro* analysis of the effects of NADH on Sir2p as well as human SIRT1 showed that NADH can act as a competitive inhibitor of both enzymes' catalytic activities.

In order to examine whether this is applicable *in vivo*, Lin *et al.* overexpressed two NADH dehydrogenases to induce a decrease of cellular NADH (Lin *et al.*, 2004). These cells exhibited increased life-span in the presence of 2% glucose similar to wildtype cells under caloric restriction (0.5% glucose) and this life-span extension was not further enhanced by lowering glucose levels. Finally, they overexpressed Nnt1p to reduce the levels of nicotinamide and found that caloric restriction would still increase life-span in a mutant *Pnc1*

background suggesting. The authors interpreted this as an indication that in mutant *Pnc1* strains, elevated nicotinamide levels mask the beneficial effects of decreased NADH. However it is important to note that this conclusion is the result of alternative interpretation of the experiments by Anderson *et al.* rather than hardcore experimental evidence to support Lin *et al.*'s claims. Furthermore, despite their life-span measurements, Lin *et al.* provided no evidence as to the actual downstream effects such as ERC accumulation or silencing function of Sir2p.

Recent genetic studies by Kaeberlein *et al.* have put both these models under scrutiny. To begin with, Kaeberlein *et al.* could show that caloric restriction is able to actually increase life-span in the absence of Sir2p (Kaeberlein *et al.*, 2004). Subsequently, they used respiratory-deficient yeast strains with a broader range of glucose concentrations than used before to show that even in the original strain background used by Lin *et al.* Sir2p is dispensable for the caloric restriction-induced extension of longevity (Kaeberlein *et al.*, 2005a). Caloric restriction did not alter Sir2p activity based on an experiment assaying telomeric silencing efficiency. In addition they could show that in the absence of Sir2p, nicotinamide was able to reduce replicative life-span while this was also true for the effects of caloric restriction. Surprisingly, they found that although in strains lacking Sir2p nicotinamide did not reduce life-span consistent with the notion that it targets Sir2p, when these strains where subjected to caloric restriction nicotinamide only partially inhibited life-span extension.

These data implicated that there exist at least one Sir2p-independent pathway mediating longevity in response to caloric restriction and that nicotinamide inhibits both pathways. Indeed, using a large-scale screen for genes that mediate longevity in yeast, Kaeberlein *et al.* subsequently identified genes whose deletion increased life-span. Among them were components of the yeast *TOR1* pathway and *SCH9* kinase the latter being an orthologue of mammalian PKB (Akt) kinase. Consistent with their previous conclusions *TOR1* and *SCH9* deletions extended yeast life-span in the absense of *Sir2* (Kaeberlein *et al.*, 2005b).

The works presented above exemplify one of the many controversies present in the field of Sir2 biology. Clearly, some differences in the conclusion drawn from each study may be accounted for due to differences in yeast strains and experimental conditions used. While Sir2p overexpression can extend replicative life-span, whether it also plays a role in life-span extension upon caloric restriction remains at best uncertain. Current evidence supports a

model where Sir2 acts in parallel with another independent pathway which is driven by caloric restriction, yet the molecular mechanisms of this remain to be identified.

2.3.2.5 Homologues of Sir2 (Hst) proteins

Apart from Sir2p only limited information exists about the other four yeast sirtuins, Hst1-4 (for homologues of Sir two 1-4) (FIGURE 2-16). Brachmann *et al.* showed that Hst1p can rescue the silencing defect of *Sir2* mutants while an *Hst3* and *Hst4* double mutant strain exhibits telomeric silencing defects. Furthermore, they could demonstrate that *Hst3* and *Hst4* contribute to genomic stability. Interestingly, in an advanced genetic screen for synthetic lethality, Pan *et al.* discovered a broad genetic interaction between *Hst3* and replication machinery components. *Hst3* and *Hst4* also interact genetically with eachother albeit the latter does not share the synthetic lethal interactions of the former with replication components (Pan *et al.*, 2006).

Bedalov *et al.* provided evidence supporting a role for Hst1p as a cellular NAD^+ sensor. They could show that whereas the NAD^+ salvage pathway is constitutively active, low intracellular NAD^+ concentrations potently induce the *de novo* pathway and that Hst1p is a transcriptional repressor of these genes (Bedalov *et al.*, 2003). Consistent with the notion that Hst1p senses NAD^+ levels, they could show that Hst1p has higher K_m for NAD^+ than other yeast sirtuins.

Halme *et al.* identified Hst1p and Hst2p as the deacetylases responsible for the epigenetic silencing of *FLO10* (Halme *et al.*, 2004). *FLO* genes express cell-wall glycoproteins that regulate cell adhesion. Only one family member is expressed while the others are silenced due to their proximity near telomeres. Expression of the silenced genes allows for variation of cell-surface properties which is functionally important for processes such as pseudohyphal growth in response to nutrient limitation. Thus Hst1p and Hst2p can influence the cell-surface properties of a yeast cell through epigenetic regulation of *FLO* gene epxression. In the light of the results presented by Bedalov *et al.* it would be of interest to investigate whether a drop in intracellular NAD^+ levels can function as an indicator of the metabolic status of the cell and thus influence *FLO* gene expression contributing to the phenotypic changes accompanying nutritional availability.

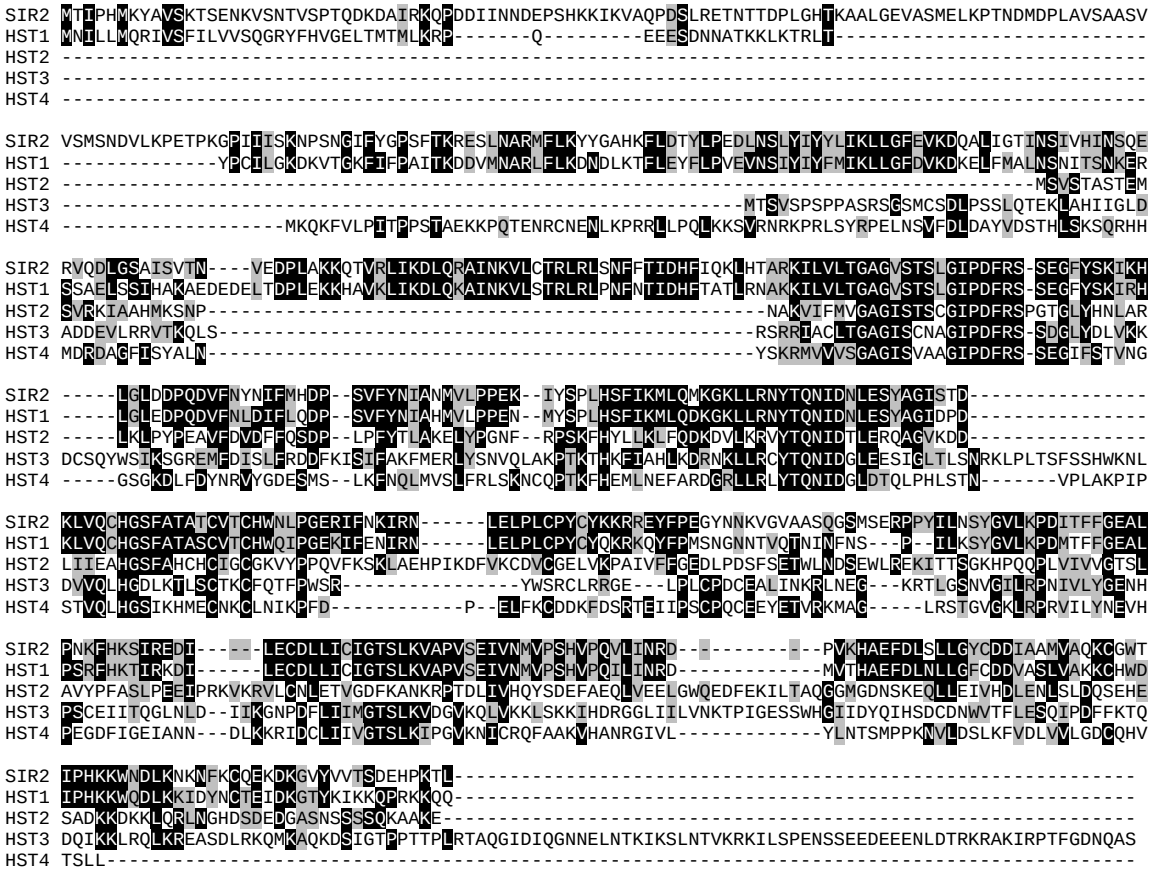


FIGURE 2-16. Multiple sequence alignment of the yeast sirtuin family members. Accession numbers of the sequences used are repsectively from top to bottom: P06700, P53685, P53686, P53687, P53688, P0A2F3, O28597.

Perrod *et al.* showed that Hst1p and Hst2p are not required for rDNA and for either rDNA or telomeric silencing respectively (Perrod *et al.*, 2001). Even though Hst2p is not able to compensate for the silencing defects upon *Sir2* loss, it seems to have a dominant negative effect on telomeric silencing upon overexpression. Conversely, overexpression of Hst2p enhances rDNA silencing in a wild-type *Sir2* background. Strikingly, Hst2p is a cytoplasmic protein. This led Perrod *et al.* to hypothesise the presence of a Sir2p interacting partner that is required for telomeric silencing and undergoes nucleocytoplasmic shuttling. This partner is sequestered by Hst2p inducing the translocation of Sir2p to the rDNA locus where it enhances silencing.

In a recent study, Lamming *et al.* showed that Hst2p is required for caloric restriction-induced life-span extension by supressing rDNA recombination in the absence of *Sir2* providing further support to the notion that despite the observed non-redundancy between

them, yeast sirtuins may share some functional roles (Lamming *et al.*, 2005). The potential significance of this will be analysed in a later part of this thesis.

2.3.3 *Caenorhabditis elegans* sirtuins

The nematode *C. elegans* sirtuin family comprises four members. *C. elegans* provided an excellent multicellular model organism for the study of *sir2.1* effects on longevity. In *C. elegans*, a pathway orthologous to the insulin signalling system has been identified (FIGURE 2-17) (Kenyon, 2001). Downregulating the levels of the insulin/IGF-1 receptor orthologue DAF2 results in doubling the life-span of these animals. In addition, mutation in the *age-1* (PI-3 kinase orthologue) and *pdk1* (phosphoinositide-dependent kinase orthologue) genes also extend life-span. Life-span extension in these mutants is abolished in the background of *daf-16* mutations, a gene encoding a forkhead transcription factor. Furthermore, this pathway regulates the developmental arrest of worms induced by food deprivation, a state known as dauer (Kenyon, 2005). This can only occur prior to reproductive maturation of the animal as adult worms do not form dauers upon caloric restriction. Dauers are resistant to oxidative stress and long-lived and can resume development and progress to adulthood upon repletion of nutrients.

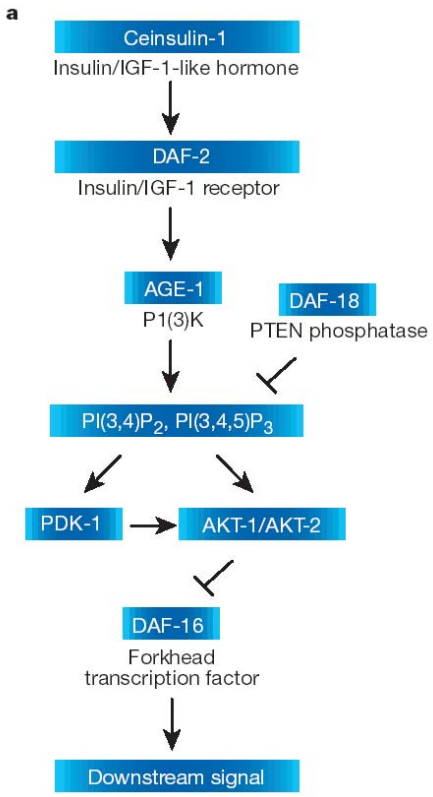


FIGURE 2-17. The IGF signaling pathway in *C. elegans*. *See* accompanying text and Chapter 1 for details.

Adapted from Guarente and Kenyon, 2000

Tissenbaum and Guarente screened several *C. elegans* strains carrying chromosomal duplications and identified one which exhibited significant extension in life-span (up to 50%) (Tissenbaum and Guarente, 2001). This strain contained a duplication of a chromosome IV region spanning the *sir2.1* locus. Transgenic lines created by injection of the *sir2.1* genomic fragment also lived longer suggesting that the *sir2.1* gene product mediated this effect. *daf-16* mutant worms have shorter life-spans that could not be extended by overxpression of

sir2.1. Furthermore, *sir2.1* overexpression did not extent further the life-span of worms carrying mutations in the *daf-2* gene. These experiments firmly established SIR2.1 as a mediator of life-span within the *C. elegans* insulin signalling system.

Mutations in the insulin signalling pathway synergise with mutations in the TGF-β pathway to affect life-span. *daf-1* and *daf-4* encode two types of TGF-β receptors and mutations in either of these genes cause a temperature-sensitive constitutive dauer phenotype. *sir2.1* overexpression alone did not induce dauer formation but in the background of either *daf-1* and *daf-4* mutations it increased markedly the proportion of animals that entered the dauer stage. These experiments further supported the notion that by modulating the *C. elegans* insulin pathway, SIR2.1 regulates longevity and that as in the unicellular *S. cerevisiae*, it holds an evolutionarily conserved function.

A later study by Wang and Tissenbaum further investigated the role of *sir2.1* in the worm by employing a loss-of-function approach. They used a strain with a deletion in the *sir2.1* locus that eliminates half of the sirtuin core domain and is likely to result in a null allele (Wang and Tissenbaum, 2006). Worms mutated for *sir2.1* showed a slight decrease in mean life-span which was further exuberated when these worms were exposed to stresses such as heat-shock, H_2O_2 and UV irradiation. Next, they tested for genetic interactions with *daf-2* mutants to establish the exclusivity as well as the topology of the pathway with respect to life-span. Interestingly, worms mutated in both *daf-2* and *sir2.1* had the same life-span as worms mutated in *daf-2* only suggesting that *sir2.1* is upstream of the insulin receptor or alternatively in a parallel pathway.

unc-13, a gene positioned genetically upstream of *daf-2*, is a regulator of neurotransmiter release and when mutated it results in a moderate life-span extension in a *daf-16* dependent manner. Mutations in *sir2.1* could partially suppress the long-lived phenotype of *unc13* mutant worms. When in this double-mutant background a mutation of *daf-16* was added, *unc-13* life-extension could be completely suppressed suggesting that *sir2.1* and *daf-16* have distinct functions in life-span determination in *C. elegans*. Interestingly, one of the many phenotypes of the *unc-13* mutants a defect in food ingestion implying that life-span extenstion in these mutants is a by-product of caloric restriction.

Mutations in the *eat-2* gene are thought to mimick caloric restriction because of pharyngeal defects leading to slower and irregular pumping. Accordingly, *eat-2* mutant

worms live longer. Worms carrying mutations in both *eat-2* and *sir2.1* have wild-type life-spans suggesting that the effects of caloric restriction on longevity are dependent on the *sir2.1* gene (Wang and Tissenbaum, 2006). These data provided experimental evidence for the involvement of *sir2.1* in regulating caloric restriction-driven extension of worm life-span and that these effects are only partially overlapping with those of *daf-16*.

Underlying the validity of these findings, Viswanathan *et al.* exploited the compound resveratrol, previously shown to enhance the activity of SIRT1, to probe the signalling pathways mediating longevity in *C. elegans*. Resveratrol was found to increase life-span in a manner dependent on *sir2.1* but independent of *daf-16* (Viswanathan *et al.*, 2005). Furthermore, resveratrol treatment in combination with *sir2.1* overexpression had additive effects on life-span, which the authors interpreted as an indication that resveratrol increases life-span *via* a *sir2.1*-independent pathway. This result could also be interpreted as super-activation of already overexpressed sir2.1 by resveratrol according to the proposed role of the compound as an activator of sirtuins, a notion supported by data in the same work which show that the effects of resveratrol on longevity are abolished in a *sir2.1* mutant strain. On the other hand, unlike resveratrol, *sir2.1* overexpression increases life-span in a manner fully dependent on *daf-16*.

Wanting to explore the molecular mechanisms involved in resveratrol-induced increase in life-span, Viswanathan *et al.*, analysed gene expression profiles of worms treated with this compound. A class of genes that was prominently induced by resveratrol treatment encodes PQN [prion-like asparagine(Q)/glutamine(N)-rich] and its subgroup ABU (activated in blocked unfolded protein response) which, as the name implies, act in response to ER stresses when the canonical UPR (unfolded protein response) pathway malfunctions. ER responses dictate cell survival upon different stress conditions so resveratrol may affect life-span by activating this pathway to promote survival. RNAi-mediated downregulation of PQN/ABU proteins reduced or even completely abolished (in the case of ABU-11) the resveratrol-induced increase in longevity. Consistent with the notion that *pqn* gene activation correlates with increased life-span, transgenic animals overxpressing ABU-11 showed life-span extension up to 28% in a manner dependent on ABU-11 expression levels.

Resveratrol was expected to activate SIR2.1 which could in turn induce transcription of the *pgn/abu* genes. Surprisingly, *sir2.1* mutant worms showed higher levels of *abu-11*

mRNA and other *pqn* genes while SIR2.1 overxpression had the reverse effect implying that SIR2.1 is a repressor of these genes. Importantly, gene expression profiles dictated by resveratrol treatment did not include genes activated in response to other stresses such as heat-shock, oxidative stress or ethanol and resveratrol did not induce a general ER stress response as evidenced by its inability to affect expression of the UPR pathway genes (Viswanathan *et al.*, 2005).

This work, while it opens a completely new *vista* on pathways mediating longevity in *C. elegans*, also raises questions on the action of SIR2.1 therein. Conspicuously, *sir2.1* is required for resveratrol-induced life-span extension which in turn is associated with elevated *pqn* gene expression. At the same time resveratrol is proposed to be a sirtuin activator and SIR2.1 ovexpression suffices to increase life-span in a manner that can be further stimulated by resveratrol. On the other hand SIR2.1 seems to repress *pqn* gene expression. To explain these disrepancies, drawing from the studies of Borra *et al.* (Borra *et al.*, 2005) and Kaeberlein *et al.*, (Kaeberlein *et al.*, 2005) Viswanathan *et al.* propose that resveratrol is either an activator or inhibitor of sirtuins depending on the substrate the enzyme encounters. In the context of their experiments resveratrol would be an inhibitor of SIR2.1 (FIGURE 2-18). On the other hand, while *abu-11* overexpression is sufficient to increase life-span, *sir2.1* mutants, which have elevated ABU-11, do not live longer. It is possible that a third *sir2.1*-dependent pathway which is not inhibited by resveratrol acts in parallel to the *pqn* pathway to promote longevity. It is also conceivable that, like in the case of nuclear receptor-driven transcription, SIR2.1 can either activate or repress transcription depending on the context of protein complexes it participates in or even modulate the content of such complexes itself, while resveratrol can act exclusively as an activator of SIR2.1. Notwithstanding this added complexity, these novel observations further triggered our thinking on the mode of action of SIR2.1 in *C. elegans* and is likely to fuel the quest to find parallels in mammalian cells.

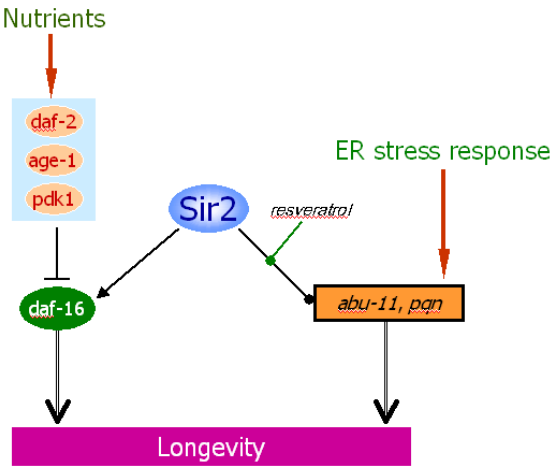


FIGURE 2-18. Proposed roles of SIR2.1 in determination of life-span in *C. elegans*. *See* text for details.

2.3.4 *Drosophila melanogaster* sirtuins

Five orthologues of the yeast Sir2 have been identified in *Drosophila* (CG5216, CG5085, G3187, CG6284, and CG11305, FlyBase), among which dSir2 (CG5216) shares the highest homology with and was actually shown to exhibit NAD^+-dependent deacetylase activity. dSir2 localises primarily in the nucleus in cultured *Drosophila* cells and the animal but is found to be exclusively cytoplasmic in the syncytial blastoderm and both cytoplasmic and nuclear during late embryogenesis. The dSir2 mRNA is present throughout emryogenesis, is markedly downregulated in larvae and returns to higher levels in the pupal and adult stages. In particular, the presence of *dSir2* transcripts within the first hours of emryogenesis imply maternal effect (Rosenberg and Parkhurst, 2002).

Indeed, embrya derived from mothers with reduced dSir2 contribution exhibit segmentation defects due to aberant pair rule gene expression as hinted by the derepression of the secondary pair rule gene *fushi tarazu* (*ftz*). This phenocopies reduced funtion of a primary pair rule gene, *hairy*, which is a member of the basic helix-loop-helix (bHLH) family of repressors Idiscussed in more detail below) and behaves genetically as a repressor of *ftz*. Consistent with this hypothesis, *dSir2* and *hairy* interact genetically to determine embryonic segmentation as well as physically in a biochemical binding assay via a basic aminoacid motif conserved in bHLH proteins (Rosenberg and Parkhurst, 2002). In fact, another bHLH protein, Deadpan, also interacted with dSir2. Deadpan is involved in sex determination by participating in the expression of a gender-specific transcipt *sex lethal* (*sxl*). In agreement to this, lower dSir2 resulted in male-specific lethality due to aberrant dosage compensation providing evidence for the physiological significance of the observed biochemical interaction with Deadpan (Rosenberg and Parkhurst, 2002).

More recently, a report by Astrom *et al.* has challenged the above conclusions based on the fact that the *dSir2* mutant strain used for these experiments could be fully complemented by a strain they generated which lacked most of the *dSir2* ORF (Astrom *et al.*, 2003). Using this strain, Astrom *et al.* also demonstrated that viability of *Sxl* null male flies is not sensitive to dSir2 levels suggesting that the effects observed by Roseneberg and Parkhurst reflected extraneous mutations in the strain used in their studies.

Another well characterised occurrence of gene silencing in *Drosophila* is mediated by the polycomb group proteins (PcG), best known for their involvement in the spatial regulation of homeobox gene expression during development. PcG comprises several chromatin-modifying proteins that are recruited to the DNA by means of specialised regulatory elements called polycomb response elements (PREs).

dSir2 deletion was found to enhance the PcG mutant phenotype "extra sex combs" *via* a PRE-dependent mechanism (Furuyama *et al.*, 2004). Consistent with a role in polycomb silencing, dSir2 interacts directly and co-localises with the PcG protein E(Z) while it co-fractionates with other members of a previously known E(Z) complex from larval extracts. One of these proteins, Rpd3 is also a deacetylase found to affect the expression of a largely distinct set of genes. Disruption of *dSir2* does not affect the localisation of PcG components to chromatin suggesting that the role of dSir2 in this context is not exerted through regulation of PcG protein recruitment. Thus the functional significance of dSir2 in this distinct E(Z) complex remains to be elucidated. Further insighst into the potential role of sirtuins in polycomb-mediated repression were provided by the identification of a mammalian complex comprising the mammalian enhancer of zeste orthologue Ezh2 and SIRT1 and will be discussed in section 2.3.5.1.3.

dSir2 associates with both heterochromatin and euchromatin. Several groups reported that *dSir2* mutations affect heterochromatin silencing a phenomenon known in *Drosophila* as position effect variegation (PEV). Assaying for PEV is attained in reporter fly strains by placing the *white* gene in the context of DNA derived from genomic regions known to be subject to silencing. When mutated, a gene thought to mediate PEV would alleviate silencing of the reporter gene and the eyes of these animals would show red patches corresponding to areas with defective silencing. Using such an assay, Rosenberg and Parkhurst suggested that dSir2 participates in heterochromatic but not telomeric silening (Rosenberg and Parkhurst, 2002). Newman *et al.* speculated that loss of dSir2 function results in impairment of heterochromatin formation or maintainance, surprisingly, though, they have omitted to substantiate this by studying the banding pattern of salivary gland chromosomes (Newman *et al.*, 2002). In the same study, dSir2 was found to share similar distribution pattern with dCBP, an acetyltransferase which was found to be associated with SIRT1 is mammalian cells (*see* later) and was also implicated in heterochromatin formation.

dSir2 loss-of-function mutations reduce *Drosophila* life-span when animals with matched genetic backgrounds are compared (Rogina and Helfand, 2004). Furthermore, when dSir2 was overexpressed under the regulation of a strong promoter, an extension of up to 57% was observed. dSir2 was shown to be highly expressed in neuronal tissues and neuronal-specific *dSir2* overexpression was sufficient to induce an increase in life-span. Significantly, calorie restriction could not further increase longevity in dSir2-overexpressing animals suggesting that the effects of caloric restriction on longevity in *Drosophila* are mediated by dSir2 (Rogina and Helfand, 2004).

2.3.5 The mammalian sirtuin family

2.3.5.1 SIRT1

2.3.5.1.1 Expression and genetic ablation of SIRT1 in the mouse

Since the description of the mammalian sirtuin family, SIRT1 (also referred to as mSir2α or Sir2α in the mouse) has attracted most of research efforts to elucidate its function as it has been considered the functional orthologue of scSir2. This was based on the fact that it exhibits the highest homology compared to the other mammalian sirtuins, it is localised in the nucleus and was shown to complement the silencing defects of *sir2*-defective strains. Furthermore, SIRT1 shares the characteristic N- and C-terminal extensions flanking the conserved sirtuin core domain laso present in scSir2. A multiple sequence alignment of SIRT1 with the other six human famiy members is shown in FIGURE 2-18.

SIRT1 mRNA is ubiquitously expressed in all mouse tissues tested (Sakamoto *et al.*, 2003; McBurney *et al.*, 2003a). Quantitative expression analysis indicated that SIRT1 is highly expressed in the mouse embryo early during development but progressively declines to lower amounts which remain constant into adulthood. The highest levels of SIRT1 mRNA were found in the lung and testes (Sakamoto *et al.*, 2003). Immunohistochemical studies also showed ubiquitous expression of SIRT1 with predominant presence of the protein in the heart, brain, spinal cord and dorsal root ganglia at E10.5-13.5 (Sakamoto *et al.*, 2003). McBurney *et al.* also showed that SIRT1 protein is highly expressed in mouse emryonic stem (ES) cells

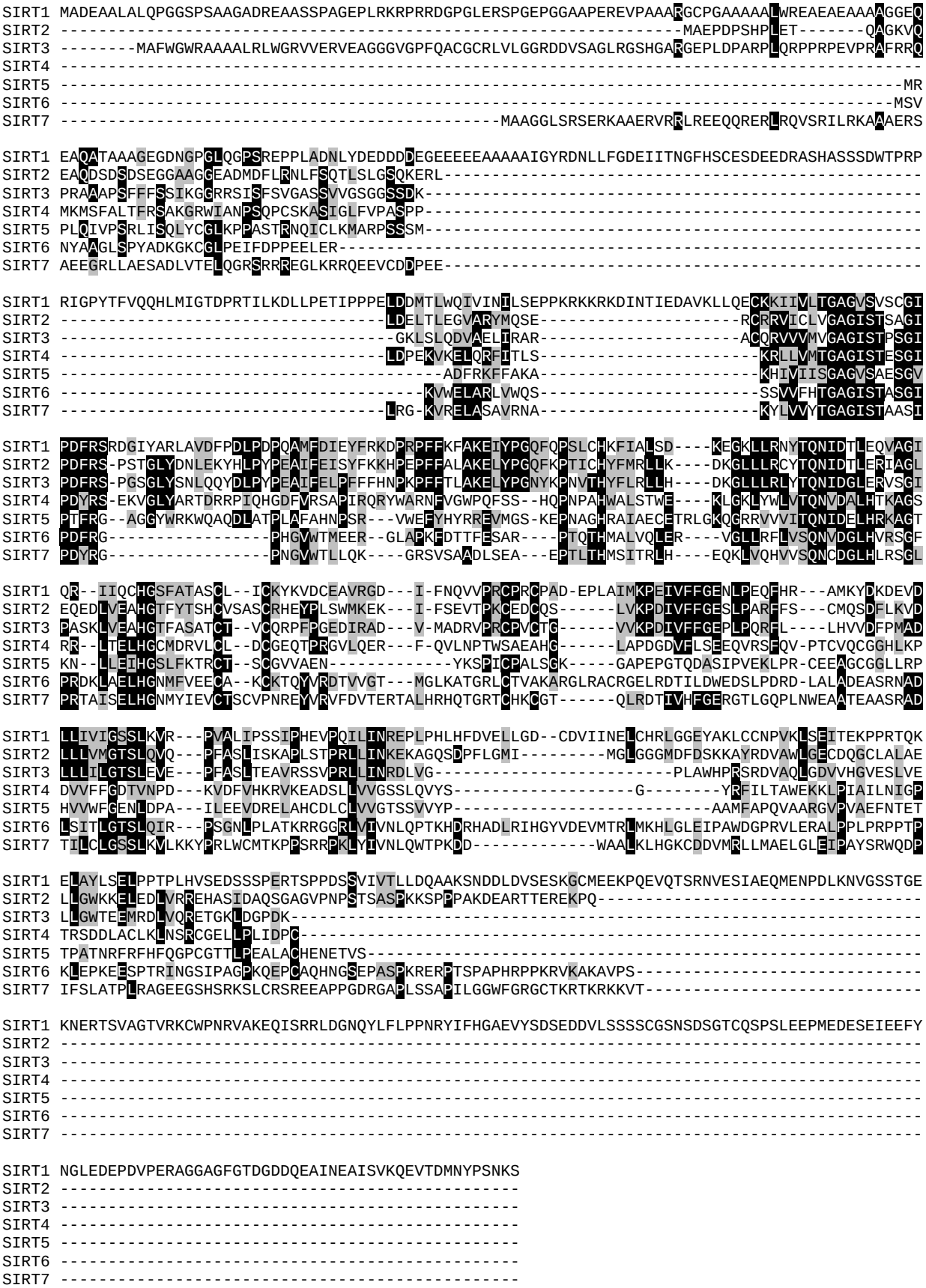


FIGURE 2-19. Multiple sequence alignment of sirtuin members from different species. Accession numbers of the sequences used are respectively from top to bottom: Q96EB6, Q8IXJ6, Q9NTG7, Q9Y6E7, Q9NXA8, Q8N6T7, Q9NRC8.

and during spermatogenesis, particularly in the nuclei of spermatogonia, spermatocytes and round spermatids (McBurney *et al.*, 2003a; McBurney *et al.*, 2003b).

Consistent with SIRT1 expression in embryonic tissues, mice with genetic ablation of the both *SIRT1* alleles die soon after birth, although in an outbred background animals can survive to sterile adults which are smaller than their littermates (McBurney *et al.*, 2003a). The sterility was attributed to defects in spermatogenesis with increased apoptosis in the seminiferous tubules and associated failure of spermatozoa to mature properly in agreement with the high expression of SIRT1 during spermatogenesis. Furthermore, *SIRT1*$^{-/-}$ mice had eye abnormalities which were attributed to an eyelid opening failure seen consistently in these animals (McBurney *et al.*, 2003a).

SIRT1$^{-/-}$ mice exhibit no global defects in silencing of either endogenous genes or reporter β-galactosidase transgene suggesting that if SIRT1 functions in silencing similarly to its yeast orthologue it does so in a gene-specific rather than genome-wide manner (McBurney *et al.*, 2003a). Furthermore, ES cells derived from *SIRT1*$^{-/-}$ mice are able to differentiate *in vitro* and exhibit no global changes in histone acetylation patterns (McBurney *et al.*, 2003b).

The *SIRT1*$^{-/-}$ mice used in the studies of McBurney *et al.* were generated by targeted deletion of exons 5 and 6 of the murine *SIRT1* gene which encode for a large part of the catalytic subunit (McBurney *et al.*, 2003a). In an independent study, Cheng *et al.* used targeted homologous recombination to delete the entire region encoding for SIRT1. They also created a mouse line with a conditional deletion of exon4 in the SIRT1 gene which encodes 51 aminoacids of the SIRT1 conserved domain (Cheng *et al.*, 2003).

These animals exhibited cardiac abnormalities, including ventricular and atrial septal defects and elongated atrioventricular valves, all of which were absent in *SIRT1*$^{-/-}$ mice surviving into adulthood. In addition, the retinas of post-natal *SIRT1*$^{-/-}$ mice showed severe defects with many of the characteristic retinal layers being thinner and disorganised. This correlated with abnormal closure of the optic fissure found in *SIRT1*$^{-/-}$ embrya providing evidence that the retinal abnormalities are attributable to a developmental defect rather than a secondary effect of eyelid closure abnormalities as proposed by McBurney *et al.* (McBurney *et al.*, 2003a).

Several studies mainly in tissue culture systems have implicated SIRT1 in various cellular processes by means of its interaction with other proteins (TABLE 2-2). There is a

varying degree of experimental evidence provided that supports a relevance of these observations in animal physiology. Thus some molecular aspects of SIRT1 biology are described in detail while there exist physiological processes where SIRT1 has been implicated in that await elucidation of the underlying molecular mechanisms.

TABLE 2-2. Substrates, biochemical and functional interactions of mammalian SIRT1.

Target protein	Endogenous interaction	Minimal determined binding site on SIRT1	Deacetylation of binding partner	Transcriptional effect of SIRT1	AT[§] involved	Reference(s)
p53	+	n.d.	+	repression	p300	Vaziri *et al.*, 2001; Luo *et al.*, 2001; Langley *et al.*, 2002
FOXO1	+	208-409	+	activation/ repression	CBP	Daitokou *et al.*, 2004; Motta *et al.*, 2004
FOXO3a	+	n.d.	+	activation/ repression	p300	Brunet *et al.*, 2004; Motta *et al.*, 2004; van der Horst *et al.*, 2004
FOXO4	n.d.	n.d.	+	activation	CBP	van der Horst *et al.*, 2004
Ku70	+	n.d.	+	n.a.	CBP	Cohen *et al.*, 2004a, b
p300	+	n.d.	+	repression	n.a.	Bouras *et al.*, 2005 and present study
FHL2	n.d.	n.d.	n.d.[∂]	repression	n.d	Yang *et al.*, 2005
Histone H1	+	1-268	+	repression	n.d	Vaquero *et al.* 2004
PPARγ	+	n.d	n.d	repression	n.d	Picard *et al.*, 2004
NCoR	+	214-541	n.d	repression	n.d	Picard *et al.*, 2004
MyoD	+	n.d.	+	repression	PCAF	Fulco *et al.*, 2003
PCAF	+	236-510	+	repression	n.a.	Fulco *et al.*, 2003
RelA/p65	+	n.d.	+	repression	p300	Yeung *et al.*, 2004
Su(z)12	+[¥]	n.d.	n.d.	repression?	n.d.	Kuzmichev *et al.*, 2005
TAF_I68	n.d.	n.d.	+	repression	PCAF	Muth *et al.*, 2001
PML	+	n.d.	n.d.	n.a.	CBP	Langley *et al.*, 2002
HIC1	+	n.d.	n.d.	n.a.	n.a.	Chen *et al.*, 2005
Histone H4	n.d.	n.d.	+	repression?	n.d.	Imai *et al.*, 2000
Bcl-6 (*)	n.d.	n.d.	+	repression	p300	Bereshchenko *et al.*, 2002
CTIP2	+	214-441	?/histones	repression	n.d.	Senawong *et al.*, 2003
Hes1/Hey2	n.d.	n.d.	n.d.	repression	n.d.	Takata and Ishikawa, 2003
HIV Tat	+ (Tat exog.)	n.d.	+	activation	p300/GCN 5	Pagans *et al.*, 2005

n.d.: not determined; n.a.: not applicable

[§] acetyltransferase. N.B.: all ATs implicated from the experiments in the corresponding publications are listed ; not all were shown to be the relevant endogenous enzymes.

* SIRT1 only implicated in Bcl-6 regulation based on evidence from nicotinamide sensitivity of Bcl-6 acetylation

[∂] FHL2 recruits SIRT1 to FOXO which is a SIRT1 substrate

[¥] SIRT1 was found to inderact with other components of the PRC4 complex, including Ezh2

For this reason, the reported functions of SIRT1 will be critically presented in two broad categories. In the first of them, data linking SIRT1 to the regulation of protein function with no experimental evidence for a physiological relevance of these interactions at the

organismal level will be discussed. In the second category work implicating SIRT1 in physiological processes irrespective of the degree of understanding of the molecular mechanisms involved will be presented. An integrative review of this work is provided in Chapter 5.

2.3.5.1.2 Regulation of chromatin structure by SIRT1

Histone H1

The fact that *Saccharomyces cerevisiae* Sir2 was shown to affect histone acetylation prompted Vaquero *et al.* to investigate whether this function was conserved in mammalian cells. In an *in vitro* deacetylase assay using recombinant SIRT1 they observed that all four core histones could be deacetylated albeit with different kinetics and an apparent specificity for histones H3 and H4 (Vaquero *et al.*, 2004). Using antibodies against specific acetylated residues of these histones they could demonstrate specificity for SIRT1 against H4-K16 *in vitro*, while *in vivo* downregulation of SIRT1 by siRNA resulted in increasing levels of H4-K16 acetylation and decrease in H4-K20 methylation, a mark for repressed chromatin.

To further investigate the function of SIRT1 on chromatin remodelling, Vaquero *et al.* isolated chromatographically SIRT1-associated proteins from cells stably expressing Flag-tagged SIRT1 and found a specific interaction with histone H1b, a histone H1 isoform implicated in heterochromatin formation. The authors next asked whether H1 is regulated by acetylation and identified H1-K26 as the residue targetted for deacetylation. To investigate the functional significance of these observations, SIRT1 was fused to the DNA binding domain of GAL4 and used in transcriptional reporter assays. GAL4-SIRT1 could induce a 94% decrease in reporter activity while a catalytically inactive mutant only 36% indicating that part of the SIRT1 repressive activity was independent of its enzymatic activity and likely to depend on its ineraction with histone H1 since an N-terminal deletion of SIRT1 was also partially defective in repressing reporter activity. This region corresponded to the H1 interacting site.

To further analyse the effect of SIRT1 on chromatin modification, the authors established a 293-based cell line with a stably-integrated luciferase reporter under the control

of GAL4-binding sites. The same line carried a stable integration of a tetracycline-regulated GAL4-SIRT1 contruct which upon induction was detected in the luciferase promoter and repressed it. This coincided with histone H1 recruitment and H4-K16 deacetylation exclusively in the promoter. Upon examination of other histone modifications by ChIP analysis, tetracycline induction also resulted in increased H4-monoMeK20 and H3-triMeK9 but these modifications also spread in the coding region of the reporter. H3-diMeK79, a modification marking the boundary between active and inactive chromatin was conversely downregulated.

In unicellular eucaryotes including *S. cerevisiae*, histone H1 depletion does not affect survival but it shortens lifespan. Histone H1 has also been shown to hold important roles in germline proliferation and differentiation in multicellular eucaryotes such as *C. elegans* where it participates in chromatin silencing. In mice, severe developmental defects are apparent only when several histone H1 variants are simultaneously deleted while experiments indicate that although required for maintainance of higher order chromatin, H1 is not likely to be needed for its formation. It is also postulated that H1-mediated changes in higher chromatin structure may affect the binding of other chromatin regulating proteins with ultimate effects on gene expression.

The work of Vaquero *et al.* provided evidence that in conjunction with histone H1, SIRT1 activity can influence the establishment of heterochromatin either through direct deacetylation of H1 and thus chromatin compaction or through the deacetylation of other histones possibly in combination with enzymatic activities recruited to chromatin through histone H1. It would be predicted that in such a role, SIRT1 could influence global chromatin structure. The consequences of this and the interlink to lifespan under the light of global heterochromatinisation observed in ageing remain to be elucidated.

SIRT1 in polycomb repressing complexes

As discussed in section 1.2.1.3.2, histone lysine modifications are important elements of the epigenetic machinery regulating chromatin structure and gene expression. Lysine methylation is catalysed by lysine-specific histone methyltransferases (HKMT) which are part of multisubunit complexes. The human enhancer of zeste orhologue 2 (Ezh2) is a SET

domain methyltransferase that forms different complexes known as polycomb repressive complexes (or PRCs) mainly defined by the isoform of Eed, the human Extra Sex Combs orthologue, that they contain. Each Eed isoform confers specificity as to the lysine residue targeted by the corresponding PRC complex.

PRC4 was purified as a novel polycomb repressive complex on account of its exclusive content of Eed2 and its specificity towards histone H1b K26 (Kuzmichev *et al.*, 2005). Equipped with the knowledge that H1b-K26 is deacetylated by SIRT1, Kuzmichev *et al.* determined that SIRT1 is a component of PRC4 most probably through its association with Su(z)12 (suppressor of zeste 12). Interestingly, a catalytically inactive SIRT1 mutant could associate with Eed but not with Ezh2.

Because PRC component abundance changes during differentiation and in cancer, the authors examined the levels of PRC4 components in these conditions. Upon induction to differentiate, mouse ES cells showed progressively decreasing levels of Ezh2, Eed2 and mSIRT1 while breast and colon cancer tissues showed similar levels of expression of PRC4 components to transformed cell lines which were significantly higher compared to normal tissues. To investigate the significance of this observation, Kuzmichev *et al.* used a mouse model of prostate cancer that carries monoallelic deletions of both the prostate-specific homeobox gene *Nkx3.1* and the tumour suppressor lipid phosphatase *Pten*. As tumours emerged in these animals, the protein levels of SIRT1 and Ezh2 as well as the mRNA levels of Ezh2, Su(z)12 and Eed were elevated. Concomitant to this, PRC target genes were accordingly affected.

Thus SIRT1 participates in a complex with chromatin modifying activity that may contribute to some aspects of the transformed phenotype (Kuzmichev *et al.*, 2005). Although not demonstrated, the targetting of H1b-K26 by the deacetylase activity of SIRT1 may allow its methylation by PRC4 in a similar fashion as previously described for HDAC1 in H3-K9 methylation. Recently, a report by Viré *et al.* showed that Ezh2 can dictate DNA CpG methylation by the direct recruitment of DNA methyltransferase activity providing an integrative model for two major epigenetic mechanisms (Viré *et al.,* 2006). It would be of interest to know whether SIRT1 activity can modulate Ezh2-associated DNA methylation.

Thus these findings further enhance the notion that a member of mammalian sirtuins participates in epigenetic regulation of gene expression and provide evidence for a conservation of the *Drosophila* dSir2 interaction with PcG proteins in mammals.

2.3.5.1.3 Regulation of transcription by SIRT1

HIV Tat

Human immunodeficiency virus-1 (HIV-1) replication depends among the others on the Tat protein, a transcriptional activator. Tat forms a ternary complex with cyclinT1 and a *trans*-acting response element (TAR) found in the 5' of all viral mRNAs (FIGURE 2-20). This complex recruits CDK9 which in turn phosphorylates RNAPolII to substantially enhance the processivity of the enzyme. Acetylation of Tat at K50 by p300 during the intermediate stages of transcription prevents the formation of the Tat/cyclinT1/TAR complex. Acetylated Tat is then transferred to the elongating polymerase to recruit PCAF by means of the later's bromodomain. At the early stages of viral replication, Tat protein is limiting yet crucial for driving transcription of more Tat mRNAs until a critical concentration that can further sustain viral replication is achieved. Based on the above model of Tat action (FIGURE 2-20), deacetylation of Tat at the early stages of viral replication is crucial. Furthermore, following completion of the transcription cycle, the fate of acetylated Tat was unclear.

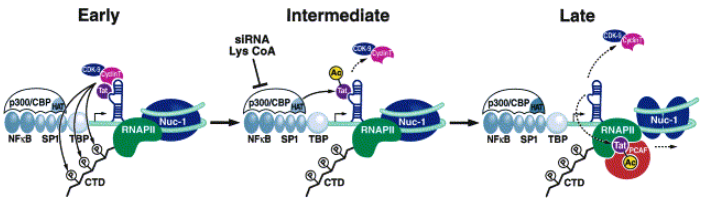


FIGURE 2-20. Regulation of HIV Tat transcriptional activity by acetylation.
Adapted from Kaehlcke *et al.*, 2003

Pagans *et al.* identified all SIRT1, SIRT2 and SIRT3 as Tat deacetylases *in vitro* (Pagans *et al.*, 2005). SIRT2 and SIRT3 are excluded from the nucleus and given the nuclear localisation of both Tat and SIRT1 they probed the functional link between these two proteins. They found that Tat and SIRT1 can associate both in *in vitro* and when co-expressed in cells. Furthermore, transcriptional reporter assays indicated that SIRT1 could enhance Tat-driven transcription in a manner dependent on SIRT1 enzymatic activity and independent of the promoter's NFκB sites (*see* below on SIRT1 and NFκB interaction).

Conversely, siRNA targeting SIRT1 reduced Tat transcriptional activity. Tat-driven transcription dramatically decreased i *Sirt1*$^{-/-}$ MEFs, and reconstitution of SIRT1 can reverse this effect. Additionally, small molecule inhibition of SIRT1 by nicotinamide or the splitomicin derivative HR73 recapitulated the effects of SIRT1 siRNA on Tat-driven transcription. To test whether SIRT1 inhibition could be used as a target for therapeutic intervention against HIV infection, Pagans *et al.* treated HIV-infected Jurkat cells with HR73 and found that HIV gene transcription was approx. 5-fold decreased. This ultimate experiment provided proof of principle that the identified role of cellular SIRT1 in the replication of HIV can be a site of pharmacological targeting in the future.

CTIP1 and CTIP2

Chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting proteins 1 and 2 (CTIP1and CTIP2), are transcription factors that interact with and emhance COUP-TF-mediated transcriptonal repression. It has also been proposed that they may mediate transcriptional repression independently of COUP-TF since they can bind directly to a defined DNA sequence and they are expressed in hematopoietic cells of lymphoid origin which do not express COUP-TF. Loss-of-function analysis in mice as well as data showing genetic aberations the *CTIP1* and *CTIP2* loci, have provided significant correlations between CTIP1and CTIP2 dysregulation and hematopoietic malignancies.

Both CTIP1- and CTIP2-driven repression are independent of class I/II HDACs based on their sensitivity to TSA. Senawong *et al.* could show that nicotinamide could alleviate CTIP2-induced repression in reporter assays as well as demostrate by ChIP that the effects of nicotinamide correlated with increased H3/H4 acetylation in an exogenous chromatinised template (Senawong *et al.*, 2003). Co-expression of SIRT1 recapitulates these findings in a manner dependent on the latter's enzymatic activity. suggesting that SIRT1 participates in CTIP2-driven repression. This effect stems from the ability of SIRT1 to interact *via* its sirtuin core domain with CTIP2 both *in vitro* and *in vivo*. A follow-up study demonstrated that the closely related protein CTIP1 behaves in a very similar manner as CTIP2 in the assays employed (Senawong *et al.*, 2005).

Apart from the discovery of SIRT1 as the deacetylase that potentially mediates the TSA-insensitive repressive activities of CTIP1/2, the physiological importance of these findings remains elucive. It would be of interest to investigate whether SIRT1 can be exploited for pharmaceutical intervention within the context of leukemias that carry aberrant CTIP1/2 chromosomal loci.

TAF_I68

Apart from PolII-mediated gene transcription, chromatin remodelling has also been implicated in the regulation of PolI-transcribed genes which predominantly encode for ribosomal DNA. Transcription termination factor-I (TTF-I) is required for the recruitment of chromatin remodelling complexes to PolI promoters to render chromatin accessible to the transcriptional machinery. Targeted promoter binding of TTF-I is achieved by a promoter-proximal terminator element named T_0 160 bp upstream the transcription start site.

Muth *et al.* showed that TTF-I is able to bind the acetyltransferase PCAF, thus they investigated whether components of the transcription initiation machinery are acetylation substrates. They found that PCAF specifically acetylates the TAF_I68 (TATA-box binding protein-associated factor I of 68 kDa) subunit of the promoter selectivity factor TIF-IB/SL-1 both *in vivo* and *in vitro*. Similar to other acetylated proteins, TAF_I68 can also bind to PCAF (Muth *et al.*, 2001). Importantly, TAF_I68 was also shown to be acetylated *in vivo* and that this acetylation can occur when it is bound to the TIF-IB/SL-1 holocomplex.

Furthermore, electrophoretic mobility-shift assays (EMSAs) demonstrated that acetylation of TAF_I68 increased its binding to rDNA sequences consistent with previous observations for many other transcription factors. In an *in vitro* reconstituted transcription system, acetylated TAF_I68 is able to drive transcription more powerfully compared to the unacetylated form suggesting that the increased binding of TAF_I68 to DNA *via* acetylation may regulate transcription of rDNA genes. Interestingly, treatment of cells with TSA did not affect rDNA transcription assayed by Norhtern blot of pre-rRNA transcripts suggesting that class I/II HDACs are not the physiological deacetylases regulating rDNA transcription. Conversely, SIRT1 can deacetylate TAF_I68 *in vitro* while incubation of the previously employed *in vitro* transcription system with SIRT1 reduced rDNA transcription suggesting

that SIRT1 may be the relevant deacetylase. Notably, the DNA template used in this system is devoid of histones suggesting that SIRT1 exerts this regulatory role independent of chromatin.

rDNA silencing was one of the first described functions for yeast Sir2. This work provided the first evidence that a mammalian sirtuin can modulate rDNA transcription. Ribosomal DNA synthesis is essential for ribosomal biogenesis which correlates with a cell's translational capacity. Protein translation is required for proliferation as demonstrated by cell cycle arrest by means of small molecule inhibitors of translation (e.g. mimosine) and the cytostatic effects of ribosome-targeting antibiotics. Furthermore, it has been suggested that there exists a checkpoint that only allows cell division to occur under conditions that optimal growth has been achieved (Thomas, 2000). Consequently, the role of SIRT1 in rDNA transcription as documented in the work of Muth *et al.* is likely to reveal a deeper role for this protein in cellular homeostasis.

Regulation of Hes1/Hey2 bHLH repressors

The bHLH family of transcription factors comprise both activators and repressors which differ in the presence of distinct regulatory motifs. These motifs are thought to mediate the interaction with co-repressors which in turn mediate the recruitment of chromatin modifying factors such as deacetylases to implement transcriptional repression. The mammalian counterpart of Hairy is Hes1 and is found to recruit co-repressors through a C-terminal WRPW motif. Another member of the Hairy family of repressors, the *Drosophila* Hey and its mammalian orthologue Hey2 show a distinct mechanism of repressor recruitment through the bHLH domain itself implying diversity in the mode of action of this family of transcriptional regulators.

Driven by the discovery of Rosenberg and Parkhurst in *Drosophila* that dSIRT1 interacts with the bHLH factor Hairy, Takata *et al.* examined the potential interaction between the mammalian counterparts of these proteins (Takata *et al.*, 2003). Although no endogenous interactions were observed between these proteins, co-transfection experiments demonstrated that both protein could immunoprecipitate each other. *In vitro* binding assays mapped the SIRT1 binding site to the bHLH domain of both human HES1 and HEY2 protein.

Reporter assays employing GAL4 fusions of Hes1 and Hey2 contransfected with either wt or catalytically inactive SIRT1 showed that SIRT1 augments the repression driven by either factor and that its enzymatic activity is required for this effect. Taken together these data suggested that SIRT1 may be a novel regulator of human bHLH factor transcriptional repression activity. It would be of interest to examine whether acetylation of bHLH factors is also involved in their regulation as for the majority of transcription factors interacting with SIRT1.

Te interaction of SIRT1 with bHLH repressors is important with respect to recent evidence suggesting that Hes1 regulates directly the transcription of the cell cycle inhibitor p27. *Hes1*$^{-/-}$ mice exhibit developmental defects attributed to reduced proliferation which correlated with increased p27 mRNA levels. Indeed, Hes1 can bind elements in the p27 promoter and repress its activity (Murata *et al.*, 2005). Interestingly, *SIRT1*$^{-/-}$ mice exhibit cardiac as well as retinal development defects similar to *Hey2*- and *Hes1*-deficient mice respectively (Cheng *et al.*, 2003). It would be of interest to investigate whether SIRT1 is required for the repressive effect on p27 expression and if any of the observed phenotypes of the *SIRT1*$^{-/-}$ mice can be attributed to the proposed function of SIRT1 on Hes1/Hey2 factors.

Bcl-6

BCL6 is a protooncogene that encodes a nuclear protein of the BTB/POZ (bric-à-brac, tramtrac, broad complex/Pox virus zinc fingers) family of proteins. It is required for germline centre formation and it has been implicated in the pathogenesis of B-cell lymphomas. Bcl-6 acts as a transcriptional repressor by recruiting HDAC-containing co-repressor complexes including SMRT and Sin3A. This function depends on the zinc finger DNA binding domain, the N-terminal POZ domain and an additional repression domain located in the middle of the molecule. Bereshchenko *et al.* showed that Bcl-6 repressional activity is regulated by acetylation (Bereshchenko *et al.*, 2003).

In particular, Bcl-6 interacts and gets acetylated by the acetyltransferase p300. This acetylation markedly decreases its transcriptional repressor activity on a luciferase reporter and induces the dissociation of Bcl-6 from HDAC2. Accordingly, mutation of the Bcl-6 acetylation sites abolishes p300-mediated regulation and diminishes the transformation

capacity of Bcl-6 based on anchorage-independent growth assays. TSA and nicotinamide treatment have additive effects in inhibiting Bcl-6 transcriptional activity and its association to HDAC2 suggesting a synergistic role of both HDACs and sirtuins in the regulation of Bcl-6.

As the only evidence that sirtuins are involved in the regulation of Bcl-6 acetylation was based on nicotinamide sensitivity, it can only by presumed that SIRT1 is the relevant sirtuin. Interestingly, though, Bcl-6 has been shown to directly repress p53 target genes and thus inhibit p53-mediated apoptosis and cell cycle arrest hinting that these proteins may be co-ordinately regulated by acetylation. Moreover, this work provided evidence that sirtuins can induce transcriptional repression by deacetylating and thus activating transcriptional repressors such as Bcl-6 (Bereshchenko *et al.*, 2003).

2.3.5.1.4 Regulation of survival by SIRT1

SIRT1 regulation of p53 and p53-mediated tumourigenesis

The *p53* gene encodes a transcription factor that has been shown to be mutated in more than 50% of human tumours and has a firmly established role as a tumour suppressor (Vogelstein *et al.*, 2000). Mice lacking both *p53* alleles showed normal development but exhibited a broad array of spontaneous tumours by 6 months of age confirming that p53 is a gatekeeper against neoplasia (Donehower *et al.*, 1992).

p53 is subject to proteasome-mediated degradation by means of at least an E3 ubiquitin ligase encoded by the gene *Mdm2*. Upon genotoxic stresses, p53 is stabilised and mediates transcription programmes that dictate processes such as cell cycle arrest, apoptosis and DNA repair (Vogelstein *et al.*, 2000). p53

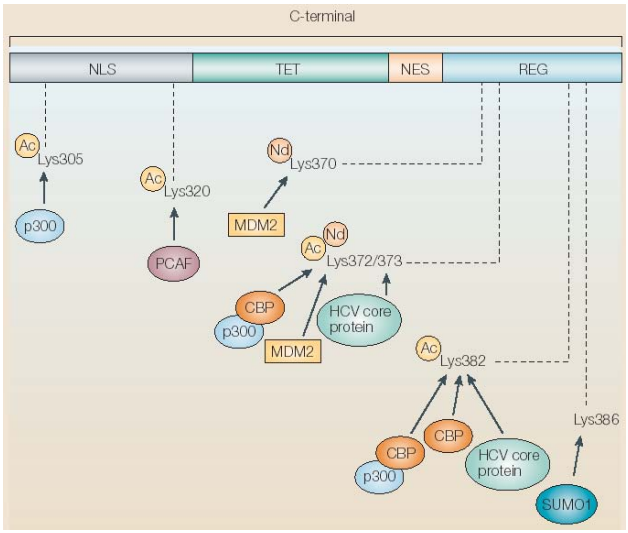


FIGURE 2-21. Acetylation sites in the C-terminus of p53.

Adapted from Bode and Dong, 2004

stabilisation is not sufficient, though for full p53 activity. Concomitant to the occurrence of protein stabilisation, an array of post-translational modifications is required for p53 to fully exert its roles which include phosphorylation, sumoylation, ubiquitination, neddylation, acetylation and methylation. These modifications span the entire region of the protein and affect protein stability, transcriptional activity, hetero- and homo-dimerisation and localisation.

Acetylation of p53 has been shown to increase protein stability and transactivation capacity (Figure 2-21). It is believed that acetylation prevents ubiquitination, but also neddylation or sumoylation of lysine residues, a proposal fitting to the observation that increased deacetylase activities found in many cancers correlate with destabilisation of p53. In parallel, acetylation of the C-terminus of p53 increases its DNA binding affinity both *in vivo* and *in vitro*. Thus, modification of p53 by acetylation modulates its activity at multiple levels and emerges as an important regulatory mechanism in tumour progression.

Acetylation of p53 upon DNA damage can be enhanced by treatment of cells with HDAC inhibitors but TSA-independent deacetylation can also occur (Luo *et al.*, 2001). Two reports by Luo *et al.* and Vaziri *et al.* first demonstrated that the TSA-insensitive p53 deacetylase activity could be attributed to SIRT1 (Luo *et al.*, 2001; Vaziri *et al.*, 2001). SIRT1 interacts *in vivo* and *in vitro* with p53 and shows an apparent specificity for acetylated K382. Overxpression of SIRT1 results in decrease of both basal as well as p300 overexpression-induced and ionising radiation-or etoposide-induced K382 acetylation. Transfection of a catalytically inactive mutant of SIRT1 had a dominant-negative effect in that, while deficient in p53 deacetylation *in vitro*, it could induce p53 hyperacetylation in response to ionising radiation. Furthermore, nicotinamide, a proposed inhibitor of sirtuins, was sufficient to abolish most of p300-induced p53 acetylation. Importantly, SIRT1 inhibited p53-driven transcription in both *in vivo* targets (p21) and in reporter assays. Matching these, SIRT1 overexpression inhibited apoptosis in response to ionising radiation, etoposide treatment or indeed p53 overexpression alone but not Fas-induced apoptosis pointing to a specific effect on the p53 apoptotic pathway.

In an extension of these studies, Langley *et al.* provided evidence that p53 deacetylation by SIRT1 occurs in the context of promyelocytic leukemia (PML) nuclear bodies (Langley *et al.*, 2002). PML bodies are distinct foci in the cell nucleus with high concentrations of the PML protein and are known to be sites of regulation of transcription,

apoptosis, tumour suppression and the anti-viral response. They are dynamic structures that respond to a variety of cellular stresses and comprise cellular depots for several factors including the small ubiquitin-like modifier SUMO-1, the acetyltransferase CBP and the tumour suppressors pRb and p53. Upon overexpression of the PML isoform PML-IV, p53 is recruited to PML bodies, a translocation which correlates with acetylation of p53 in K382, its subsequent activation and induction of premature cellular senescence.

SIRT1 was found to be associated with PML-IV and localise to PML bodies in both primary and transformed cells that overxpressed PML-IV (Langley *et al.*, 2002). This localisation appeared to bear functional significance because PML-IV-induced cellular senesence was attenuated by co-expression of SIRT1, which correlated with decreased p53 acetylation at K382. This report supported the notion that SIRT1 may regulate cellular life-span through a pathway involving p53.

Fibroblasts derived from mice deficient for mSIRT1 show increased levels of p53 acetylation in response to DNA damaging agents. However, it was also found that by using acetylation-specific antibodies higher acetylation of residues other than K379, the mouse equivalent of K382, could also be observed suggesting an expanded specificity for SIRT1. Furthermore, despite hyperacetylation of p53, adriamycin fails to induce higher levels of p21, a p53 target, in *Sirt1*$^{-/-}$ MEFs a finding contradictory to the ones of Luo *et al.* and Vaziri *et al.*. Consistent with these, *Sirt1*$^{-/-}$ MEFs were equally sensitive to death induced by adriamycin and UV. In contrast, thymocytes of *Sirt1*$^{-/-}$ animals were hypersensitive to ionising radiation (IR)-induced cell death and this correlated with p53 hyperacetylation. Of note, *p53*$^{-/-}$ thymocytes were resistant to IR-induced death. This implied that the hypersensitivity of *Sirt1*$^{-/-}$ thymocytes was due to hypeactivation of the p53 pathway. However, the levels of p53 target genes were not assayed in this context leaving open the issue of whether p53 hyperacetylation in *Sirt1*$^{-/-}$ animals/cells actually affects a downstream transcription program.

SIRT1 is highly expressed in mouse ES cells (McBurney *et al.*, 2003b). Interestingly, ES cells fail to undergo G1 arrest upon treatment with ionising radiation despite the presence of p53 protein raising the possibility that p53 function is impaired due to presumed concomitantly high SIRT1 activity. This, however is not the case as *SIRT1*$^{-/-}$ ES cells show cell cycle profiles which are intistinguishable from those of *SIRT1*$^{+/+}$ ES cells (McBurney *et al.*, 2003b). Although the discrepancies between studies can be attributed to the different nature

of the cell systems employed, they also illustrate that the regulation of p53 by SIRT1 in the physiological context of an organism remains poorly understood.

This issue has been addressed in a recent study by Kamel *et al.* who generated mice defective in both *Sirt1* and *p53* genes (Kamel *et al.*, 2006). These animals had an overall phenotype indistinguishable from *Sirt1*$^{-/-}$ mice, including early post-natal lethality, eyelid closure defects and reduced size. Furthermore, although they could confirm the interaction of the two proteins and the effects on p53 acetylation, the authors could obtain no evidence of changes in p53-mediated gene transcription nor differential sensitivity of thymocytes/splenocytes to ionising radiation.

These results are in agreement with the study of Solomon *et al.* who identified compound EX-527 in a high-throughput screen for SIRT1 catalytic inhibitors and used it to acutely downregulate SIRT1 activity (Solomon *et al.*, 2006). EX-527 enhances p53 K382 acetylation upon different genotoxic stimuli (etoposide, adriamycin, hydroxyurea, H_2O_2) in a variety of human cell lines [NCI-H460 (large-cell lung carcinoma), U2-OS (osteosarcoma), MCF7 (breast carcinoma), HMEC (human mammary epithelial cells)]. This is not accompanied, however, by a concomitant change in p21 expression, nor cell viability or growth rate. Conversely, although TSA acted additively to EX-527 to induce p53 hyperacetylation, it also decreased stress-induced viability under the same experimental conditions.

Overall these studies suggested that SIRT1-mediated deacetylation as a potential novel mechanism of p53 regulation. This is an exciting observation because it would imply that in tumours that are treated with DNA-damaging agents, SIRT1 inhibition may potentiate apoptosis by allowing hyperactivation of the p53 apoptotic response and thus providing the basis for ameliorated cancer therapies. However, given the absense of effects on p53-mediated transcription and the accumulating evidence that SIRT1 inhibition may not affect viability in response to genotoxic stress, it would be of great importance to determine the functional relevance of SIRT1-catalysed p53 deacetylation.

SIRT1 autoregulation through HIC1 and p53 tumour suppressors

p53 functionally cooperates with a multiplicity of pathways that ultimately contribute to cancer initiation and progression. One such pathway of relevance here is regulated by the tumour supressor *HIC1* (hypermethylated in cancer 1). Monoallelic *HIC1* gene disruption results in a broad spectrum of cancers in mice in which the other allele of the gene is hypermethylated. This reflects the situation in human tumours where epigenetic inactivation of the gene occurs rather than mutation. Genetic evidence also suggests that *HIC1* and *p53* synergise in tumour suppression given that animals heterozygous for both genes have a distinct spectrum of tumours, with earlier appearance, increased prevalence and aggressiveness. Furthermore, *Hic1*$^{-/-}$ MEFs are more resistant to etoposide-induced death compared to wt MEFs. Conversely, MCF-7 cells overexpressing HIC1 are more sensitive to DNA damage-induced apoptosis.

The gene product of *HIC1* encodes a zinc-finger transcriptional repressor. In HIC1 protein, an N-terminal POZ domain and a central proline-rich domain mediate transcriptional repression in a manner both independent and dependent of HDAC1 respectively with correlating TSA sensitivities. Given the functional link to p53, an enzymatic target of the TSA-insensitive deacetylase SIRT1, Chen *et al.* investigated whether SIRT1 is the enzyme mediating HIC1 POZ-domain transcriptional repression. Indeed, SIRT1 was found to interact with the POZ domain of HIC1 (Chen *et al.*, 2005). Deletion of this domain partially abolishes the hypersensitivity of cells to etoposide-induced apoptosis.

Interestingly, *Hic1*$^{-/-}$ MEFs exhibit elevated levels of SIRT1 and tumors from *Hic1*$^{+/-}$ animals with inactivation of the second *HIC1* allele also show elevated levels of SIRT1. Both

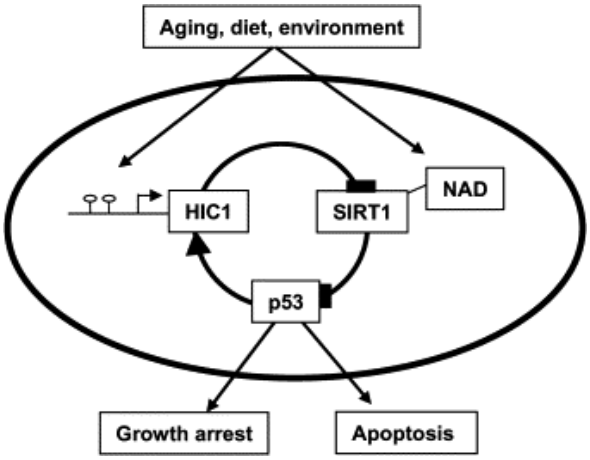


FIGURE 2-22. A Model for the Roles of HIC1 in Tumor Suppression. A circular regulation of HIC1, SIRT1, and p53 is proposed for modulation of cellular responses to DNA damage. HIC1 represses the transcription of SIRT1, SIRT1 deacetylates p53 post-transcriptionally, and p53 *trans*-activates HIC1.

Adapted from Chen *et al.*, 2005

reporter and ChIP assays confirmed that HIC1 but not a ΔPOZ species binds directly to the SIRT1 promoter *via* two $^{5'}$TGCC(A/C)$^{3'}$ sites in the same orientation. This implied that SIRT1 may regulate its own expression by complexing with HIC1 which proved to be the case since consecutive ChIP experiments confirmed the presence of both proteins on the *Sirt1* promoter. Importantly, transfection of dominant negative SIRT1 in *Hic1*$^{-/-}$ MEFs resulted in reduced resistance to apoptosis presumably by blunting the elevated levels of SIRT1 encountered in these cells. Furthermore, despite its accumulation in etoposide-treated cells, p53 acetylation was only evident in HIC1-overexpressing cells and, in reverse, siRNA-mediated downregulation of HIC1 resulted in SIRT1 accumulation and p53 hypoacetylation.

These data provided evidence for SIRT1 as a key effector of tumourigenesis in response to HIC1 inactivation and pointed to a molecular link between HIC1 and p53 tumour supressor pathways (FIGURE 2-22).

Regulation of forkhead transcription factors

The insulin signalling pathway has been described as a phylogenetically conserved signalling module in the determination of life-span. In *Drosophila* and *C. elegans*, the forkhead transcription factor *daf-16*, which is negatively regulated by the insulin pathway, is important for this effect on longevity (Giannakou *et al.*, 2004).

In mammals the functional orthologues of *daf-16* are believed to be the forkhead or winged helix family of transcription factors (TFs) which comprises four members, FOXO1 (or FKHR), FOXO3 (or FKHRL1), FOXO4 (or AFX) and FOXO6. Phosphorylation of these factors by PKB leads to their exclusion from the nucleus under conditions of growth factor availability. The identification of SIRT1 as a transcription factor deacetylase provided an entry point for the experimental validation of the hypothesis that mammalian sirtuins and forkhead factors functionally interact.

The acetyltransferase CBP enhances acetylation of both FOXO1 and FOXO4 when co-transfected in cultured cells while both p300 and PCAF have the same effect on FOXO3. In all cases complexes between FOXO TFs and the corresponding acetyltransferases were observed. Furthermore, FOXO TF acetylation increases upon some genotoxic stresses. Brunet *et al.* found that H_2O_2 and to a certain extend heat shock but not UV induce FOXO3

hyperacetylation in 293T cells (Brunet *et al.*, 2004). Increased FOXO3 acetylation correlated with enhanced binding to the acetyltransferase PCAF. In HeLa cells, Motta *et al.* also observed UV-induced FOXO3 hyperacetylation (Motta *et al.*, 2004). Consistent with these van der Horst *et al.* found that FOXO4 is hyperacetylated in response to H_2O_2 in HEK293T cells and this event also correlated with increased binding to acetyltransferase CBP. A more recent study by Kitamura *et al.* validated the acetylation-dependent binding of FOXO1 too to CBP in the pancreatic β-cell line βTC3.

FOXO3 acetylation was not altered by treatment of either TSA or nicotinamide alone but the combination of the two drugs resulted in a marked hyperacetylation of the protein suggesting that it is subject to regulation by both class I/II and class III deacetylases (Brunet *et al.*, 2004). Co-transfection experiments showed that FOXO1, FOXO3 and FOXO4 all interact with SIRT1. This interaction was observed mainly in response to H_2O_2 (Brunet *et al.*, 2004; van der Horst *et al.*, 2004) or after serum starvation (Daitoku *et al.*, 2004), conditions that were shown to result in nuclear accumulation of FOXO but in the case of FOXO3, Motta *et al.* could also observe the interaction in unstimulated cells in co-transfection experiments. Moreover, Yang *et al.* showed that the LIM-only protein FHL2 (four-and-a-half LIM 2) tethers SIRT1 to FOXO1 to induce its deacetylation (Yang *et al.*, 2005).

Mutation of the FOXO sites phosphorylated by PKB to alanines results in the constitutive translocation of FOXOs to the nucleus. Such a mutant species cannot interact with SIRT1 in the absense of stress stimuli suggesting that this interaction probably depends in additional modifications confered upon the protein(s) in response to such stimuli (Brunet *et al.*, 2004). Brunet *et al.* identified several stress-induced phosphorylation and acetylation sites in transfected FOXO3 which might contribute to this interaction.

These observations raised the question whether acetylation of play a role in the regulation of FOXO activity. FOXO1 and FOXO3 reporter activities were enhanced by contransfection of CBP and p300 respectively. This effect could arise either due to acetylation of histones or direct activation of FOXOs. Daitoku *et al.* identified putative acetylation sites on FOXO1 by similarity to known histone H2B acetylation sites and mutated them to arginine to create non-acetylatable species (Daitoku *et al.*, 2004). Surprisingly, some of these mutants either alone or in combination resulted in increased FOXO1 transcriptional activity. Comparison of the relative activation induced by either wild-type or non-acetylatable

mutants showed that although CBP could induce transcription driven by both species, it would do so more profoundly in the non-acetylatable mutants. This led to the conclusion that in this context CBP co-activated FOXO1-driven transcription by presumably deacetylating histones but would subsequently attenuate FOXO1 activity through its acetylation. Consistent with these conclusions, van der Horst *et al.* discovered that CBP inhibited the transcriptional activity of FOXO4. At present, it is not clear whether this applies to FOXO3 too, yet it demonstrates the potential pitfalls and complexity in interpreting such results.

Conversely, in order to determine whether SIRT1-driven deacetylation of FOXOs could alter their activity a variety of assays were employed. SIRT1 was shown to inhibit reporter acivity driven by wild-type and a constitutively nuclear FOXO3 as well as in cells overexpressing the lipid phosphatase PTEN and thus are expected to have low PKB activity and allow translocation of exogenous FOXO3 to the nucleus. This effect depended on SIRT1 deacetylase activity and was sensitive to nicotinamide only when SIRT1 was co-expressed (Motta *et al.*, 2004). SIRT1 could also inhibit reporter activity driven by constitutively nuclear FOXO1 and FOXO4 (Motta *et al.*, 2004). Although conceptually agreeable these results are surprising under the light of the observation that simple translocation of FOXO3 to the nucleus does not suffice to sustain an interaction with SIRT1 raising the question as to the mode of action of SIRT1 under these experimental conditions. On the other hand, Daitoku *et al.* could show that SIRT1 deacetylase activity enhanced CBP-driven FOXO1 co-activation in an additive manner.

In the light of these contradictory results it is interesting to know how SIRT1 affects FOXO target genes. In agreement with their reporter assay results, upon treatment of HEK293 cells with nicotinamide Daitoku *et al.* observed a decrease in the protein levels of the cell cylce inhibitor p27 and maganese superoxide dismutase (MnSOD), an enzyme involved in the detoxification of reactive oxygen species both FOXO1 target genes. Similarly, overexpression of SIRT1 but not a catalytically inactive mutant significantly enhanced the levels of these proteins in response to growth factor withdrawal. They also demonstrated the presense of SIRT1 along with CBP and FOXO1 in the promoters of *p27* and *MnSOD* genes. In agreement with the above conclusions is the study of van der Horst *et al.*, who found that FOXO4-driven *p27* and *MnSOD* expression is suppressed when SIRT1 are downregulated by RNAi. Interestingly, using the same cell line (HEK293) Motta *et al.* observed the opposite

effect on p27 protein expression when they transfected SIRT1 along with FOXO3. They also found enhanced activity of a reporter gene driven by a FOXO consensus DNA binding site in *Sirt1*$^{-/-}$ ES cells both in the presence and absense of exogenous FOXO3, supporting their findings of SIRT1 as a repressor of FOXO transcriptional activity.

Brunet *et al.* used a Rat-1 cell line derivative that was engineered to express a fusion of FOXO3 to the ligand binding domain of the oestrogen receptor (Brunet *et al.*, 2004). Upon addition of 4-hydroxytamoxifen (4-OHT), the fusion protein dimerises and translocates to the nucleus and is competent in driving transcription of FOXO target genes such as *p27* and *GADD45*. In these settings, the combination of TSA and nicotinamide suppresses *p27* and *GADD45* induction but does not affect another FOXO target gene *BIM* which is known to promote cell death. Accordingly, Brunet *et al.* note that SIRT1 also repressed FOXO-dependent transcription of a reporter under the control of the death cytokine *Fas* ligand gene promoter. They could also extend their findings in *Sirt1*$^{-/-}$ MEFs where expression of *GADD45* was impaired in response to treatment with the PI-3 kinase inhibitor LY294002, which allows FOXO translocation to the nucleus. Conversely, *BIM* expression was unaltered in *Sirt1*$^{-/-}$ MEFs. Motta *et al.* went further to investigate FOXO target gene expression in the liver and kidney of *Sirt1*$^{-/-}$ mice where they found enhanced expression of *PEPCK* and *IGFBP1*. Accordingly, p300, FOXO1, FOXO3 and SIRT1 itself are bound to the *IGFBP1* gene promoter in wildtype MEFs, as well as in *Sirt1*$^{-/-}$ MEFs despite the absense of SIRT1 protein from this site which suggests that SIRT1 does not prevent transcription factor recruitment (Motta *et al.*, 2004).

To investigate the functional consequences of FOXO coactivation by SIRT1 of *p27*, Brunet *et al.* used their FOXO3-ER Rat1 fibroblasts and could show that, in agreement with their observations on *p27* expression, SIRT1 enhanced the G1 cell cycle arrest driven by nuclear translocation of FOXO3. In agreement to this, exogenous *FOXO3* expression in *Sirt1*$^{-/-}$ MEFs was impaired in eliciting a G1 arrest compared to wild-type MEFs. Similar effects of SIRT1 on cell cycle progression were reported by van der Horst *et al.* in the DLD-1 human colon carcinoma cell line derivative DL-23 that inducibly expresses constitutively active FOXO3 as well as in transient transfections of constitutively active FOXO3 in A14 cells which are NIH3T3 mouse fibroblast derivatives that overexpress the human insulin receptor.

van der Horst *et al.* could also demonstrate a functional link for the observed SIRT1 effects on *MnSOD* expression (van der Horst *et al.*, 2004). They used a probe whose fluorescence intensity is sensitive to its oxidation status which in turn reflects cellular oxidative stress. Although FOXO3 alone could protect cells from H_2O_2-evoked oxidative stress, nicotinamide suppressed this effect. Brunet *et al.* provided further evidence supporting the cytoprotective role of SIRT1 against oxidative stress by showing that *Sirt1*$^{-/-}$ MEFs were more sensitive to H_2O_2 treatment. They could also show that FOXO-induced cell death is attenuated by SIRT1. Cerebellar granule neurons contransfected with FOXO3 and SIRT1 showed less apoptosis compared to cells expressing only FOXO3. Also, in their FOXO3-ER Rat1 fibroblasts the DNA damaging agent etoposide was less potent in inducing cell death in the presence of exogenous SIRT1.

Overall these data suggest a gene-specific role for SIRT1 in the regulation of FOXO-driven transcription. SIRT1 supports the function of FOXO in G1 cell cycle arrest by co-activating transcription of *p27* and supports its anti-oxidative capacity presumably through *MnSOD* and probalby other genes. On the other hand SIRT1 opposes the pro-apoptotic role of FOXOs. In the context of a cell under cytotoxic stress, SIRT1 could thus modulate FOXO-driven transcriptional responses in a way that promotes cell cycle arrest but opposes death allowing time for the cell to repair damage and eventually promote survival.

The inconsistent effects of SIRT1 on different FOXO target genes are not conpletely surprising. As discussed in the introduction, Ramaswamy *et al.* have identified distinct classes of FOXO target genes with accordingly different influence on cell cycle arrest and apoptosis (Ramaswamy *et al.*, 2002). Thus the observation of Motta *et al.* that SIRT1 can negatively regulate FOXO-driven expression of *IGFBP1* and *PEPCK*, as opposed to what was shown by other groups for *p27* and *GADD45* may reflect the fact that these genes exhibit different promoter requirements for their FOXO-mediated activation. Interestingly, the study of Ramaswamy *et al.* also showed that downregulation of *p27* is not necessary for FOXO-induced cell cycle arrest which rather driven by FOXO-mediated repression of cyclinD1 (Ramaswamy *et al.*, 2002). It would be of interest to investigate whether SIRT1 mediates cell cycle arrest exclusively through *p27* or is also shared by the cyclinD1 branch of FOXO-driven transcription.

Recently, a molecular link between FOXO1 and PML, both of which are binding partners of SIRT1, was discovered and provided a physiological setting where these interactions might hold a major role. In particular, they investigated the role of FOXO1 in the protection of pancreatic β-cells from glucose-induced toxicity. Chronic exposure to high glucose such as in diabetic patients, is thought to overload β-cells' glycolytic capacity ultimately resulting in the production of excess reactive oxygen species (ROS). By nature, β-cells have reduced capacity to deal with these agents which in turn have been proposed to impair expression of the *Insulin2* (*Ins2*) gene trascription factor Pdx1 leading to increased apoptosis.

Kitamura *et al.* found that induction of ROS leads to relocation of FOXO1 to nuclear PML bodies, an event shown to depend on its acetylation (Kitamura *et al.*, 2005). Concomitant to this relocation, FOXO1 activates the transcription of two *Ins2* transcription factors, MafA and NeuroD to protect β-cells from death due to hyperglycaemia. In contrast to its requirement for localisation to PML bodies, the transcriptional activity of FOXO1 requires its deacetylation. Hypoacetylated FOXO1 is highly active in transcription but has a high ubiquitin-mediated turnover. FOXO1 ubiquitination is increased in cells overexpressing SIRT1. In this respect, it is implied, although not experimentally proven, that SIRT1-driven deacetylation promotes the transcriptional activity of FOXO1 while inducing its increased turnover. Thus, SIRT1 may have a cytoprotective role for pancreatic β-cells *in vivo* by co-activating FOXO1-driven expression of *MafA* and *NeuroD*.

Transcriptional regulation of *SIRT1* by p53 and FOXO

All the evidence described above supports a role for SIRT1 as a regulator of FOXO function. Another study by Nemoto *et al.* showed that FOXO may be itself a regulator of SIRT1 expression (Nemoto *et al.*, 2004). They could show that the promoter of the *Sirt1* gene mediates the induction of a reporter gene transcription in response to glucose and growth factor starvation in PC12 cells. Under these conditions, FOXO was found in the nucleus and showed increased transcriptional activity. siRNA-mediated decrease of FOXO3 by siRNA could suppress the activity of the *SIRT1* promoter under starvation suggesting that FOXO3 transcriptionally regulates *SIRT1*. Interestingly, SIRT1 mRNA levels increased upon

overnight fasting in the muscle and liver but not in the heart of mice. This is in contrast to the findings of Rodgers *et al.* who found increased SIRT1 protein levels in mouse liver upon fasting without concomitant increase of mRNA levels (*see* below) (Rodgers *et al.* 2005).

Nemoto *et al.* found that the region between nucleotides -202 and -91 from *SIRT1* transcription start site is important for starvation-inducible induction of the gene but could identify no consensus forkhead binding sites within it. However, they determined two p53 consensus sites that when mutated alone or together they could abolish FOXO3-stimulated *SIRT1* promoter activity. This suggested that FOXO3 and p53 synergise in the regulation of *SIRT1* gene transcription. FOXO3 and p53 were shown to physically interact both *in vitro* and when co-transfected *in vivo* and this interaction increased upon growth factor withdrawal. p53 inhibited the transcription of a reporter gene driven by the *SIRT1* promoter p53 binding element and this was aleviated by co-expression of FOXO3. In parallel, FOXO3 could also inhibit p53-driven reporter activity driven by an alternative p53 binding element suggesting that the physical interaction of FOXO3 with p53 in promoters had antagonistic effects on the latter's transcriptional activity. In accordance to these results, basal *SIRT1* mRNA levels were elevated in the adipose tissue of $p53^{-/-}$ mice but the starvation-induced induction of its transcription was abolished.

Taken together, these data suggested that in response to nutrient limitation, SIRT1 is upregulated *via* a transcriptional mechanism involving the alleviation of p53-mediated repression of its promoter by FOXO3.

Overall, there is compelling evidence that SIRT1 interacts physically and functionally with several forkhead transcripton factors in a reciprocal manner. Furthermore, the recent findings that p53 can also participate in the determination of organismal longevity and the interconnection between PML, which by itself regulates senescence, forkhead transcription factors and SIRT1 implicates a complex regulatory mechanism that may govern cellular and organismal life-span.

NFκB

The transcription factor NFκB is known to hold important roles in acute inflammatory responses and cell survival by mediating the expression of genes with of a broad spectrum of

functions including cytokines and apoptotic inhibitors (Chen and Greene, 2004). The NFκB family comprises NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel and act as homo- or hetero-dimers in a variatey of subunit combinations. NF-κB1 and NF-κB2 are synthesized as large precursors, p105 and p100, that are post-translationally processed to the DNA-binding subunits p50 and p52, respectively, which by themselves lack transcriptional activity. A transcriptional activation domain is present in the Rel members of the family. In unchallenged cells, NFκB dimers are bound to inhibitor of NFκB (IκB) and are held in the cytoplasm. Two pathways that lead to the activation of NFκB transcription have been described, known as the classical and the alternative pathway (FIGURE 2-23). In the classical pathway, activation of cellular receptors by signals such as pro-inflammatory cytokines and bacterial cell-wall components activate the IκB kinase (IKK) which comprises three subunits: α and β with catalytic activity and the regulatory γ subunit. Phosphorylation of IκB by IKKβ leads to its proteasome-mediated degradation and subsequent release of NFκB heterodimers which can translocate to the nucleus and activate target genes. The alternative pathway is independent of IKKβ and IKKγ. IKKα homodimers are activated upon receptor stimuation by the upstream NFκB inducing kinase (NIK) and in turn phosphorylate and lead to the proteasome-mediated processing of p100. The processed p52 subunit acts as a heterodimer with RelB to translocate to the nucleus and activate transcription.

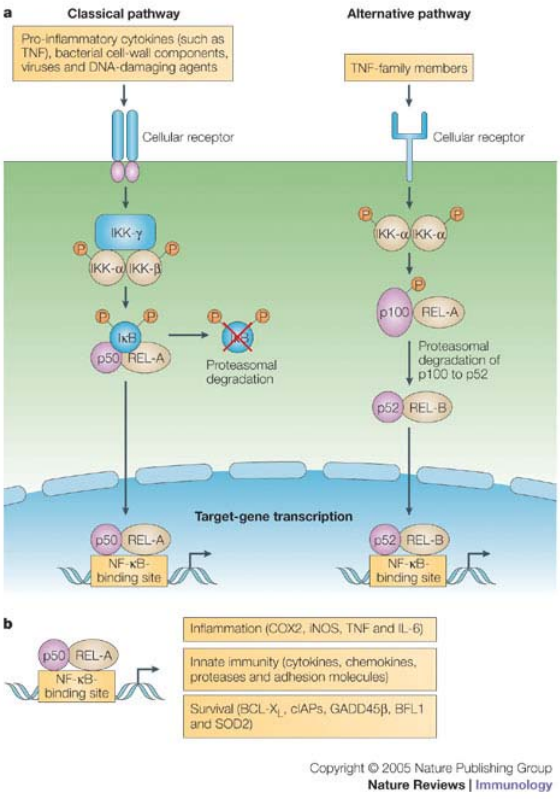


FIGURE 2-23. Regulation of HIV Tat transcriptional activity by acetylation.
Adapted from Chen and Greene, 2003

As in every multistep cellular process, the NFκB pathway offers multiple levels for regulatory inputs but the transcription factor itself is subject to a variety of modulatory events. Phosphorylation of RelA/p65 by PKA, MAP kinases and PKCζ allows the phosphorylation-dependent binding of co-activators including CREB binding protein (CBP) and p300 both of which exhibit histone acetyltransferase (HAT) activity. Acetylation of RelA/p65 at specific lysines affects both DNA binding and transcriptional activity. Recent reports also suggest that RelA/p65 acetylation reduces its affinity for IκB and deacetylation by class I HDACs promotes its nuclear export in a IκB-dependent manner.

The observation that resveratrol, a sirtuin activator, is also a potent NFκB transcriptional inhibitor, led Yeung *et al.* to investigate whether SIRT1 is required for this inhibitory effect. They confirmed that pharmacological activation of sirtuin activity with resveratrol suppressed TNFα-induced NFκB activation of reporter activity while the sirtuin inhibitors splitomycin and nicotinamide had the reverse effect (Yeung *et al.*, 2004). Interestingly, the latter compounds also increased basal reporter activity in the absence of TNFα, a fact not discussed by the authors but that could signify a role for sirtuin-mediated inhibition of basal transcription as observed in the work of Vaquero *et al.* discussed earlier. SIRT1 co-transfection inhibited TNFα-induced NFκB reporter activity suggesting that the observed effects of the pharmacological study were mediated by this sirtuin.

The authors went on to determine that it was the transcriptional activity of the RelA/p65 subunit that was sensitive to SIRT1 dose pointing at a potential regulatory site for SIRT1 on NFκB dimers. Indeed, co-transfection as well as endigenous co-immunoprecipitation experiments showed that SIRT1 and p65 interact. Importantly, resveratrol treatment did not alter IκB degradation kinetics, nuclear accumulation kinetics of neither p65 nor p50, nor did it affect their DNA binding capacity. These suggested that SIRT1 may modulate this factor at the level of its transcriptional activity.

It could subsequently be shown that SIRT1 acts through the transactivation domain of p65 by deacetylating it at lysine 310. Furthermore, a p65 transactivation domain mutant for the acetylateable residue (K310) was no longer subject to resveratrol- and SIRT1-mediated repression confirming that NFκB activity is modulated by SIRT1 through deacetylation of the transactivation domain. Importantly, these events are significant for cellular homeostasis

because NFκB inhibition by resveratrol sensitised cells to TNFα-induced apoptosis in a SIRT1-dependent manner. As expected, this was due to suppression of TNFα-induced expression of NFκB anti-apoptotic target genes, such as cIAP and Bcl-X_L. Interestingly, resveratrol seems to potentiate SIRT1 occupancy of target promoters which correlated well with the observed repression of target genes.

This study brought forward an aspect of SIRT1 biology which conflicted previous reports supporting its role as an antiapoptotic factor, e.g. in the context of p53 and FOXO. This is not entirely surprising since the signalling cascades involved in the activation of these pathways are seemingly divergent. Thus while DNA damage would engage the p53 response, inflammatory stimuli such as TNFα would signal through the NFκB pathway. However, it is also increasingly appreciated that under specific contexts, there is extensive cross-talk between these pathways which establishes an elaborate mechanism determining cell survival (Janes *et al.*, 2006; Janes *et al.*, 2005).

Ku70

Ku70 is a protein involved in the DNA repair pathway and has been shown to be a substrate for the DNA-dependent protein kinase DNA-PK. As a heterodimer with Ku80 it participates in non-homologous end-joining (NHEJ), V(D)J recombination and telomere maintenance. Consequently, Ku70 null knockout mice exhibit higher sensitivity to ionising radiation, are immunocompromised and show high rates of apoptosis in the developing nervous system. The latter phenomenon is attributed to the ability of Ku70 to associate with the pro-apoptotic factor Bax in the cytosol in a complex devoid of Ku80 and sequester Bax from mitochondria. In response to apoptotic stimuli Bax translocates to the mitochondrial outer membrane, oligomerises and renders it permeable eliciting the release of cytochrome c and other death-promoting factors.

The interaction between Ku70 and Bax is regulated by the acetylation status of Ku70. When Ku70 is acetylated by the acetyltransferases CBP and PCAF it dissociates from Bax allowing it to translocate to mitochondria (Cohen *et al.*, 2004a). Several lysines were shown to be acetylated *in vivo* but K539 and K542 appeared to be critical for this regulation given that

cells expressing Ku70 with either of these lysines substituted with glutamines to mimic acetylation, were more sensitive to apoptosis.

Sirtuins were implicated in the regulation of Bax-mediated apoptosis on account of the fact that Ku70 acetylation is sensitive to both TSA and nicotinamide, a sirtuin inhibitor. In this way, deacetylase-mediated sequestration of Bax by Ku70 would promote survival. In a later report, Cohen *et al.* showed that SIRT1 is a Ku70 deacetylase which led them to propose that caloric restriction promotes survival at the cellular level and increased life-span at the organismal level by supressing Bax-mediated apoptosis in a manner dependend on SIRT1 (Cohen *et al.*, 2004b).

2.3.5.1.5 SIRT1 regulation of transcription factors involved in muscle differentiation

Repression of MyoD by SIRT1

Intracellular redox state is finely regulated in muscle cells in particular during differentiation when a drop in the $[NAD^+]/[NADH]$ ratio is observed. This prompted Fulco *et al.* to investigate the role of SIRT1 in muscle differentiation due to the NAD^+ dependence of the enzyme (Fulco *et al.*, 2003). MyoD is a basic helix-loop-helix (bHLH) transcription factor which collaborates with the MEF2 (myocyte enhancer factor 2) transcription factor to induce gene expression programmes that dictate muscle differentiation.

Inhibitor studies indicated that mSIRT1 supressed transcription driven by MyoD and MEF2 and attenuated muscle differentiation by global downregulation of muscle-specific genes. mSIRT1 was found in a ternary complex with PCAF and MyoD and was able to deacetylate both factors *in vitro* and *in vivo*. Since MyoD acetylation is important for its full transcriptional activity, it is possible that deacetylation of MyoD by SIRT1 mediates the effects of the later on muscle differentiation (Fulco *et al.*, 2003). Conversely, the authors demonstrated that mSIRT1 activity regulates histone H3 K9 and K14 acetylation in muscle-specific gene promoters implying that SIRT1 may regulate promoter accessibility directly through chromatin modifications. Importantly, by modulating the intracellular $[NAD^+]/[NADH]$ ratio coupled to RNA interference-mediates mSIRT1 downregulation the authors

demostrated the effects of intracellular redox state on muscle differentiation are mediated through mSIRT1.

Functional interaction of SIRT1 with HDAC4 to regulate MEF2 repression

The class II deacetylase HDAC4 has been shown to interact with MEF2 *via* an N-terminal MITR (MEF2-interacting transcriptional repression) homology domain and repress muscle-specific gene expression. This function has been assumed to be performed via promoter specific HDAC4-mediated histone deacetylation. Nevertheless, deacetylase-independent transcriptional repression activity of HDAC4 has also been reported.

SUMO (small ubiquitin-like modifier) is a small protein that can be covalently linked to lysine residues of other proteins and has been implicated in transcriptional repression. Similar to ubiquitination, protein sumoylation requires an E1 activating enzyme, an E2 SUMO conjugating enzyme (Ubc9) and a SUMO E3 ligase several of which have been identified.

In a yeast two-hybrid screen, Ubc9 was found to bind to an N-terminal coiled-coil region of HDAC4 previously shown to mediate interaction with MEF2 (Zhao *et al.*, 2005). HDAC4 was shown to be sumoylated on K559, however mutations in this residue failed to reveal an effect on the ability of HDAC4 to repress MEF2 transcriptional activity. Zhao *et al.* subsequently showed that MEF2 is itself sumoylated at K424 and HDAC4 could enhance MEF2 sumoylation both when co-transfected in cultured cells or in *in vitro* sumoylation assays. This suggested that HDAC4 recruits the SUMO conjugating complex to modify MEF2.

Interestingly, MEF2 K424 is also acetylated directly by the MEF2 co-activator CBP yet this acetylation is not sensitive neither to HDAC4 overexpression nor to TSA treatment. MEF2 K424 is, however, sensitive to the sirtuin inhibitor nicotinamide and SIRT1 was shown to interact with HDAC4 and reverse CBP-mediated MEF2 acetylation in co-transfection experiments.

As MEF2 acetylation increases during myocyte differentiation Zhao *et al.* predicted that K424 sumoylation would have an inhibitory effect on MEF2 function. Indeed, fusion of SUMO to MEF2 impedes its ability to interact with MyoD in activation of reporter gene

transcription while a sumoylation-deficient mutant of MEF2 shows higher activity in the same assay.

These data led the authors to propose a model where HDAC4 and SIRT1 synergise to induce MEF2 respression. SIRT1 deacetylates MEF2 at K424 to allow its sumoylation by the SUMO-conjugating activity recruited by HDAC4. This is a first indication that SIRT1 acts in concert with another HDAC to repress gene expression in a mechanism other than histone deacetylation.

2.3.5.1.6 Genetic and biochemical interactions of SIRT1 with proteins regulating metabolism

The implication of sirtuins in regulation of life-span upon caloric restriction in lower eucaryotes led to the hypothesis that they may mediate metabolic changes which in turn promote longevity. In this respect genetic ablation of the insulin receptor in mice leads to 18% increase in mean life-span while as discussed *C. elegans sir2.1* interacts genetically with the insulin signalling pathway. Recent studies have shed light into potential mechanisms that employ SIRT1 to regulate cellular metabolic pathways.

SIRT1 and the regulation of fat metabolism

Picard *et al.* used the mouse cell line 3T3-L1 which can be induced to differentiate into cells with adipocytic characteristics including expression of adipose-specific differentiation markers and triglyceride accumulation in cytoplasmic lipid droplets (Picard *et al.*, 2004). Upon differentiation, 3T3-L1 cells with downregulated levels of mSIRT1 by means of a vector-driven short hairpin RNA showed increased accumulation of intracellular triglycerides (TG) while the converse was observed in cells overepxpressing SIRT1. This behaviour was paralleled by the levels of adipogenic factors such as PPARγ, C/EBPδ, C/EBPα and aP2 respectively. This implied a role of SIRT1 in adipogenesis.

To investigate whether SIRT1 affects fat metabolism in differentiated adipocytes, the authors used the sirtuin activator resveratrol to stimulate the activity of mSIRT1 and also

observed suppression of TG accumulation while the levels of free fatty acids (FFA) in the medium were elevated. Picard *et al.* could recapitulate these findings in primary rat adipocytes treated with resveratrol. To investigate the molecular mechanism underlying this behaviour, chromatin immunopreciptiation (ChIP) was employed. mSIRT1 was found bound to the aP2 and PPARγ promoters implying that it may exert its adipogenesis-suppressing role by inhibiting the action of adipogenesis-promoting factors. Indeed, mSIRT1 can directly bind to both PPARγ and its transcriptional repressor NCoR in the same promoters where PPARγ is found. Importantly, NCoR depletion by shRNA abrogated the suppressive action of SIRT1 on fat accumulation. The authors extended their findings in the mouse and showed that mSIRT1 is bound to PPARγ and aP2 promoters in mice that had been fasted, a condition characterised by high FFA serum content. Consistent with this, *Sirt1*$^{+/-}$ animals exhibited lower FFA serum levels upon fasting than *Sirt1*$^{+/+}$ animals and adipocytes from *Sirt1*$^{+/-}$ animals also had suppressed FFA mobilisation upon adrenergic stimulation. In all, these findings suggested that SIRT1 modulates circulating FFA levels by suppressing the function of adipogenic transcription factors.

Although the data in this study are convincing and well-controllled with respect to the experimental setups employed, there are two issues worth of further investigation. Firstly, the authors showed that upon induction of 3T3-L1 cells to differentiate, endogenous mSIRT1 levels increase. This behaviour is reminiscent of that of the adipogenesis-*promoting* factor PPARγ and contradicts the proposed role of SIRT1 as an *inhibitor* of adipogenesis. Secondly, the proposed mechanism of SIRT1 regulation of fat metabolism would suggest that suppression of adipogenesis, which is characterised by accumulation of TG, is sufficient to induce the release of FFA. To the best of my knowledge little evidence exists to support this. It is possible that absence of SIRT1 supresses FFA production upon fasting by a machanism related to TG turnover such as lipolysis an aspect worth investigating, in particular through assaying the activity of TG mobilising enzymes such as hormone-sensitive lipase (HSL) and the recently discovered adipose triglyceride lipase (ATGL)(Zehner *et al.*, 2005; Zimmermann *et al.*, 2004).

SIRT1 and the regulation of glucose homeostasis

Blood glucose levels are tightly regulated through elaborate molecular mechanisms involving several tissues. This guarantees that tissues such as the brain and red blood cells where glucose is the primary fuel used for energy production are in constant supply.

SIRT1 regulation of fasting hepatic gluconeogenesis. Another important regulator of energy homeostasis is peroxisome proiferator-activated receptor-γ coactivator 1α (PGC-1α). PGC-1α is required for a diverse set of metabolic processes such as mitochondrial oxidative metabolism in brown adipose tissue, fiber-type switching in skeletal muscle and gluconeogenesis and stimulation of fatty acid oxidation in the liver upon fasting. Fasting liver metabolism is dictated by the actions of hormones as well as nutrients the mode of action of the latter being largely elucive.

As some aspects of fasting (controlled by PGC-1α) recapitulate some of the effects of caloric restriction (proposed to be regulated by sirtuins), Rodgers *et al.* examined the potential role of SIRT1 in glucose homeostasis. They found that although mSIRT1 mRNA levels do not change in the livers of fasted animals, protein levels increase (Rodgers *et al.* 2005). They also observed that fasting livers have elevated NAD^+ levels. These observations indicated that mSIRT1 has a role in fasting liver responses.

The authors went on to show that the observed elevation of mSIRT1 protein is due to increased translation and that it can be positively regulated by the levels of pyruvate, a metabolite derived from blood alanine by the fasting liver and used as a substrate for gluconeogenesis. Conversely, glucose causes a decrease in mSIRT1 protein levels. PGC-1α activates the gluconeogenic programme through its interaction with HNF4α. SIRT1 is found in a complex with both proteins and regulates the acetylation status of PGC-1α. Acetylated PGC-1α has decreased capacity to co-activate HNF4α in response to pyruvate and downregulation of mSIRT1 by siRNA abolishes the ability of PGC-1α to induce gluconeogenic genes such as *PEPCK* and *Glucose-6-phosphatase*. Interestingly, this SIRT1 dependence is not extended to the mitochondrial genes regulated by PGC-1α. PGC-1α and SIRT1 also synergise to suppress glycolysis and in combination with the co-ordinated enhancement of gluconeogenesis they result in elevation of glucose production in

hepatocytes. Taken together these data provided solid evidence for a role of SIRT1 in regulating glucose production in the liver through the transcriptional co-activator PGC-1α (Rodgers *et al.* 2005).

SIRT1 regulation of insulin secretion. A major role in acute glucose regulation at the organismal level is played by the pancreas. The exocrine pancreas which comprises the bulk of the organ is largely involved in the excretion of digestive enzymes. Embedded within this exocrine tissue are clusters of cells known as the islets of Langerhans which are involved in the endocrine functions of the organ. Three major cell types are found in the islets of Langerhans and are the sites of secretion of the major hormones produced therein. The α-cells, which secrete glucagon, the β-cells which secrete insulin and the δ cells which secrete somatostatin. Defects in pancreatic function, expecially in β-cells as encountered in disease such as diabetes have detrimental effects on animal physiology.

Pancreatic β-cells respond to elevated levels of blood glucose to secrete insulin which acts in peripheral tissues such as the muscle to stimulate glucose uptake and adipose tissue to increase energy storage in the form of triglycerides. The basic components of glucose-stimulated insulin secretion in β-cells have been described. Glucose catabolism results in increased ATP/ADP ratios that lead to the closing of ATP-dependent K^+ channels. This causes membrane depolarisation which in turn activates voltage-gated Ca^{2+} and induces calcium influx that then allows intracellular vesicles containing insulin to fuse with the plasma membrane and secrete their contents. Proteins that modify this pathway have been described, notably uncoupling protein 2 (UCP2) which uncouples proton entry to mitochondria from ATP production. The action of UCP2 results in lower ATP/ADP levels and decreased insulin secretion. Indeed *Ucp2*$^{-/-}$ knockout animals show increased islet ATP and increased glucose-stimulated insulin secretion. UCP2 levels were elevated in *ob/ob* mice, a genetic model of obesity-induced diabetes, and this elevation correlated with increased insulin lelvels and hypoglycemia.

Moynihan *et al.* (Moynihan *et al.*, 2005) and Bordone *et al.* (Bordone *et al.*, 2005) independently described a role for mSIRT1 in β-cell function. Both groups' work was triggered by the observation that mSIRT1 is expressed in the pancreatic islets. Moyihan *et*

al., clearly showed that mSIRT1 shows higher expression in α-cells where it localises to the cytoplasm while a weak β-cell specific staining was also observed.

To investigate the physiological significance of this observation, Moyihan *et al.* created a transgenic mouse that expressed mSIRT1 cDNA under the control of the human insulin promoter (Moynihan *et al.*, 2005). They termed these animals BESTO for β-cell-specific *Sirt1*-overexpressing. Conversely, Bordone *et al.* made use of the *Sirt1*$^{-/-}$ animals generated by McBurney *et al.* as a loss-of-function model (Bordone *et al.*, 2005).

BESTO mice exhibited normal insulin and glucose levels in both fed and fasted state but when challenged with high glucose they showed better glucose tolerance attributed to elevated insulin levels. *Sirt1*$^{-/-}$ animals had constitutively lower levels of insulin and completely impaired insulin secretion upon glucose injection. Surprisingly, glucose levels in *Sirt1*$^{-/-}$ mice were lower compared to wild-type controls while these mice also consumed more food, suggesting that they have improved insulin sensitivity. In their respective models, both groups confirmed that islet morphology and gross mass did not differ from control preparations. Islets isolated from *Sirt1*$^{-/-}$ animals, however were defective in glucose-induced insulin secretion while pancreata of BESTO mice showed elevated insulin levels when perfused with glucose.

In an attempt to understand the impaired insulin secretion phenotype of *Sirt1*$^{-/-}$ islets, Bordone *et al.* used INS-1 cells which are rat pancreatic cell lines where they downregulated SIRT1 by means of shRNA to exclude differential expression of proteins involved in glucose uptake and insulin secretion. However, they observed suppressed levels of ATP/ADP in the knockdown cells which implied that respiration in these cells is more uncoupled compared to control cells. Upon examination the levels of UCP2 protein were found to be higher in both INS-1 knockdown cells and the pancreata of *Sirt1*$^{-/-}$ mice. Furthermore, ChIP experiments showed that mSIRT1 binds directly the *Ucp2* promoter to suppress expression of the gene. Using gene expression profiling of derivatives of MIN6, a mouse β-cell line, where mSIRT1 levels were modulated, Moynihan *et al.* reached largely the same conclusions. Interestingly, Bordone *et al.* measured the NAD^+/NADH in the pancreata of starved animals and found in to be decreased. This would lead to a decrease in SIRT1 activity. In agreement to this, UCP2 levels were high in starved wild-type animals.

β-cell disfunction is associated with diseases such as diabetes and positively correlates with old age. mSIRT1 expression in the pancreas was clearly demonstrated although the focus of the above work was on β-cells despite the significant presence of mSIRT1 in α-cells too. However, the above data are significant because they provide the first evidence of a physiological system where modulation of SIRT1 activity can be considered as a drug target and paved the way for further investigation of its role in metabolic regulation which the major effect of caloric restriction is thought to be exerted upon.

2.3.5.1.7 Neuroprotection and cardioprotection by SIRT1

Degeneration of neuronal axons is a feature of many neuropathies such as Parkinson's and Alzheimer's disease. In Wallerian degeneration slow (w*ld*^{s}) mice, a spontaneous dominant mutation results in the overexpression of Wld^s, a chimaeric nuclear protein comprising the N-terminal 70 aminoacids of Ufd2a (ubiquitin fusion degradation protein 2a) which participates in ubiquitin chain assembly and the entire sequence of Nmnat1 which participates in nuclear NAD^+ biosynthesis (*see* section 2.2.2.1). These mice exhibit delayed axonal degeneration.

Araki *et al.* determined that the enzymatic activity of Nmnat1 is required for the neuroprotective effects of Wld^s in response to various damaging agents such as ischaemia and actin poisons as well as mechanical ablation of axons suggesting a role of nuclear NAD^+ in this process which they called NAD^+-dependent axonal protection (NDAP) (Araki *et al.*, 2004). In agreement to this, exogenously provided NAD^+ also had a protective effect in neuronal cultures. Araki *et al.* hypothesised that *de novo* gene expression is required for NDAP as these were evident only after >8 hours of pretreatment with NAD^+.

Using sirtinol and resveratrol, an inhibitor and activator of sirtuins respectively as well as lentivirus-expressed shRNA against SIRT1, Araki *et al.* could demonstrate that NDAP required SIRT1 consistent with the dependence of its activity on NAD^+. Interestingly, they could observe no change in cellular NAD^+ concentrations suggesting that because of the nuclear localisation of Wld^s it is only the nuclear pool of NAD^+ that is relevant in NDAP (Araki *et al.* 2004).

Similarly, pharmacological evidence suggests a role for sirtuins in cardioprotection and cardiac hypertrophy as SIRT1 overexpression protected cardiomyocytes from serum deprivation-induced death and casued an increased cell size (Alcendor *et al.*, 2004). Conversely, sirtuin inhibition induced cell death in isolated neonatal rat cardiomyocytes. These effects are likely mediated by SIRT1 since in a dog model of heart failure, SIRT1 was specifically found to be upregulated (Crow, 2004).

2.3.5.1.8 SIRT1 and caloric restriction in rodents

As sirtuins in lower organisms mediate in part the effects of caloric restriction on longevity, it is of great interest to examine whether this constitutes a conserved function of mammalian sirtuins. At least two pieces of evidence indicate that this is the case.

Caloric restriction in mice can be implemented by reducing food availability to 60% of normal food intake. Under these conditions, complex physiological and behavioural changes are observed such as a drop in blood plasma triglyceride, glucose and IGF-1 levels and increase in animal movement. In response to caloric restriction, mice carrying a genetic ablation of *SIRT1* alleles, exhibit reduced or absent increases of physical activity (Chen *et al.*, 2005). This is in spite of changes in plasma markers of caloric restriction comparable to wildtype animals and enhanced performance in movement capacity assays such as accelerating treadmil or rotarod. This suggests that SIRT1 mediates at least some behavioural effects of caloric restriction, possibly through its action in movement centers of the central nervous system where SIRT1 has been shown to be strognly expressed (Sakamoto *et al.*, 2003).

In a separate study, Nisolo *et al.* observed a marked increase of mitochondrial biogenesis and respiration in mice under a caloric restriction regimen (Nisolo *et al.*, 2005). This correlated with increased expression of key mitochonrial biogenesis regulators such as PGC-1α, nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (Tfam) in WAT. This response was markedly reduced in mice null for endothelial nitric oxide synthase gene (*eNOS*$^{-/-}$) while eNOS mRNA levels were induced upon caloric restriction in wild-type mice uggesting that it mediates the effects of caloric restriction on mitochondrial biogenesis.

Interestingly, SIRT1 mRNA and protein level changes paralleled those of mitochondrial biogenesis genes and were also shown to depend on the presence of eNOS in WAT as well as other tissues. The same could be recapitulated in cultured white adipocytes where treatment with NO donors or a cGMP analogue (mimicking a second messenger downstream of eNOS) induced a significant increase in SIRT1 protein levels. Thus SIRT1 expression is upregulated in WAT upon caloric restriction by a mechanism involving eNOS-mediated signaling.

In this study, the expression of both PGC-1α and SIRT1 in induced upon caloric restriction suggesting a potential functional interaction of the two proteins in WAT similar to the one seen in the liver (Rodgers *et al.*, 2005). However, in the latter case Rodgers *et al.* showed that SIRT1 synergises with PGC-1α for the expression of genes involved in hepatic gluconeogenesis but not mitochondrial biogenesis (Rodgers *et al.*, 2005). It is possible that the interaction of SIRT1 with PGC-1α in WAT does occur and is distinct from that in the liver owing to the differing function of the two tissues upon fasting.

2.3.5.2 SIRT2

SIRT2 localises to the cytoplasm and in particular is found associated with microtubules. Concomitant to this property, SIRT2 is the only sirtuin that deacetylates tubulin while it shares this function with a member of class II deacetylases, HDAC6 (Hubbert *et al.*, 2002). SIRT2 and HDAC6 interact *in vivo* and siRNA-mediated knock-down of either enzyme alone is sufficient to induce tubulin hyperacetylation (North *et al.*, 2003). Crucially, the authors did not investigate the effect of both SIRT2 and HDAC6 protein knock-down which would shed light on the interdependency of their activities on tubulin. Interestingly though, SIRT2 and HDAC6 differ in that among the two only SIRT2 can deacetylate microtubules purified from cell lysates while both enzymes can deacetylate *in vitro*-assembled microtubules. Also, as predicted, the tubulin deacetylase activity of SIRT2 is NAD^+-dependent. These observations imply the presence of an HDAC6-inhibitory microtubule-associated protein(s) (MAPs) and point to differential regulation of these tubulin deacetylases.

Microtubule dynamics play central role in cell motility and in settings where cell movement is thought to participate in disease, such as cancer metastasis. HDAC6

overexpressing cells showed increased motility in transwell migration assays, a property thought to be conferred by decreased levels of stable microtubules due to higher deacetylation rates (Hubbert *et al.*, 2002). However, although high acetylation levels of tubulin correlate with stabilised microtubules, it has been an issue of debate whether microtubule acetylation is a cause or a consequence of microtubule stability. In another report, Palazzo *et al.* showed that HDAC6 inhibition by TSA, while inducing tubulin hyperacetylation, fails to induce tubulin detyrosynation (Palazzo *et al.*, 2003). Tubulin detyrosynation by itself does not induce microtubule stabilisation, rather stable microtubules accumulate this modification and thus it is considered a *bona fide* marker of microtubule stability. Based on this, it was proposed that the effects of microtubule acetylation changes on cell motility are not due to influences on microtubule stability but on the activity of other factors such as MAPs and motor proteins which in turn might influence such events as endocytic vesicle recycling and recruitment of intermediate filaments to microtubules.

Dryden *et al.* reported that SIRT2 abundance increases during mitosis and that SIRT2 is subject to a λ-phosphatase-sensitive modification occuring in cells arrested with nocodazole (Dryden *et al.*, 2003). Stable overexpression of SIRT2 but not of a catalytically inactive mutant delayed exit from a nocodazole-induced mitotic arrest. In *S. cereviciae* exit from mitosis depends on a signalling network known as the mitotic exit network (MEN). A key molecule in this system is the phosphatase Cdc14p, which dephosphorylates the mitosis-specific kinase Cdc28/Clb.

Two orthologues of the yeast Cdc14 have been described in humans, Cdc14A and Cdc14B (Mailand *et al.*, 2002). Transfection of Cdc14B in cells stably expressing SIRT2 led to decrease in SIRT2 levels while a catalytically inactive mutant of the phosphatase failed to have the same effect (Dryden *et al.*, 2003). The loss of SIRT2 was shown to be reversible by treatment with proteasome inhibitors suggesting that phosphorylation may regulate SIRT2 stability during cell cycle progression. Corroborating to this was the fact that co-transfection of SIRT2 with ubiquitin allowed the detection of ubiquitinated SIRT2. It would be of great interest to investigate whether the proposed role of SIRT2 in mitosis is exerted through its microtubule deacetylation activity.

Some light to this aspect of SIRT2 function was shed by the study of Bae *et al.* who analysed the proteomes of human gliomas and showed that SIRT2 is markedly

downregulated in these cancers (Bae *et al.*, 2004). Interestingly, the *SIRT2* locus is located in chromosomal region 19q13.2 which is frequently deleted in gliomas. Gliomas are also characterised by aneuploidy suggesting that defects in the spindle checkpoint may underlie tumour development and may reflect the proposed function of SIRT2 in mitosis.

In an attempt to identify SIRT2 interacting partners by a yeast two-hybrid assay, Hiratsuka *et al.* showed that SIRT2 interacts both *in vitro* and *in vivo* with the homeobox transcription factor HOXA10 (Hiratsuka *et al.*, 2003). Interestinlgy, HOXA10 was shown to regulate the transcription of the cell cycle inhibitor p21 in the context of differentiation. This is reminiscent of the SIRT1 interacting partners Hes1/Hey2 that regulate another cell cycle inhibitor, p27. It remains unclear whether these interactions are of physiological significance while little is known about the mechanism of action of SIRT2 in the experimental settings employed in these studies.

2.3.5.3 SIRT3

SIRT3 localises to mitochondria by means of a predicted amphipathic α-helix comprising the first 25 aminoacids of the protein (Schwer *et al.*, 2002). Immunoblotting analysis of cell extracts revealed two immunoreacetive bands, one at the predicted size of ~44 kDa and one at ~28kDa. The 28 kDa species was shown to be a soluble mitochondrial matrix protein derived proteolytically from the inner mitochondrial membrane-associated 44 kDa species predominantly through the action of mitochondrial matrix processing peptidase (MPP). Interestingly, the full-length protein lacks detectable enzymatic activity while the processed form exhibits NAD^+-dependend deacetylase activity.

Aside their role in energy production, mitochondria are also major players in the dissipation of energy in the form of heat, a process known as adaptive thermogenesis, which is important for maintaining energy balance in response to environmental stimuli such as diet and temperature. In rodents, brown adipose tissue (BAT) is the primary site of adaptive thermogenesis a fact linked to their high content of mitochondria. The production of heat by mitochondria is attained primarily through the action of uncoupling protein 1 (UCP1) which uncouples fuel oxidation from ATP production by inducing proton leakage across the mitochondrial inner membrane.

Shi *et al.* observed that mSIRT3 mRNAs are enriched in mouse BAT but not in white adipose tissue (WAT) (Shi *et al.*, 2005). In addition, mSIRT3 transcripts were induced in response to cold exposure and brown adipocytes overexpressing SIRT3 by means of retroviral transduction induced the expression of UCP1 along with other mitochondrial genes (ATP synthase, cytochrome c oxidase subunits II and IV) and PGC-1α, a coactivator shown to have major metabolic regulatory functions including mitochondrial biogenesis. Importantly, the authors demonstrated that these effects required both the deacetylase and ADP-ribosyltransferase activities of mSIRT3. mSIRT3-induced UCP1 induction was shown to depend on PGC-1α and correlated with phosphorylation of CREB, a known PGC-1α activator. Consistent to these, mSIRT3 stimulated both mitochondrial electron transport and uncoupling and reduced mitochondrial membrane potential and ROS production.

Upon examination of mSIRT3 transcript levels in mouse models of obesity, mSIRT3 was shown to be reduced in BAT, in agreement with its defective thermogenesis capacity in obesity. Interestingly, given the proposed role of PGC-1α in diabetes (Mootha *et al.*, 2003), Yechoor *et al.* observed a downregulation of SIRT3 mRNA levels in the skeletal muscle of a streptozotocin-induced mouse model of diabetes a phenomenon further exuberated by muscle-specific deletion of the insulin receptor (MIRKO mice) (Yechoor *et al.*, 2004). Moreover, as ROS production has been linked to life-span regulation, it remains to be investigated whether the proposed roles of SIRT3 mediate organismal longevity.

Indeed there is evidence that in humans specific *SIRT3* polymorphisms are found in long-lived individuals. The *SIRT3* genomic locus maps to the chromosomal region 11p15.5 where four other genes potentially associated with longevity are also located (HRAS1, Insulin-like Growth Factor 2, Proinsulin, and Tyrosine Hydroxylase). A genetic-demographic study identified a silent nucleotide transversion that correlated with increased life-span in males but not in females of the examined cohort. Linkage-disequilibrium analysis revealed a potential role of this SIRT3 polymorphism or a tightly linked gene in longevity independent of the other longevity-associated genes in the locus (Rose *et al.*, 2003).

Further analysis identified a allele-length polymorphism in intron 5 of the gene that was highly linked to this silent variant. A nucleotide variability within this polymorphism was found to convert a GATA3 site to a deltaEF1 site lacking enhancer activity in reporter assays. Intriguingly, this allele was completely absent in old male subjects while allele length was

also shown to correlate with specific age groups (Bellizzi *et al.*, 2005). How different haplotypes dictate downstream molecular events and how these are linked to longevity remains to be elucidated.

2.3.5.4 SIRT6

FISH studies located the *SIRT6* locus in chromosomal region 19p13.3. In the mouse, it is expressed in both embryonic and adult tissues, with higher mRNA levels detected in the brain, heart and liver. lacZ reporter gene replacement of the endogenous *mSIRT6* locus confirmed ubiquitous expression of the protein in the mouse. Interestingly, in the muscle mSIRT6 shows the highest protein expression levels despite low mRNA abundance relative to other tissues tested (Liszt *et al.*, 2005).

SIRT6 is reported to be a nuclear protein, excluded from nucleoli (Michishita *et al.*, 2005; Liszt *et al.*, 2005) while showing a moderate concentration in regions of high Hoechst staining, implying that it is associated with heterochromatin. To this effect, it has been shown by biochemical fractionation of chromatin- and nuclear matrix-bound proteins that, contrary to SIRT1, SIRT6 associates with chromatin.

SIRT6 shows no *in vitro* deacetylase activity against an acetylated histone H4 (K16) or acetylated p53 (K382) peptide. Consistent with this, p53 K382 acetylation was markedly suppressed in normal human prostate epithelial cells transduced with a SIRT1 but not with SIRT6 recombinant retrovirus. This implied either that the specificity of SIRT6 for the tested substrates differed from that of SIRT1 or that SIRT6 has no deacetylase activity.

Biochemical studies probing the enzymatic activity of mouse SIRT6 (mSIRT6) showed that bacterially expressed mSIRT6 is able to transfer robustly ^{32}P from ^{32}P-NAD^+ but not to core histones as substrate suggesting that it has auto-ADP-ribosyltransferase activity (Liszt *et al.*, 2005). Moreover, this was shown to be an intramolecular reaction since co-incubation with differently tagged mSIRT6 failed to label an mSIRT6 species mutated in the catalytic site previously demonstrated to be important for the ADP-ribosyltranferase activity. Although no substrates of this SIRT6 activity have been reported to-date, it is thought that auto-ADP ribosylation may serve as an autoregulatory mechanism for the enzyme based on previous reports from other proteins.

In an attempt to understand the physiological functions of SIRT6, Mostoslavsky *et al.* created a knock-out mouse lacking SIRT6 by means of homologous recombination (Mostoslavsky *et al.*, 2006). ES and MEF cells derived from these animals showed reduced BrdU incorporation and S-phase cells implying a proliferation defect. These cells also axhibited a higher sensitivity to ionising radiation treatment but not ultraviolet radiation indicating an intact nucleotide excision repair (NER) pathway. Karyotype analysis revealed a multiplicity of chromosomal abnormalities pointing to a defect in genomic stability maintainance pathways. To address this issue, Mostoslavsky *et al.* analysed the ability of SIRT6$^{-/-}$ cells to arrest in response to IR and found it to be intact. In addition, mSIRT6 deficiency did not compromise double-strand break (DSB) repair pathways as judged by an extrachromosomal plasmid-based non-homologous end joining (NHEJ) assay, chromosomal DSB repair rates and formation/clearance rates of γ-H2AX foci, an early marker of DSBs.

Conversely, both SIRT6$^{-/-}$ MEFs and ES cells were found to be more sensitive to treatment with the alkylating agent MMS and the ROS inducer H_2O_2, which create DNA lesions repaired by the BER pathway. Importantly these phenotypes were shown to be reversible by re-expression of recombinant SIRT6 but not a catalytically inactive mutant. Furthermore, the MMS, IR and H_2O_2 sensitivity was rescued by overexpression of the 5'-deoxyribose phosphate (dRP) lyase domain of Polβ, a protein exclusively operating in the BER pathway, confirming that SIRT6 mediates genomic stability by modulation of BER repair mechanisms. These effects are not thought to directly affect BER factors, since their expression is not altered in SIRT6$^{-/-}$ cells, nor does mSIRT6 colocalise with these components upon MMS and H_2O_2 treatment. Interestingly, however, extracts of SIRT6$^{-/-}$ cells could perform as wildtype cells in *in vitro* BER assays.

SIRT6$^{-/-}$ mice were born at the expected Mendelian frequency from an intercross between SIRT6 heterozygous animals and remained apparently normal and viable albeit weighing less than their wild-type litermates (Mostoslavsky *et al.*, 2006). However, SIRT6$^{-/-}$ mice invariably died at around 3 weeks of age following the onset of several degenerative processes such as subcutaneous fat loss, lordokyphosis due to osteopenia, colitis, lymphopenia, phenotypes associated with ageing. The lymphopenia could be attributed to increased lymphocyte apoptosis which correlated with decreased $CD4^+/CD8^+$ double positive thymocytes and decrease in splenic lymphocytes and bone marrow B cell progenitors. The

latter phenotypes were shown to be due to systemic effects on lymphocytes, since both donor bone marrow SIRT6$^{-/-}$ cells and wt competitor cells contributed equally to lymphocyte repopulation when co-injected into lethally irradiated acceptor mice. Along these lines, SIRT6$^{-/-}$ mice exhibited markedly reduced IGF-1 serum levels. Concomitantly, plasma glucose of these animals steadily decreased after P12 reaching undetectable levels before death. IGF-1 is thought to contribute to lymphocyte resistance to apoptosis and reduction of lymphocyte population associated with ageing correlates with low IGF-1 serum levels.

Although little is known about the molecular mechanisms or, indeed, the actual SIRT6 enzymatic activity that mediates the observed effects, genomic stability and consequently ageing, this work comprises the most convincing evidence to-date that in higher eucaryotic organisms a member of the sirtuin family mediates processes involved in determining life-span.

2.4 Aim of the project

In relation to our interests in understanding cellular responses to nutrient availability we selected to study sirtuins as potential candidates linking the metabolic status of the cell expressed by the levels of NAD^+ and transcription through their deacetylase activity.

The identification of the NAD^+-dependent enzymatic activity of sirtuins fuelled a lot of speculation as to whether intracellular NAD^+/NADH levels could act as regulators of their activity. This is a particuarly attractive hypothesis since yeast sirtuins are known mediators of the effects of caloric restriction on longevity. However, experimental verification of this hypothesis is hintered by the lack of direct methods of assaying sirtuin activity in native conditions since cell disruption automatically dictates the provision of exogenous NAD^+ for the enzymatic reaction. Consequently, sirtuin activity can only be indirectly predicted by correlation with the (measurable) intracellular [NAD^+]/[NADH] or assessment of the acetylation status of known substrates.

Post-translational modification (PTM) is a ubiquitous mode of protein regulation by means of affecting biomolecular interactions or enzymatic activity. Thus, PTMs have been broadly employed as a means to investigate the behaviour of proteins and their influence upon the biological systems they participate in. To-date, no PTMs have been identified for sirtuins.

The aim of this project was to identify PTMs that influence sirtuin activity, focusing on SIRT1. We employed an broad range of approaches towards this goal and to understand the functional significance of the newly-discovered modifications.

CHAPTER 3

MATERIALS AND METHODS

3.1 **BUFFERS** (in alphabetical order)

Apoptotic extract buffer A

20 mM	HEPES-KOH pH 7.5
10 mM	KCl
1.5 mM	$MgCl_2$
1.0 mM	EDTA
1.0 mM	EGTA

Used with freshly added DTT (1mM) protease inhibitors as described in the text.

ATP for luciferase assays

100 mM dissolved in H_2O adjusted to pH 7.8 with NaOH

2xBBS (Borate-buffered saline)

50 mM	BES [N-N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] pH 6.96
280 mM	NaCl
1.5 mM	Na_2HPO_4

β-galactosidase assay buffer

60 mM	Na phosphate
10 mM	KCl
1 mM	$MgCl_2$
50 mM	β-mercaptoethanol (added fresh prior to use)
0.7 mg/ml	ONPG (*o*-nitrophenyl-β-D-galactopyranoside)

60 mM Na phosphate was prepared by mixing (for 1 litre):

93.2 ml	1M Na_2HPO_4
6.8 ml	1M NaH_2PO_4

Gel Filtration buffer

50 mM	Hepes pH 7.5
150 mM	NaCl
10 mM	$MgCl_2$
0.1%	NaN_3

Gel Filtration lysis buffer

50 mM	Tris-HCl pH 8.0
150 mM	NaCl
1%	Triton X-100
20 mM	NaF

Used with freshly added DTT (1mM) protease and phosphatase inhibitors as described in the text.

Glutathione elution buffer

15 mM	Glutathione
50 mM	Tris pH 8.0

Stored at -20 °C

6.7xGlycylglycine buffer

150 mM	$MgSO_4$
250 mM	Glycylglycine
pH 7.8	

HAT (histone acetyltransferase) buffer

50 mM	Tris-HCl pH 8.0
10 mM	Na butyrate
0.2 mM	acetyl-CoA
10 %	glycerol

Used with freshly added DTT (1mM)

2xHBS (HEPES-buffered saline)

50 mM	HEPES pH 7.05
140 mM	NaCl
1.5 mM	Na_2HPO_4

In vitro transcription/translation (IVT) reaction mix

25 μl	rabbit reticulocyte lysate (RRL)
2 μl	TNT buffer
1 μl	aminoacid mix (w/o methionine)
1 μl	RNasine RNase inhibitor
2 μl	DNA (at 1 μg/μl starting concentration)
16 μl	H_2O
1 μl	TNT T7 RNA polymerase
2 μl	^{35}S-methionine (30 μCi)
50 μl	FINAL VOLUME

Kinase assay buffer

20 mM	Tris-HCl pH 7.5
50 mM	KCl
10 mM	$MgCl_2$
2-10 μM	ATP
10 μCi	^{32}P-γ-ATP

λ-phosphatase lysis buffer

50 mM	Tris-HCl pH7.5
250 mM	NaCl
0.1 mM	EDTA
0.5 %	NP-40

Used with freshly added DTT (1mM) and protease inhibitors

LB medium

1 %	(w/v)	Bacto-tryptone (BD)
0.5 %	(w/v)	Bacto-Yeast extract (BD)
1%	(w/v)	NaCl

pH 7.0

LB agar

15g of agar in 1 l LB medium

6.7x Luciferin

2mM in 1x Glycylglycine buffer

Luciferase assay buffer

1x Glycylglycine buffer
1x Luciferin
5 mM ATP

Luciferase extraction buffer

0.1 M K-phosphate buffer pH 7.8 [10 parts of 1 M K_2HPO_4 mixed with 1 part 1M KH_2PO_4]
0.1% Triton X-100

Used with freshly added DTT (1mM)

Maltose elution buffer

20 mM	Tris-HCl pH 8.0
200 mM	NaCl
1 mM	EDTA
10 mM	β-mercaptoethanol (freshly added)
10 mM	Maltose

NETN lysis buffer

20 mM	Tris-HCl pH 8.0
100 mM	NaCl
1 mM	EDTA
0.5%	NP-40

Used with freshly added DTT (1mM) and protease inhibitors as described in the text.

Paraformaldehyde (PFA) fixation solution

3.7% PFA/2% sucrose

18.5 g PFA dissolved in 400 ml of H_2O by heating and addition of 400 μl 10N NaOH. To this solution add 50 ml of 10x PBS and 10g sucrose. Adjust pH to 7.4, make up to 500 ml with H_2O and aliquot in Falcon tubes. Stored at -20 °C.

PBS (Phosphate-buffered saline)

137 mM	NaCl
2.7 mM	KCl
10 mM	Na_2HPO_4
2mM	KH_2PO_4
pH 7.4	

Peptide/protein affinity column blocking buffer

0.1 M	Tris-HCl pH 8.0

Peptide/protein affinity column coupling buffer

0.1 M	$NaHCO_3$
0.5 M	NaCl

pH 8.0

Peptide/protein affinity column washing buffer A

0.1 M	CH_3COONa
0.5 M	NaCl

pH 4.0

Peptide/protein affinity column washing buffer B

0.1 M	Tris-HCl
0.5 M	NaCl

pH 8.0

SDS running buffer

25 mM Tris-HCl
250 mM glycine
0.1% SDS
pH 8.3

SIRT1 deacetylation assay buffer

25 mM	Tris-HCl pH 8.0
137 mM	NaCl
2.7 mM	KCl
1 mM	$MgCl_2$
1 mg/ml	BSA

Sodium borate (NaB) solution for antibody coupling

0.2 M sodium borate pH 8.0

1 part	0.2 M H_3BO_3 (Boric acid)
10 parts	0.05 M $Na_2B_4O_7$

TBE

89 mM Tris-HCl pH 8.0
89 mM Boric acid
2 mM EDTA

TBST

50 mM	Tris-HCl pH 8.0
150 mM	NaCl
0.1 %	Tween-20

TNN lysis buffer

50 mM	Tris-HCl pH 7.5
250 mM	NaCl (high salt) or 120 mM (low salt)
5 mM	EDTA
50 mM	Sodium Fluoride (NaF)
0.5%	NP-40 (high NP-40) or 0.1% (low NP-40)

Used with freshly added DTT (1mM) protease and phosphatase inhibitors as described in the text.

WCE (Whole Cell Extract) lysis buffer

20 mM	HEPES-KOH pH 7.5
400 mM	NaCl
25%	Glycerol
1mM	EDTA
5 mM	NaF
0.1%	NP-40

Used with freshly added DTT (1mM) protease and phosphatase inhibitors as described in the text.

3.2 METHODS

3.2.1 BIOINFORMATICS RESOURCES

Multiple sequence alignments: ClustalW 1.8 (Baylor College of Medicine)
http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html

Aligned sequence editing: BoxShade (Pasteur Institute)
http://bioweb.pasteur.fr/seqanal/interfaces/boxshade-simple.html

PEST motif identification: PESTFind (University of Vienna)
https://emb1.bcc.univie.ac.at/toolbox/pestfind/pestfind-analysis-webtool.htm

Phosphorylation site searches: Scansite Motif Scanner (MIT)
http://scansite.mit.edu/motifscanner/motifscan1.phtml?database=_SWS_

3.2.2 MOLECULAR BIOLOGICAL TECHNIQUES

Construction of plasmids expressing SIRT1 and deletion/point mutants thereof

Two vectors containing the cDNA encoding for human SIRT1 and that of a catalytically inactive mutant (Vaziri *et al.*, 2001) were kindly provided by Prof. Robert Weinberg (Whitehead Institute, MIT, USA). These vectors were named pYESir2 and pYESir2HY respectively and were based on a pBabePuro retroviral expression vector backbone. The SIRT1 cDNA was excised with BamHI/SnaBI and subcloned into pcDNA3 and pcDNA3-HA vectors in the BamHI/EcoRV sites.

All SIRT1 point mutants were created by the two-step PCR method, using either the Pwo polymerase kit (Roche), or GC-rich PCR system (Roche) for amplification of regions corresponding to the N-terminus of the protein, according to manufacturer's protocols. Mutations proximal to the N-terminus of SIRT1 were introduced to BamHI/PacI-digested

pcDNA3-HA-SIRT1 plasmid while mutations proximal to the C-terminus were introduced to PacI/XbaI-digested pcDNA3-HA-SIRT1 plasmid. The XbaI site is part of the 3' UTR of SIRT1 mRNA which was present in the original pYESir2 vector. Truncation mutants were cloned following the same strategy. All mutations were confirmed by sequencing of the relevant region. The mutated cDNAs as well as other SIRT1 fragments were subsequently subcloned to various vectors as follows:

pcDNA3-based vectors:	Into the BamHI/XbaI sites
pBabePuro vector:	Into the BamHI/SnaBI sites (following 3' Klenow fill-in)
pAcGST vector:	Into the BamHI/SmaI sites
pGEX-2TK vector:	Into the BamH/EcoRI sites
pMAL-c2 vector:	Into the BamHI/XbaI sites

Other plasmids used in this study were obtained as follows:

GST-Pin1:	Gianni Del Sal, LNCIB, Trieste, Italy (Zacchi *et al.*, 2002)
GST-p300(HAT):	Richard Eckner, New Jersey Medical School, New Jersey, USA
Flag-β-TrCP:	Giulio Draetta, European Inst. of Oncology, Milan (Busino *et al.*, 2003)

Origin of SIRT1 knock-out mice

Mice of the 129/SvJ strain containing a targeted deletion of the *SIRT1* locus were provided by Prof. F. Alt, Harvard University, Boston USA (Cheng *et al.*, 2003).

Genotyping of SIRT1 knock-out mice and MEFs

Genotyping was performed by standard Taq polymerase PCR methods using the following primers recommended by the provider of the mice:

SIRT1SKO-F: 5'-CTTGCACTTCAAGGGACCAA-3'
SIRT1SKO-R1: 5'-GTATACCCACCACATCTGAG-3'
SIRT1SKO-R2: 5'-CTACCACTCCTGGCTACCAA-3'

All three primers were uses simultaneously giving a band of ~ 800 bp for the knockout allele and a band of ~500 bp for the wildtype allele.

Specific PCR conditions were: 33 cycles of 94 °C, 40"; 55 °C 50"; and 72 °C 1.2'.

3.2.3 CELL CULTURE METHODS

3.2.3.1 Mammalian cell culture

NB: When the term "**standard medium**" is used in the context of cell culture applications, the following formulation should be assumed:

> 500 ml Dulbecco's MEM (Invitrogen, #10938-025) supplemented with 50 ml FCS (final: 10%) 5ml of 200mM L-Glutamine (final: 2mM) and 5ml 10,000 U/ml penicillin/10,000μg/ml streptomycin mix (final: 100U and 100μg respectively).

MEF medium: standard medium + 200 μM β-mercaptoethanol
Sf9 insect cell medium: Grace's insect medium (Invitrogen, #11605-045) + 10 % FCS
Other media formulations are indicated were appropriate

Transfection of mammalian cells with small interfering RNAs (siRNAs)

At least 24 hours prior to transfection, cells were seeded in antibiotic-free standard medium so that they were approx. 50% confluent on the day of transfection.

For a 10-cm tissue culture plate, 18 μl of 20 μM siRNA (40 nM final concentration unless othewise indicated) were added to 892 μl OPTIMEM serum-free medium in an Eppendorf

tube and mixed by pipetting. In a separate tube, 18 μl of Oligofectamine transfection reagent were added to 72μl OPTIMEM and gently mixed by flicking the tube 1-2 times. The lipid preparation was then allowed to sit at room temperature for 10' and it was then added to the diluted siRNA mix. In the meantime, cells were washed at least 1x with standard medium without antibiotics and without serum and 5 ml of the wash medium were added to the cells prior to return to the incubator. Following a 20-25' incubation at room temperature, the transfection mix was added to the cells in a dropwise fashion. 4-6 hours after transfection, standard medium containing 30% FCS was added to the plate to a final FCS content of 10% (typically 3 ml of this medium to the 6 ml of medium in plates). Cells were harvested at the indicated times and typically 48-72 hours after transfection.

RNAi target sequences

control non-silencing:	5'-AATTCTCCGAACGTGTCACGT-3'
Cdh1 (Donzelli *et al.*, 2002):	5'-AATGAGAAGTCTCCCAGTCAG-3'
Cdc20 (Donzelli *et al.*, 2002):	5'-AAACCTGGCGGTGACCGCTAT-3'
MPS1 (Stucke *et al.*, 2002):	5'-AACCCAGAGGACTGGTTGAGT-3'
Bub1 (Johnson *et al.*, 2004):	5'-AAATACCACAATGACCCAAGA-3'
BubR1 (Johnson *et al.*, 2004):	5'-AACGGGCATTTGAATATGAAA-3'
SIRT1-1:	5'-AATTATCACTAATGGTTTTCA-3'
SIRT1-2 (Lin and Elledge, 2003):	5'-AACTGGAGCTGGGGTGTCTGT-3'
SIRT1-3*:	5'-CAAGCGATGTTTGATATTGAA-3'

(*designed using the HiPerfmormance Design Algorithm by Qiagen)

Calcium phosphate transfection of cultured cells

For calcium phosphate transfection, cells were seeded 24h prior to transfection. 1-2 hr prior to transfection, the medium was exchanged with 9ml of fresh standard medium.

Typically 1-5 μg of plasmid DNA were combined with H_2O to a final volume of 450 μl. 50 μl of 2.5M CaCl were added to the DNA mix followed by vortexing for 10" then 500 μl of 2xBBS buffer were added followed by 30" vortexing. The tranfection mix was left at room temperature for 20' then added to the cells in a drop-wise fashion while gently rocking the plates. 16 hours post-transfection the cells were washed 3x with DMEM and supplemented with 9 ml of standard medium. Cells were harvested for further work approx. 40-46 hr, post-transfection.

Luciferase and β-galactosidase reporter assays

$1.0x10^5$-$1.5x10^5$ cells were seeded in 6-well plates at least 24 hours prior to transfection. Transfected cells were lysed in 250 μl luciferase extraction buffer, harvested with a Teflon cell scraper and transferred to an Eppendorf tube. Cellular debris was pelleted by centifugation for 10-15' at 14,000 rpm (20,800xg) at 4 °C. Cleared supernatants were transferred to white 96-well plates. 100 μl of cell lysate were used for luciferase activity assay in a MicroLumat Plus LB96V (Berthold Technologies, Bad-Woldbad, Germany) luminometer. Two injections, each 50 μl luciferase assay buffer were added per sample with a 1.6" delay and 15" integration time.

In parallel, 20 μl of each lysate were transferred to wells in transparent 96-well plates and 170 μl β-galactosidase assay buffer were added to each sample. Reactions were incubated in the dark at 37 °C until a yellow coloration started becoming visible and quantified by spectophotometry at 420 nm prior to apparent reaction saturation.

All tranfections for luciferase assays were performed in triplicate, each luciferase reading was normalised against the corresponding β-galactosidase value, data were averaged and standard deviation was calculated using Microsoft Excel.

In vivo ^{35}S-labelling of proteins

Cells at approx. 60-80% confluency were used for *in vivo* ^{35}S-labelling of newly synthesised proteins. Cells were washed twice with methionine-free medium (with L-glutamine and penicillin/streptomycin as in standard medium) without FCS and supplemented with 4 ml of the wash medium containing 10 % dialysed FCS. Cells were incubated for 15'-30' at 37 °C, the medium was subsequently replaced with 3ml of methionine-free medium (incl. penicillin/streptomycin, L-glutamine and 10 % dialysed FCS) supplemented with 100 μCi/ml radioactive labelling mix (PROMIX, Amersham Biochiences). The plates were covered with charcoal filters and incubated at 37 °C, 5% CO_2 for 3 hours after which cells were harvested for further manipulations.

Isolation of mouse embryonic fibroblasts (MEFs)

SIRT1$^{+/+}$, SIRT1$^{+/-}$ and SIRT1$^{-/-}$ MEFs were derived from E12.5 embryos from an intercross of SIRT1$^{+/-}$ animals. Observation of a vaginal plug after overnight mating was considered as E0.5. Pregnant mice were sacrificed by cervical dislocation, embryos were isolated and placed in ice-cold PBS containing 2% FCS. The head and internal organs of the embryos were removed and the head was used for genotyping. The remaining tissue was finely cut with a scalpel and incubated for up to 15' in trypsin solution at 37 °C, 5% CO_2. Trypsinised tissue was further dissociated by pipetting through a 1ml pipette tip, transferred to a 10 cm tissue culture plate and incubated at 37 °C, 5% CO_2. After reaching confluency, each 10-cm dish of MEFs was expanded into two 15-cm dishes. At confluency, MEFs were either counted and frozen in 9:1 MEF medium/DMSO or expanded for further experiments.

Retroviral infection of mammalian cells

Retroviruses were created by transfecting the appropriate recombinant retroviral transfer vector pBabePuro, that carries the puromycin selectable marker into the BOSC23 or Phoenix "packaging" cell lines.

In detail, one day prior to trasfection, the packaging cells were seeded so that at the day of transfection they were 40-60% confluent. 1-2 hr prior to transfection, the medium was exchanged with 9ml of fresh medium.

For the transfection, 20 μg of recombinant retroviral transfer vector containing the cDNA of interest were diluted with H_2O to a final volume of 440 μl. After addition of 62 μl of 2M $CaCl_2$ the mixture was vortexed for 10'' followed by addition of 500 μl of 2xHBS. The transfection mix was vortexed for 30'' and let to stand at room temperature for 20'. The transfection mix (1ml in 9ml of medium per 10 cm dish) was added dropwise to the packaging cell line and cells were incubated for approx. 16 hours (O/N) at 37°C, 5% CO_2. Cells were subsequently washed 3x with DMEM and supplemented with 9ml of standard medium. On the same day, the cells to be infected were seeded so that they were approx. 50-60% confluent on the day of infection. Approx. 40-46 hr, post-transfection, the medium of the packaging cells was harvested with a sterile syringe and filtered through a 0.45 μm syringe filter into a 15ml Falcon tube. This viral supernatant was supplemented with polybrene from a stock of 8 mg/ml to a final concentration of 4 μg/ml and added to the cells to be infected after removal of the old medium. Infection was repeated twice and 24 hr after the last infection, selection of the infected cells was initiated by replacing the medium with standard medium containing 2 μg/ml puromycin. Based on inspection of puromycin resistant colonies, typically >200-300 colonies comprised each pool (uncloned cell mass).

3.2.3.2 Insect cell culture

Production of recombinant baculoviruses

$2x10^6$ Sf9 cells were seeded in 6-cm dishes and were left to attach for approx. 20' at room temperature in a total medium volume of 4 ml. In the meantime, 0.5μg of BaculoGold® DNA (in 5 μl) were combined with 2 μg of recombinant baculovirus transfer vector, mixed gently by pipetting and let stand for 5'. Following that, 1ml of Transfection Buffer B was added to the DNA mix. The medium of Sf9 was then aspirated and cells were overlayed with 1ml Transfection Buffer A. The 1ml of Transfection Buffer B/DNA was then added dropwise to the cells, and the plates were incubated for 4 h at 27 °C. The transfection mix was then aspirated, cells were washed 2x with 4 ml and 4 ml of medium was applied to each plate. The 6-cm dishes were then placed in 15-cm dishes along with Whattman 3M paper pre-wet with H_2O to minimise culture mediun evaporation and incubated in ambient atmosphere, at 27 °C for 5 days. Supernatants were then harvested, cell debris was removed by centrifugation (1000xg, 2' at RT), the supernatant was filtered through a 0.45 μm syringe filter and used to infect $2x10^6$ Sf9 cells in 6-cm dishes in 3 ml of Grace's insect medium/10% FCS. At least 72 h post-infection, the resulting viral supernatant (1^{st} amplification) was harvested as above and 1 ml of it was used to infect $2x10^7$ Sf9 cells in T75, screw-cap tissue culture flasks with a filter lid in a medium volume of 30-40 ml. At least 72 h post-infection, the viral supernatant (2^{nd} amplification) was harvested as above and stored in the dark at 4 °C. This was used as the main viral stock and further viral amplifications were initiated from this.

Production of recombinant proteins in Sf9 insect cells

Logarithmically growing Sf9 cells were dislocated from the dish, counted and re-plated at $5x10^6$ cells per 10 cm plate. Cells were left to attach to the plate and the medium was replaced with 5 ml fresh medium (Grace's insect medium/10% FCS). 0.2-1.0 ml of recombinant baculovirus stock was used for infection. Cells were incubated at 27 °C and harvested 48 hours after infection.

3.2.3.3 Bacterial culture - Production of recombinant proteins in *E. coli*

The appropriate plasmid encoding the protein of interest was transformed into *E. coli* by the heat-shock method. Transformants were grown O/N at 37 °C. A single colony was then used to inoculate 10 ml LB medium (+antibiotic) and the culture was grown shaking (230 rpm) O/N. The following day, the culture was diluted 1:10 with fresh pre-warmed LB medium (+antibiotic) and incubated for 1.5 h as above. Protein expression was induced with 0.1mM IPTG for 3-4 hours and cells were harvested by centrifugation prior to further processing.

3.2.4 BIOCHEMICAL TECHNIQUES

Purification of GST-fusion proteins produced in Sf9 insect cells

Cells were lysed in 1ml TNN lysis buffer per cell pellet derived from a 10-cm culture plate (initial seeding density: $5x10^6$ cells infected for ~48 hours) for 30' on ice. Cell debris was removed by centrifugation (20', 12000xg, 4 °C). Glutathione bead slurry was added to the cleared supernatant and the mixture was incubated for 1 h in a rotating wheel at 4 °C. The beads were subsequently pelleted by brief centrifugation up to maximum speed at 4 °C and similarly washed 4x with lysis buffer. The beads were subsequently washed 1x with 50 mM Tris-HCl pH 8.0 and the purified proteins were either used for assays as described in the corresponding sections or eluted 2x with glutathione elution buffer for 10-20' at room temperature. The eluted material was supplemented with glycerol to a final concentration of 20% and stored at -80 °C. All steps were performed at 4 °C.

Purification of GST-fusion proteins produced in *E. coli*

All steps were performed exactly as above with the exception that bacterial cells were lysed in NETN buffer and sonicated (3x10" at maximum amplitude) on ice.

Purification of MAL-fusion proteins produced in *E. coli*

All steps were performed as described for bacterially-expressed GST-fusion proteins with the exception that amylose resin (NEB, E8021-S) was used for the purification of the recombinant proteins and following washing with the lysis buffer the bead-bound material was washed 1x with maltose elution buffer (without maltose) and eluted 2x with maltose elution buffer for 10-20' at room temperature.

Gel filtration chromatography

A frozen pellet of $5x10^9$ HeLa cells was lysed in 10 ml gel filtration lysis buffer, dounce homogenised (15 strokes) with a type B tight-fitting pestle and left for 30' on ice. The lysate was pre-cleared by centrifugation for 30', at 3000xg, 4 °C and filtered through a 0.45 μm low-protein binding syringe filter prior to gel filtration. Gel filtration was performed using a Superose 6, XK26/70 preparative column at a flow rate of 0.5 ml/min in gel filtration buffer. 50 fractions of 4 ml each were collected in total and 50 μl of each fraction were used for SDS-PAGE analysis.

***In vitro* kinase assays**

Recombinant GST or GST-SIRT fragment fusion proteins were expressed in *E. coli* and purified using glutathione beads as described. Recombinant CK2 was purchased from Cell Signalling. 500 units of CK2 were diluted in kinase assay buffer (10 μM ATP) containing ~4

μg recombinant protein substrate to a final volume of 30 μl. Reactions were incubated for 30' at 30°C, stopped by addition of Laemmli buffer and boiling followed by analysis on an SDS-PAGE gel. Gels were dried and exposed for autoradiography.

GST-CyclinB1/His-Cdk2 were co-expressed in Sf9 cells and purified by glutathione affinity chromatography. Assay conditions were as for CK2.

BubR1 was immunoprecipitated from cell extracts. Assay conditions were as for CK2.

In vitro SIRT1 deacetylase activity assay using a fluorogenic peptide

For *in vitro* determination of SIRT1 activity, SIRT1 preparation (immunoprecipitated or recombinant expressed in Sf9 cells as a GST-fusion) was incubated with 100 μM Fluor de Lys-SIRT1 acetylated peptide substrate (corresponding to residues 379-382 of p53) and 0.5 mM NAD^+ in SIRT1 deacetylation assay buffer according to manufacturer's protocol (BIOMOL HDAC fluorescent activity assay/drug discovery kit - AK-500). Final reaction volume was 50 μl. Reactions were incubated for 30' at 37 °C and stopped by addition of 1x volume of Fluor de Lys Developer II incubated for an additional 45' at 37 °C in the dark and fluorescence was measured (excitation: 360 nm, emission: 460 nm, cutoff: 455 nm) with a SPECTRAmax GEMINI 96-well plate fluorometer (Molecular Devices).

In vitro SIRT1 deacetylase activity assay using autoacetylated GST-p300(HAT)

Prior to use in the deacetylation assay, GST-p300(HAT) was expressed in *E. coli*, purified using glutathione beads, washed 3x in NETN buffer, washed 2x in HAT buffer (w/o acetyl-CoA) and the beads were resuspended in HAT buffer with acetyl-CoA. The reaction was left to proceed for 30' at 30 °C. The final reaction volume was 50 μl. This autoacetylation reaction can also be omitted with equally good results as GST-p300(HAT) shows significant autoacetylation when expressed in bacteria and can thus be readily used as a substrate for

the deacetylation reaction. Recombinant GST-SIRT1 or GST-SIRT1(H363Y) expressed in Sf9 cells was purified using glutathione beads and eluted as described. Following autoacetylation, the GST-p300(HAT)-bound beads were washed 3x in TNN buffer, 2x in SIRT1 deacetylation assay buffer, the beads were equally divided to Eppendorf tubes for the various reactions and resuspended in SIRT1 deacetylation assay buffer supplemented with 0.5 mM NAD^+ and, where indicated, 0.5 mM nicotinamide or 10 mM Na butyrate. 5 μl of SIRT1 enzyme preparation corresponding to ~2 μg of purified protein were used per reaction. The final reaction volume was 50 μl. Reactions were incubated for 1h at 37 °C, stopped with addition of Laemmli buffer, boiled and analysed by SDS-PAGE.

In vitro transcription/translation

In vitro transcription/translation was performed with the Promega TNT® coupled reticulocyte lysate system using T7 RNA polymerase. Typically, 2 μg of DNA midiprep were incubated with IVT reaction mix for 90' and used directly or stored at -20 °C.

In vitro binding assays

The appropriate fusion protein (bait) was purified as detailed above, the beads were washed 3x with 1 ml lysis buffer without additives, and resuspended in 1 ml lysis buffer with additives. 1-5 μl of IVT were added to the bead suspension and the tubes were incubated in a head-to-head rotor for 1-3 hr. The beads were subsequently washed 4x with 1 ml lysis buffer without additives and bound material was released by addition of Laemmli buffer and boiling for 5' prior to loading on a gel.

Preparation of cell-free apoptotic extracts from 293 cells

Extracts were prepared as described in (Liu *et al.*, 1996). In detail, 293 cells were grown to 90% confluency, washed 1x with ice-cold PBS and scrapped with a teflon cell scrapper in a Falcon tube and resuspended in 5x volumes of ice-cold apoptotic extract buffer A. The lysates were allowed to stand on ice for 15' then homogenised with a tight-fitting type B pestle in a Dounce homogeniser followed by centrifugation at 1000xg for 10' at 4 °C. The resulting supernatant was then centrifuged at 10^5xg (23,700 rpm) for 1h at 4 °C in a Beckman SW40 rotor. The supernatant (S-100 fraction) was aliquoted, snap-frozen in liquid N_2 and stored at -80 °C until further use. Total protein concentration in these extracts was at 5 μg/μl.

***In vitro* caspase cleavage assay**

For *in vitro* cleavage assays, S-100 extracts were thawed on ice, dATP was added to a final concentration of 1 mM where applicable and the extract was incubated at 30°C for 1h. 10 μl of extract corresponding to 5 μg total protein were added to 1-2 μl of *in vitro* translated material and incubated for the time indicated in the text. Reactions were stopped by the addition of SDS sample buffer and analysed by SDS-PAGE.

Tryptic digestion of GST-SIRT1 for phosphorylation analysis

GST-SIRT1 was expressed in Sf9 cells and purified as described, but not eluted from the beads. Following washes with lysis buffer, the sample was divided into two equal parts and either mock-treated, or treated with λ-PPase as described. The bead-bound material was then washed with lysis buffer to remove λ-PPase followed by 2x washes with 10 mM Tris pH 8.1. The beads were subsequently resuspended in 50 μl of 10 mM Tris pH 8.1 and supplemented with $CaCl_2$ to a final concentration of 2mM and sequencing-grade trypsin (Promega) at a ratio of 1:20 (w/w) trypsin:GST-SIRT1 (this corresponded to 1 μg trypsin). The samples were incubated at 37°C O/N, the supernatants were harvested and stored at -80°C until analysed by MALDI-MS.

3.2.5 IMMUNOLOGICAL TECHNIQUES

Paraformaldehyde fixation/Triton X-100 permeabilisation

Cells were seeded at least 24 and usually 48 hours prior to treatment/fixation on 18x18 mm #1 coverlips placed in the wells of a 6-well plate. The medium was aspirated and 2 ml PFA fixation solution freshly thawed and pre-warmed to 37 °C was added to each well. The plate was incubated at 37 °C for 10'. Cells were then washed 2x with PBS and permeabilised with 0.02% Triton X-100 in PBS for 10' at RT. Cells were washed 2x with PBS and proccessed for immunostaining as described below.

Methanol fixation/Acetone permeabilisation

Cells were seeded on coverslips as above, the medium was aspirated and cells were directly fixed with methanol (-20 °C) then permeabilised in an acetone bath (-20 °C). Coverslips were subsequently washed 1x in a PBS bath at RT and processed for immunostaining as described below.

Cells were seeded at least 24 and usually 48 hours prior to treatment/fixation on 18x18 mm #1 coverlips placed in the wells of a 6-well plate.

Immunostaining of cultured cells

Fixed/permeabilised cells were incubated with primary antibody mix (1-5 μg/ml of antibody in PBS/1% BSA/1% goat serum) for 1-2h at RT or O/N at 4 °C. Cells were washed 3x with PBS at RT and subsequently incubated with secondary antibodies coupled to the appropriate fluorescent dye (diluted in PBS/1% BSA/1% goat serum) for 1h at RT. In parallel DNA was counterstained with 1 μg/ml DAPI (1,4,6-diamidino-2-phenylindole). Cells were washed 3x and inverted over Vectashield medium on top of a microscope glass. Coverslips were sealed using nail varnish and either observed directly , or were stored in the dark at 4 °C until observation.

Preparation of peptide affinity columns for antibody purification

For antibodies raised against a peptide, the same antigenic peptide was used for affinity purification. In detail: Activated CH sepharose 4B (Amersham #17-0490-01) was resuspended in 20 ml of 1 mM HCl and washed for 15' through a sintered glass filter with 300-400 ml of the same solution. Washed sepharose was transferred to a 15 ml Falcon tube and washed 1x with peptide coupling buffer. Peptide was dissolved in coupling buffer in a volume equivalent to 2x volumes of swollen sepharose and was added to the sepharose bead slurry [20mg peptide per gram of sepharose (dry weight; 1 g sepharose gives ~3ml slurry following swelling)]. The suspension was incubated on a tilting shaker for 2 h at RT. The beads were subsequently washed 2x with peptide coupling buffer and unreacted moieties on the beads were blocked with blocking buffer for 1 h at RT. The beads were then washed for 3 consecutive cycles of alternating wash buffer A and B. Finally, the beads were washed with PBS and stored in PBS+0.05% NaN_3 at 4 °C.

Preparation of protein affinity columns for antibody purification

Preparation of protein affinity columns was essentially as described for peptide columns with the following modifications:

CNBr-activated sepharose 4B (Amersham #17-0430-01) was used; 1 mg of protein was coupled per gram of sepharose; 1 g sepharose gives ~3.5 ml slurry following swelling.

The relevant antigenic region was expressed as a fusion protein in bacteria and purified as described. Prior to coupling, the purified protein was dialysed against coupling buffer O/N at 4°C.

Affinity purification of rabbit polyclonal antibodies

α-SIRT1 Ctp was purified through a peptide affinity column using the same antigenic peptide. α-SIRT1 GST-S1 antibody was purified through a MAL-SIRT1(575-676) column. Affinity purification was performed identically for both antibodies.

Affinity columns were prepared as described. The storage buffer was allowed to flow through and the column was washed with 3 volumes of binding buffer (PBS+0.2% Tween-20). Typically, 3 ml of rabbit serum were diluted to 10 ml total volume with binding buffer (PBS+0.2% Tween-20) and applied to the column. Bound material was washed until the OD_{280} was equal to $OD_{PBS+0.2\%\ Tween\text{-}20}$ (usually approx. 3 column volumes). Elution was accomplished using 8-10 ml of 0.2M Glycine pH 2.2. Fractions (7-9) of 750 μl were collected directly into Eppendorf tubes containing 250 μl 1M Tris pH 8.0. The OD_{280} of all fractions was measured using (PBS+0.2% Tween-20)/Tris pH 8.0 (3:1) for the first 2 fractions and 0.2M Glycine pH 2.2/Tris pH 8.0 (3:1) for the following fractions. Fractions with the highest OD_{280} were poolled, loaded on a pre-wet dialysis bag (MWCO 6,000-8,000, Spectra/Por) and dialysed O/N in PBS+30% glycerol. The dialysate was collected, OD_{280} was determined using the dialysis buffer as blank and purified antibody concentration was determined according to the formula:

$$[\text{antibody } \mu g/\mu l] = OD_{280} \times \varepsilon$$

where ε= 0.699 and is the IgG extinction coefficient.

The purified antibody solution was supplemented with BSA at a final concentration of 1 μg/ml, aliquoted and stored at -80 °C until further use. Antibody working solutions were typically stored at 4 °C where they were stable for at least 3 months.

Immunoprecipitation

Cells were rinsed with ice-cold PBS and either lysed directly in 1ml TNN lysis buffer per 10cm dish, and hervested with a teflon cell scraper in a 1.5ml Eppendorf tube. Cells were left on ice for 30' and debris and unlysed material was pelleted by centifugation for 20-30' at 14,000 rpm (20,800xg) at 4 °C. The cleared supernatant was transferred to a new Eppendorf tube containing 35 μl of a 50% Protein-A sepharose bead slurry, or a 1:1 mixture of Protein-A:ProteinG beads where appicable and pre-cleared for 1 h on a head-to-head rotator at 4°C. The beads were subsequently pelleted for 10' at 14,000 rpm (20,800xg) at 4 °C, the supernatant was transferred to a new Eppendorf tube and supplemented with the appropriate antibody. Immunocomplexes were precipitated with the addition of 35 μl of beads as used for the preclearing step, for 1 h on a head-to-head rotator at 4°C. Beads were washed 4x in 1 ml lysis buffer without supplements by successive pelleting of the beads and following the final wash, residual buffer was aspirated using a hypodermic needle, the beads were resuspended in 2x Laemmli buffer, boiled and analysed by SDS-PAGE.

Alternatively, cells were rinsed with ice-cold PBS and collected with a teflon cell scraper in 1 ml PBS in an Eppendorf tube, centrifuged for 3' at 3,000 rpm (1000xg) at 4 °C, the supernatant was aspirated, cell pellets were snap-frozen in liquid nitrogen and stored at -80°C until use.

Antibody coupling to sepharose beads

Antibody diluted in PBS was bound to protein-A sepharose CL-4B beads (Amersham, #17-0780-01) in a rotating wheel for 1 h at 4 °C. Approx. 2 mg antibody were bound per ml of wet beads. The beads were washed 2x with 10 volumes of 0.2 M NaB (pH 9.0) and resuspended in 10 volumes of the same solution. Solid dimethylpimelimidate (DMP) was added to the suspension to a final concentration of 20 mM while ensuring that the pH of the suspension was ≥8.3. The coupling reaction was allowed to proceed for 30 min in a tilting shaker and

was stopped by washing the beads 1x with 0.2 M ethanolamine followed by a 2 h incubation in the same solution with gentle mixing at RT. The beads were finally washed 2x with PBS and stored in PBS+0.05% NaN_3 at 4 °C. Coupling efficiency was assessed by analysing equivalent volumes of bead suspension before and after coupling by SDS-PAGE.

Preparative large-scale immunoprecipitation for mass spectrometry

A frozen pellet of $5x10^9$ HeLa cells was lysed in 160 ml TNN buffer, dounce homogenised (10 strokes) with a type B tight-fitting pestle and left for 30' on ice. The lysate was cleared by centrifugation for 30', at 18000xg, 4 °C using an SS34 rotor, followed by centrifugation for 30', 100000xg at 4°C using an SW37 rotor. Rabbit IgG covalently coupled to protein-A sepharose was used to pre-clear the lysate for 1h at 4°C, then the beads were removed by centrifugation (5', 3000xg) and the bound material (unspecific, control) was eluted by addition of 300 µl 0.2M glycine pH2.2. the eluate was neutralised with 100 µl of 1M K_2HPO_4. The supernatant was incubated for 3h with α-SIRT1 Ctp antibody covalently coupled to sepharose beads. The beads were subsequently washed 4x with lysis buffer and bound material was eluted as above. Both eluates were concentrated using Centricon columns (Millipore) with a M.W.C.O. (molecular weight cut-off) of 10,000. Laemmli buffer was added to the samples to a 1x final dilution and samples were analysed by SDS-PAGE in a 6-15% gradient gel. Gels were subsequently stained using colloidal blue solution and relevant bands were excised with a surgical scalpel. Following in-gel trypsinisation, proteins were identified as described (Gstaiger *et al.*, 2003).

Antibodies

Antigen	Host & isotype	Clone	Conjugation	Cat. no.	Manufacturer
α-acetyl-lysine	mouse	Ac-K-103	-	#9681	Cell signaling
BubR1	mouse IgG2a	9	-	D 612502	Transduction Labs
Cdc20 (p55CDC)	rabbit	H175	-	sc-8358	Santa Cruz
Cdh1	mouse IgG1κ	Ab-2	-	CC43	Calbiochem
cyclin B1	rabbit	H-433	-	sc-752	Santa Cruz
cyclin B1	rabbit	GNS1	-	sc-245	Santa Cruz
Flag	mouse	M2	-	F3165	SIGMA
cdk2	goat	-	-	sc-163G	Santa Cruz
GST	rabbit	(GST-2)	-	G-1160	SIGMA
HA	mouse	Y11	-	MMS-101R	BABCO
Histone H3 pSer-10	rabbit	-	-	9706	Cell signaling
α-rabbit IgG	donkey	-	HRP	NA-934V	Amersham
α-mouse IgG	donkey	-	HRP	NA-931V	Amersham
α-rabbit IgG	goat	-	Alexa Fluor 680	A-21076	Molecular Probes
α-mouse IgG	goat	-	Alexa Fluor 680	A-21057	Molecular Probes
α-rabbit IgG	goat	-	IR Dye 800	611-132-122	Rockland
α-mouse IgG	goat	-	IR Dye 800	610-132-121	Rockland
α-goat IgG	rabbit	-	HRP	61-1620	Zymed
α-rabbit IgG	goat	-	Alexa Fluor 488	A-11070	Molecular Probes
α-mouse IgG	goat	-	Alexa Fluor 488	A-11017	Molecular Probes
rabbit IgG	rabbit	-	-	02-6102	Zymed
p21	mouse	-	-	15091-1	BD Pharmingen
p27	rabbit	N-20	-	sc-527	Santa Cruz
p300	mouse	RW128	-	-	Richard Eckner
PARP	mouse	-	-	556362	BD Pharmingen
Skp2	mouse	95.60.2	-	-	Krek Lab
Topoisomerase-IIβ	rabbit	JB-1	-	-	Dan Sullivan
α-tubulin	mouse	-	-	CP06	Calbiochem

CHAPTER 4

RESULTS: FUNCTION AND REGULATION OF SIRT1

4.1 REGULATION OF SIRT1 BY PHOSPHORYLATION

4.1.1 Production of α-SIRT1 polyclonal and monoclonal antibodies

Since at the time this project was initiated no commercial antibodies against mammalian sirtuins were available, in order to perform functional analyses of SIRT1, we developed two polyclonal antibodies raised in rabbits. One was raised against a peptide corresponding to the last 21 aminoacids of the C-terminus of SIRT1. The other was raised against an N-terminal fusion of GST to aminoacids 575-676 of SIRT1 expressed in *E. coli*. These antibodies will henceforth be referred to as Ctp and GST-S1 respectively. The latter fragment was chosen based on sufficient expression levels of the corresponding recombinant protein after a small-scale screen of GST-fusions of SIRT1 fragments spanning the N- and C-terminal regions of the protein. Given the enhanced mobility of protein domains known to harbour catalytic acivity due e.g. to induced-fit effects upon substrate binding, the sirtuin core domain was deemed potentially unsuitable for raising an antibody against it. This is because in experimental conditions where the protein's tertiary structure is conserved such as immunofluorescence microscopy studies or immunoprecipitation, such antibodies may exhibit weak affinities for the antigen.

Two rabbits were immunised with each of the antigens and the sera were tested for immunoreactivity to HA-tagged SIRT1 overexpressed in 293 cells. Based on their ability to efficiently recognise the antigen, the serum from one rabbit typically 3-5 months after

immunisation start was used to purify SIRT1-specific antibodies by affinity chromatography.

FIGURE 4-1 shows the characterisation of these antibodies. Both the Ctp and GST-S1 antibodies recognise a single band of endogenous SIRT1 with an apparent moilecular weight of ~120 kDa by Western blotting of cell extracts from a variety of cell lines. In addition, both antibodies could efficiently immunoprecipitate endogenous SIRT1. Specificity was probed by siRNA-mediated depletion of the immunoreactive band for the Ctp antibody and by blotting GST-S1 immunoprecipitates with a monoclonal antibody recognising SIRT1 (*see* FIGURE 4-3).

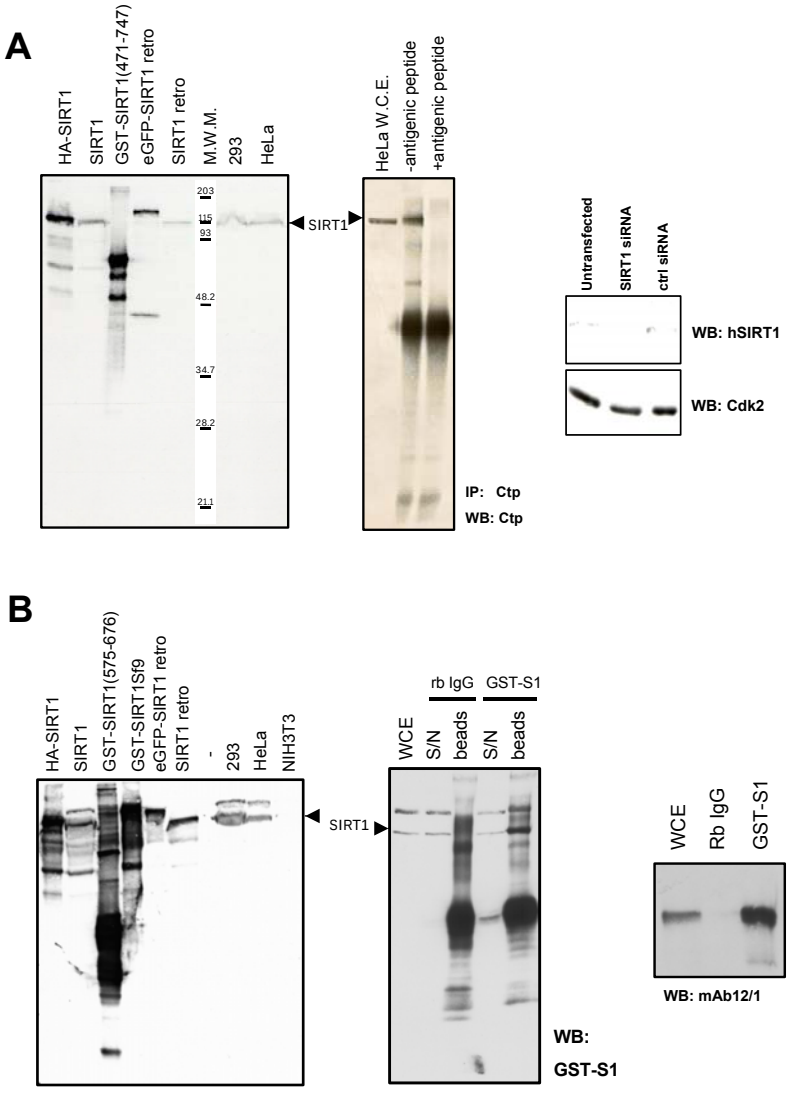


FIGURE 4-1. Characterisation of α-SIRT1 polyclonal antibodies. (A) Characterisation of Ctp, a rabbit polyclonal antibody raised against a peptide corresponding to the C-terminus of SIRT1; **(B)** Characterisation of GST-S1, a rabbit polyclonal antibody raised against residues 575-767 of SIRT1. *See* text for details.

Neither of these antibodies recognises mouse SIRT1 in NIH3T3 and primary embryonic fibroblasts (FIGURE 4-1B and not shown). This is not surprising for the Ctp antibody since the C- termini of human and mouse SIRT1, although similar, do not exhibit a stretch of ≥6 identical aminoacids which could serve as a common antigenic region. However, the region comprising aminoacids 575-676 of human SIRT1 should provide such an epitope and the reasons of this are currenlty elucive provided that fibroblasts do express detectable SIRT1 levels according to published reports and our own observations.

Immuofluorescence microscopy on PFA-fixed cells revealed a consistent nuclear staining which at times appeared speckled, especially when using the GST-S1 antibody (FIGURE 4-2). This is consistent with essentially all the studies published subsequently confirming the nuclear localisation of SIRT1. Moreover, these signals can be abolished when the SIRT1 antibodies are pre-incubated with the corresponding antigens and downregulation of SIRT1 by RNAi as confirmed by WB (FIGURE 4-2C). These data corroborate to the specificity of the signal.

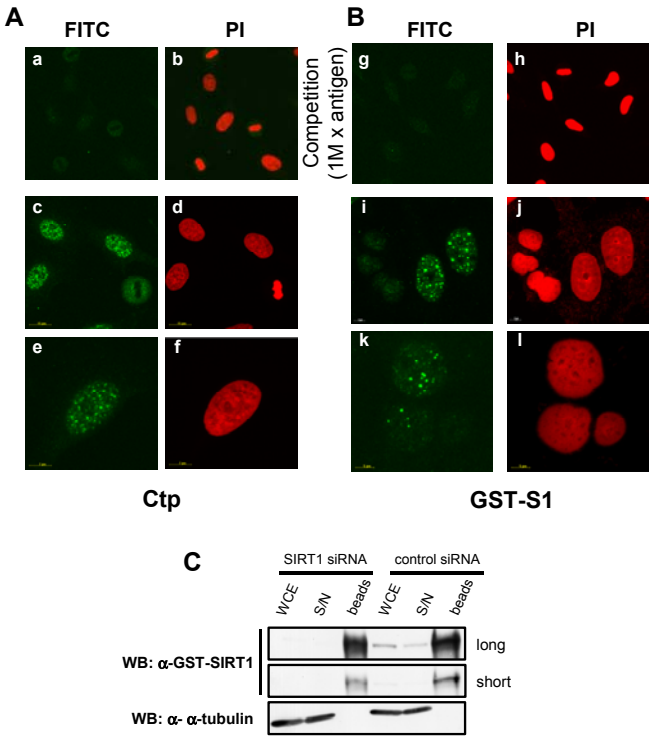


FIGURE 4-2. Subcellular localisation of SIRT1. (A) SIRT1 immunolocalisation in HeLa cells using the Ctp antibody. Scalebars represent 15 μm (c,d), 7 μm (e,f); **(B)** SIRT1 immunolocalisation in HeLa cells using the GST-S1 antibody. Scalebars represent 5 μm (i,j,k,l); **(C)** Western blot of cells used in (B) confirming siRNA-mediated downregulation of SIRT1

To enrich our SIRT1 toolkit, it was considered desirable to also generate mouse monoclonal antibodies targeting this protein. Mouse monoclonal antibodies have several advantages that complement polyclonal antibodies since they recognise a single epitope, once generated they are essentially of unlimited quantity and they do not cross-react with rabbit secondary antibodies used for immunoprecipitation to name but few. Thus monoclonal antibodies were raised in mice, the antigen used being bacterially-expressed full-length SIRT1 fused in its N-terminus with maltose binding protein (MAL). After several serial screens following sub-cloning of initially immunoreactive-clones, a single clone, that exhibited sustainable growth in culture and consistently produced α-SIRT1 immunoreactivity was isolated. This clone and henceforth the antibody that it produces are referred to as mAb12/1.

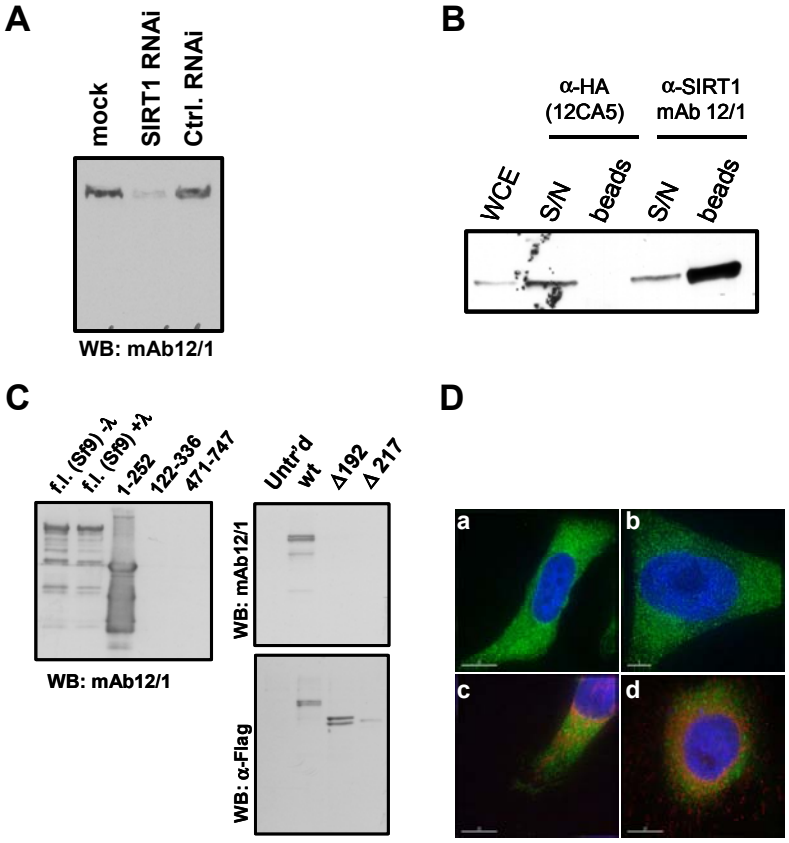


FIGURE 4-3. Characterisation of α-SIRT1 monoclonal antibody mAb12/1. (A) siRNA-mediated SIRT1 downregulation in HeLa cells to probe the specificity of mAb12/1; **(B)** Capacity of mAb12/1 to immunoprecipitate endogenous SIRT1 from HeLa cells; **(C)** Mapping of mAb12/1 immunogenic epitope; **(D)** Immunolocalisation pattern of SIRT1 using mAb12/1. **a,b**: HeLa cells fixed with methanol and permeabilised with acetone. **c,d**: HeLa cells were treated for 30' with 100 nM mitotracker dye fixed with paraformaldehyde and permeabilised with Triton X-100. All scalebars represent 5 μm. SIRT1 is in green, mitotracker in red, DAPI nuclear stain in blue.

mAb12/1 can recognise a single band in WB (FIGURE 4-3) and immunoreactivity is reduced in cells treated with siRNA targeting SIRT1. Furthermore, mAb12/1 can efficiently immunorpecipitate endogenous SIRT1 from HeLa cell lysates using Protein-A sepharose. The epitope recognised by mAb12/1 was determined to be within the first 121 aminoacids of the protein (FIGURE 4-3C) and λ-phosphatase (henceforth: λ-PPase) treatment did not affect its recognition of GST-SIRT1 expressed in Sf9 cells. Furthermore, mAb12/1 was also tested for its ability to recognise SIRT1 by indirect immunofluorescence (FIGURE 4-3D). Unlike Ctp and GST-S1, mAb12/1 gave a cytoplasmic staining pattern when using different fixation/permeabilisation methods. This cytoplasmic staining does not correspond to mitochondria as judged by co-staining with the mitochondrial marker MitoTracker. Although in contrast to both what the polyclonal antibodies of this study as well as numerous published results from other groups suggest, mAb12/1 may selectively recognise a small cytoplasmic subfraction of SIRT1 which is otherwise masked when a strong nuclear SIRT1 staining is present. mAb12/1 was subsequently shown to specifically recognise an unphosphorylated form of SIRT1, however whether this is unlikely to be connected to the observed immunolocalisation because mutants of the phosphoaminoacids in question are also nuclear (*see* Section 1.2.2, this chapter).

Given the fact that all three antibodies recognise different epitopes while they all immunoprecipitate endogenous SIRT1, their ability to recognise different SIRT1 complexes was tested. For this purpose, HeLa cells were incubated with ^{35}S-methionine to label newly synthesised proteins and subjected to immunoprecipitation with GST-S1 and mAb12/1. As shown in FIGURE 4-4A, apart from a band at ~120kDa presumably corresponding to SIRT1, the pattern of co-immunoprecipitated bands differed between the two antibodies. As SIRT1 requires NAD^+ for its enzymatic activity, NADH was proposed to be a competitive inhibitor thereof (Lin *et al.*, 2004) and because of the fact that small molecules involved in catalysis often impose a conformational change to the corresponding enzymes, the possibility that either NAD^+ or NADH can change the pattern of SIRT1 binding partners was tested. HeLa cells were labelled as above but all subsequent manipulations thereof including lysis, immunoprecipitation with mAb12/1 and washes were performed in the presence of the indicated compound. No major differences in the pattern of co-precipitated bands between the different conditions tested were observed suggesting that NAD^+ and NADH may not exert their effects on SIRT1 activity by modulation of its binding to other proteins.

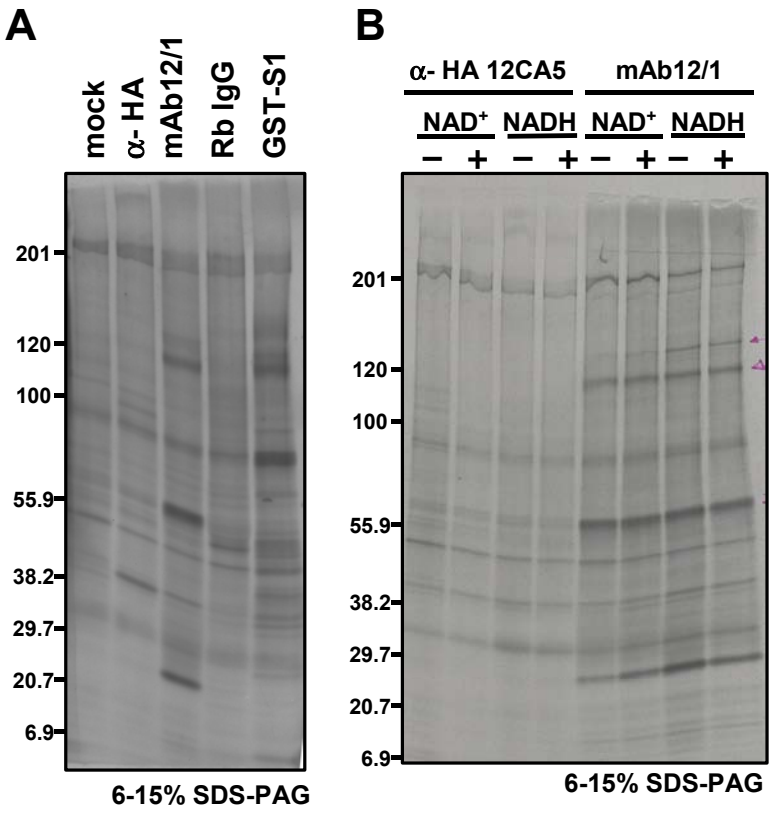


FIGURE 4-4. Comparison of protein patterns co-immunoprecipitating with SIRT1 using α-SIRT1 antibodies and effects of NAD^+, NADH. (A) SDS-PAGE analysis of SIRT1 co-immunoprecipitating protein patterns with mAb12/1 and GST-S1 antibodies; **(B)** Effects of NAD^+ and NADH in SIRT1 co-immunoprecipitating protein patterns using mAb12/1.

Finally, GST-S1 and mAb12/1 were tested for their ability to interfere with the enzymatic activity of SIRT1. This is particularly important when it comes to immunopurifying SIRT1 from cell extracts in order to assay for SIRT1 activity under particular conditions. For this, a baculovirus expressing GST-SIRT1 was constructed and used to generate recombinant protein in Sf9 insect cells. GST-SIRT1 affinity-purified using reduced glutathione was able to deacetylate a peptide based on the known p53 target sequence flanking K382 in a fluorescence-based peptide deacetylation assay (FIGURE 4-5). Thus insect cell-expressed GST-SIRT1 is an active deacetylase. When immunoprecipitated with either mAb12/1 or with GST-S1, Sf9 cell-expressed GST-SIRT1 retained its enzymatic activity, which, as expected, was sensitive to inhibition by nicotinamide, suggesting that these antibodies do not obviously interfere with its enzymatic activity. It cannot be excluded, though, that under particular conditions these antibodies may interfere with binding of a cellular regulator of SIRT1 activity.

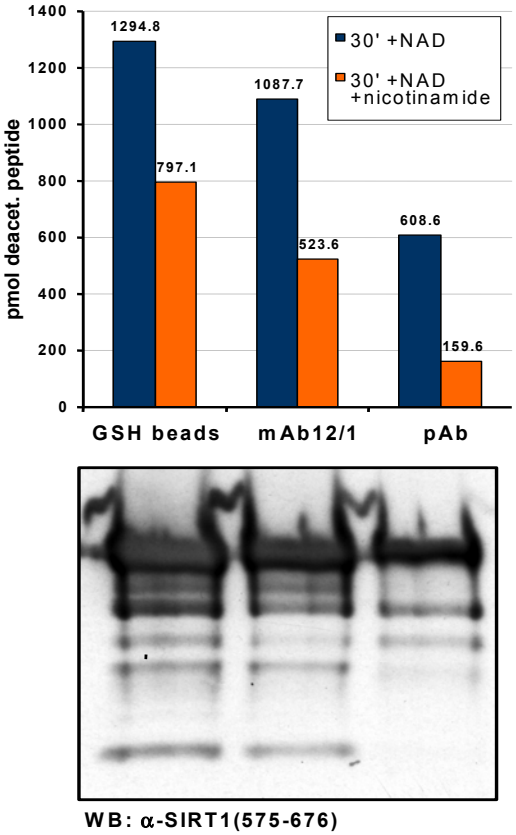


FIGURE 4-5. Lack of interference with SIRT1 enzymatic activity by α-SIRT1 antibodies. α-SIRT1 antibodies were used to immunoprecipitate GST-SIRT1 expressed in Sf9 cells, the immunoprecipitates were equally divided in two and the deacetylase activity of immunoprecipitated SIRT1 as well as the inhibitory effect of nicotinamide were quantified using a fluorogenic peptide deacetylation assay (BIOMOL). Lower panel: western blot analysis of immunoprecipitated SIRT1 corresponding to the reactions presented on the upper panel.

4.1.2 SIRT1 is a phosphoprotein

The immunofluorescence staining patterns of either the Ctp or GST-S1 antibodies revealed a characteristic loss of intensity in cells that appeared to be in the phase of mitosis based on their counter-stain with DNA-binding dies (FIGURE 4-6). This could be attributed to the diffusion of the protein following nuclear envelope break-down which occurs in mitosis. To confirm that this is indeed the case in contrast to a genuine loss of signal due to e.g. high proteolytic turnover of the protein during mitosis, total cell extracts of HeLa cells either logarithmically growing or arrested in mitosis by treatment with nocodazole were compared by Western blot (WB) (FIGURE 4-7A). Nocodazole binds and destabilises microtubules preventing the attachment of kinetochores to the mitotic spindle during cell division which results in the engagement of the mitotic spindle checkpoint and mitotic arrest. Treatment of HeLa cells with nocodazole for 16 hours was typically used in these studies. The mitotic population of adherent cells was purified by virtue of the fact

that such cells attach loosely to the cell culture dishes due to the charactersitic rounding associated with mitotic division in contrast to their non-mitotic counterparts.

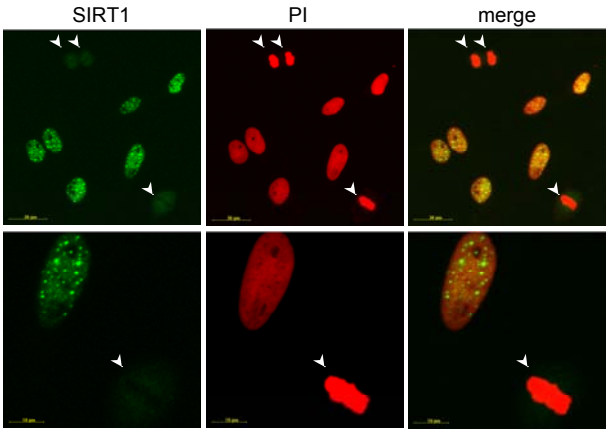


FIGURE 4-6. Changes in SIRT1 immunoreactivity between interphase and mitotic HeLa cells. PFA-fixed, Triton X-100-permeabilised HeLa cells were immunostained with GST-S1 antibody (green) and counterstained with propidium iodide (red). Mitotic cells are indicated with arrowheads. Scalebars represent 10 μm.

Immunoblotting with the GST-S1 antibody revealed that total SIRT1 protein levels were unchanged in both logarithmically growing and nocodazole-arrested cells suggesting that total SIRT1 protein levels are not subject to regulation during mitosis (FIGURE 4-7A). However, a subtle but evident retardation in the mobility of SIRT1 in nocodazole-arrested cells was observed. Such mobility retardation during SDS-PAGE is frequently seen when proteins are phosphorylated due to the increased negative charge conferred to the protein by the attached phosphate group. Thus it was hypothesised that this was also the case for SIRT1.

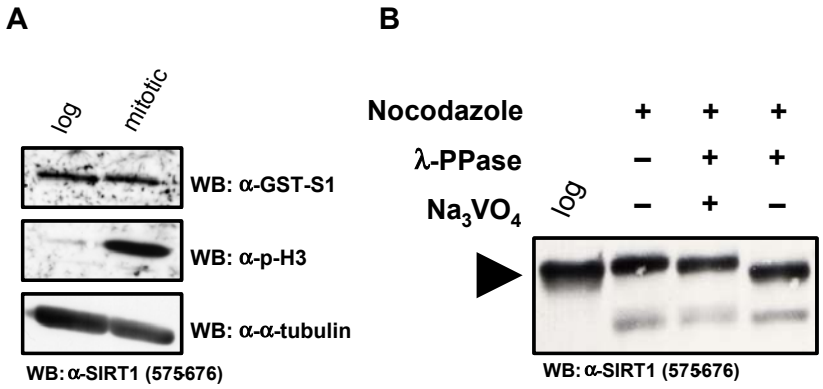


FIGURE 4-7. SIRT1 is a phosphoprotein and hyperphosphorylated in mitosis. (A) HeLa cells were arrested in mitosis with nocodazole and harvested by mitotic shake-off or left untreated (log) lysed and analysed by western blot using the indicated antibodies; **(B)** HeLa cells were treated and harvested similarly to (A). Prior to western blotting, cells were either treated with λ-PPase in the presence or absence of Na_3VO_4 or mock treated (no λ-PPase).

To test this hypothesis, cell extracts from nocodazole-arrested cells were treated with λ-PPase and subjected to immunoblotting (FIGURE 4-7B). λ-PPase is a dual specificity phosphatase encoded by the bacteriophage λ genome. As a control, a similar reaction was set in the presence of Na_3VO_4 a potent inhibitor of λ-PPase (Zhuo *et al.*, 1993). The SIRT1 immunoractive band in the λ-PPase-treated nocodazole-arrested cells migrated with increased mobility suggesting that indeed the observed band-shift was due to phosphorylation. However, it was also obvious that λ-PPase caused an even higher mobility than that of untreated cells. A more careful observation of the untreated sample SIRT1 band reveals a "comet-tail" effect suggestive of the presence of more than one SIRT1 species. Given the fact that in a typical exponentially growing population of HeLa cells 3-5% of cells are in mitosis, it is very unlikely that the observed electrophoretic retardation in interphase extracts is due to this small population. Thus, it was concluded that SIRT1 is phosphorylated in interphase and hyperphosphorylated in nocodazole-arrested mitotic cells.

Based on these results, a two-fold goal for the project was defined, namely to identify the phosphorylation site(s) of SIRT1 that cause the reduced electrophoretic mobility in (i) interphase cells and (ii) in mitotic cells and explore their effects on SIRT1 activity.

4.1.3 Phosphorylation of SIRT1 in interphase

To identify the interphase kinase and its target residues of SIRT1, a multidisciplnary approach was employed harnessing the power of bioinformatics, biochemical evidence and litterature search. These approaches as well as the functional characterisation of the identified modifications will be delineated below.

4.1.3.1 Identification of phosphorylation sites of SIRT1 expressed in Sf9 cells

Recombinant human proteins expressed in insect cells often exhibit patterns of post-translational modifications similar to the ones found in human cells. Interestingly, λ-PPase treatment of GST-SIRT1 expressed in Sf9 cells increases the electrophoretic mobility of the protein (FIGURE 4-8A) similarly to the situation in human cells suggesting that it is phosphorylated. Thus, it was hypothesised that if phosphorylation does play a role in

regulation of SIRT1, this may affect the enzymatic activity of the protein. To test this, the λ-PPase-treated GST-SIRT1 species of FIGURE 4-8A were subjected to an *in vitro* deacetylation assay using GST-p300 histone acetyltransferase (HAT) domain, which is autoacetylated when expressed in bacteria, as a substrate. Untreated GST-SIRT1 was able to robustly deacetylate GST-p300(HAT) whereas its λ-PPase-treated counterpart lost this activity (FIGURE 4-8B). These data suggest that phosphorylation of GST-SIRT1 may have an impact on its enzymatic activity, potentially reflecting a situation in human cells too.

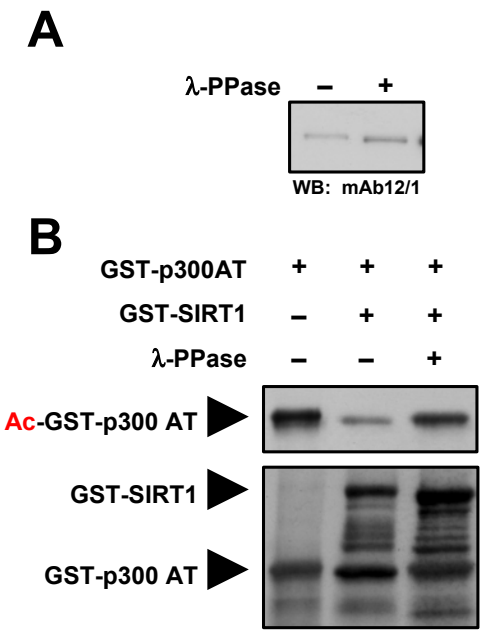


FIGURE 4-8. Dephosphorylation of GST-SIRT1 expressed in Sf9 cells abolishes its deacetylase activity. (A) GST-SIRT1 expressed in Sf9 cells was treated with λ-PPase or mock-treated and subsequently analysed by western blotting; **(B)** The GST-SIRT1 species prepared in (A) were assayed for *in vitro* deacetylase activity using bacterially-expressed autoacetylated GST-p300(HAT) as a substrate.

Thus, GST-SIRT1 purified using glutathione sepharose beads and treated with λ-PPase on-the-beads, was subsequently digested also on-the-beads with trypsin. The released tryptic peptides were subjected to matrix-assisted laser desorption ionisation mass spectrometry (MALDI - MS) to identify phosphorylated peptides. This approach, coupled to automated database searches indicated the presence of at least two phosphorylated serines, corresponding to aminoacids S27 and S47 (FIGURE 4-9).

To test whether phosphorylation of these serines conferred the characteristic mobility shift to SIRT1, Flag-tagged SIRT1 or non-phosphorylatable mutants carrying serine to alanine substitutions were transfected in 293 cells and subjected to post-lysis λ-PPase treatment. All three species exhibited similar behaviour in this experiment (FIGURE 4-10) suggesting any of the following possibilities: (i) S27 and S47 are not phosphorylated in human cells, (ii) S27 or S47 are phosphorylated but do not cause a shift or (iii) phosphorylation of either S27 or S47 suffices to cause a shift which is not abolished when only either one of these residues is mutated to alanine. To date only possibility (i) can be dismissed because during the course of these studies, a recent report identified both residues as phosphorylated in an unbiased large-scale screen of nuclear phosphoproteins from HeLa cells (Beausoleil *et al.*, 2004). In addition, two truncation mutants lacking aa's 1-

192 and 1-217 respectively showed a λ-PPase-sensitive shift suggesting that the phosphorylation sites in question are not located in the N-terminal 217 aminoacids of the protein (FIGURE 4-10 right panel).

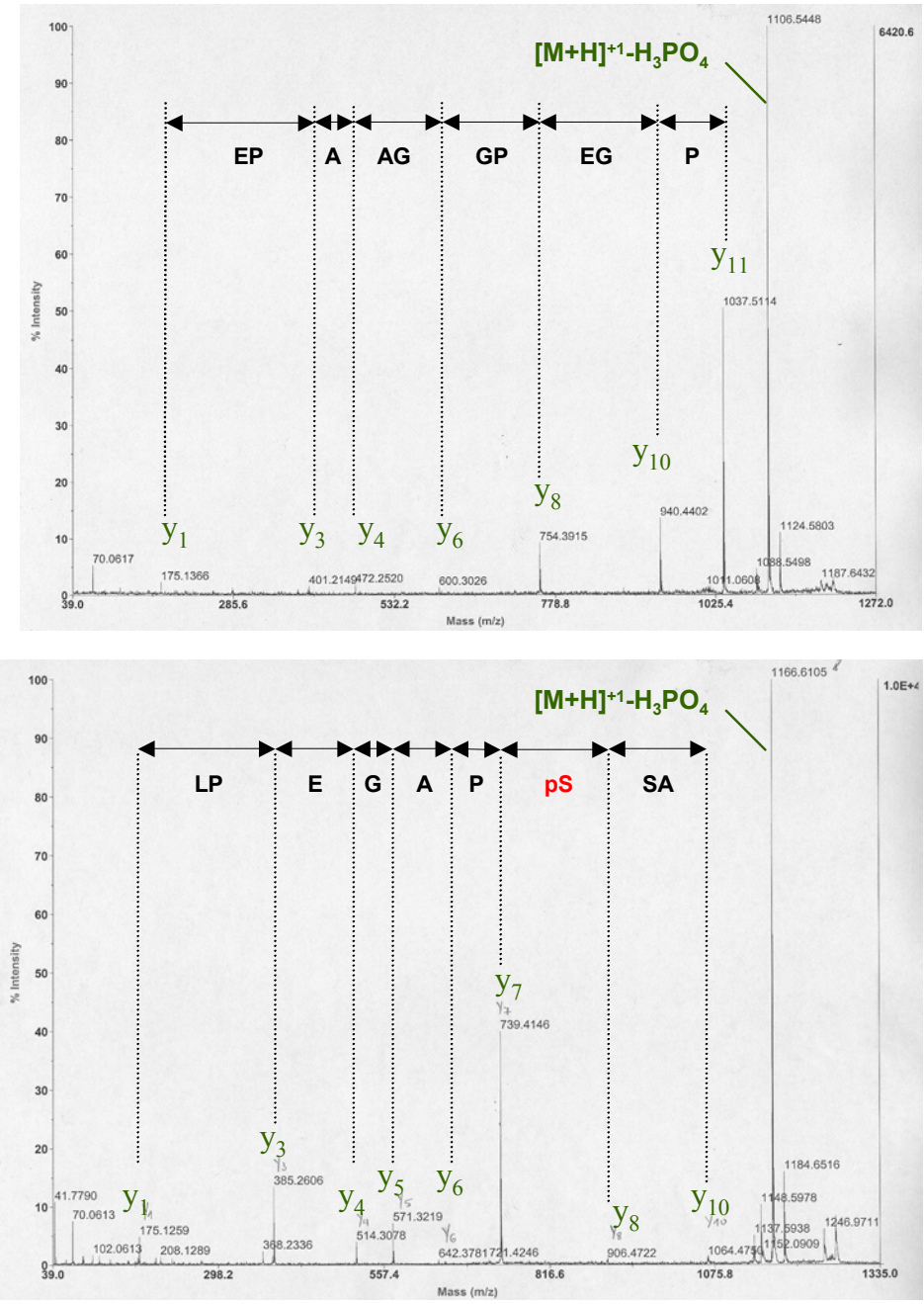


FIGURE 4-9. Identification of phosphorylation sites in GST-SIRT1 expressed in Sf9 cells. Tandem mass spectra of the two singly charged phosphopeptides found are shown along with the annotations of the aminoacids corresponding to the observed mass differences. $[M+H]^{+1}$-H_3PO_4 indicates the peak corresponding to the intact phosphopeptide that has lost H_3PO_4 following fragmentation in the mass spectrometer.

In the light of the information gained from the crystal structure of the yeast Hst2p (Zhao *et al.*, 2003) which indicated that sirtuins may form trimeric complexes by interacting through their N-termini, the possibility that SIRT1 also forms a homopolymer was investigated. Since modifications in the N-terminus of the protein could regulate this event, HeLa cells were transfected with HA- or Flag-tagged SIRT1 either alone or in combination and were subjected to immunoprecipitation with either α-HA or α-Flag antibodies. HA-SIRT1 could be detected in α-Flag immunoprecipitates only when Flag-SIRT1 was co-expressed but the reverse was not the case (FIGURE 4-11A). This suggested that human SIRT1 can homodimerise or possibly form higher order homopolymers. In a gel filtration experiment where proteins are separated according to the size of the native complexes they participate in, SIRT1 exhibits a broad profile spanning between molecular weights of ~300 kDa to 158 kDa (FIGURE 4-11B)

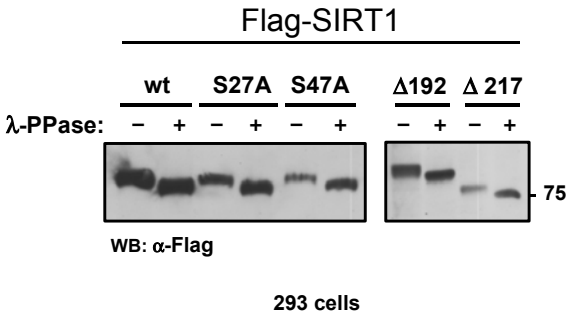


FIGURE 4-10. Alanine substitution of S27 or S47 does not abolish phosphorylation-induced electrophoretic mobility retardation of SIRT1. 293 cells were transfected with the indicated Flag-tagged SIRT1 constructs and either treated with λ-PPase or mock-treated prior to western blotting with α-Flag antibody.

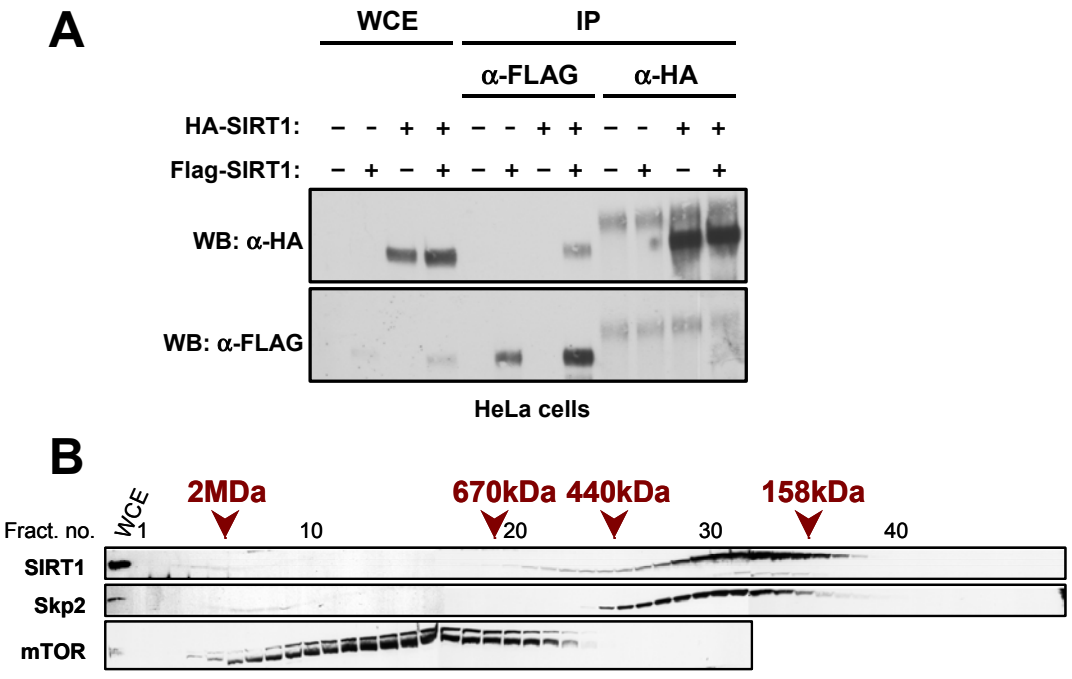


FIGURE 4-11. Evidence for SIRT1 oligomerisation. (A) HeLa cells were transfected with the indicated SIRT1 constructs and subsequently subjected to immunoprecipitation and western blotting with either α-HA or α-Flag antibodies; **(B)** Gel filtration analysis of SIRT1 derived from HeLa cells.

consistent with the presence of trimers, if these species correspond to homopolymers. This is in agreement with a subsequent report (Vaquero *et al.*, 2004) where SIRT1 was also suggested to be in a trimeric form. The above results show that SIRT1 can homopolymerise, possibly forming trimers. It remains to be tested whether this is mediated through the N-terminus of the protein and whether it is subjected to regulation by phosphorylation at S27 or S47.

As a putative nuclear localisation signal (NLS) was predicted in residues 233-238 of SIRT1 (FIGURE 4-12B and Frye, 1999; originally aa's 41-46 since the identified SIRT1 cDNA started from M193), the localisation of either of the N-terminal deletion mutants was investigated. As expected, wild-type SIRT1 was nuclear (FIGURE 4-12A). However, Δ192 exhibited equal distribution between the cytoplasm and the nucleus while Δ217 was exclusively cytoplasmic despite harbouring the putative NLS. Thus the N-terminus of SIRT1 must include another element that determines the protein's subcellular localisation. Another K/R-rich sequence that can serve as a putative NLS is found at aa's 94-99 (FIGURE 4-12B). These results raise the possiblity that SIRT1 is targeted to the nucleus by a bipartite NLS or additional modifications that reside within the N-terminus of the protein.

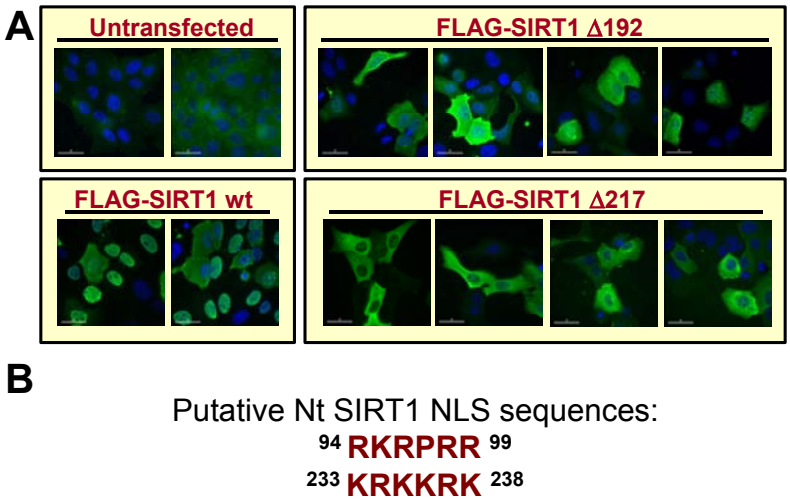


Figure 4-12. The N-terminus of SIRT1 harbours a previously unidentified NLS. (A) HeLa cells were transfected with the indicated SIRT1 constructs and subsequently subjected to immunocytochemical analysis with α-Flag antibodies; **(B)** Two sequences within the N-terminus of SIRT1 which can potentially serve as NLS.

4.1.3.2 Identification of the mobility-shift inducing phosphorylation sites

The observed mobility shift of SIRT1 in interphase cells shown to be due to phosphorylation was used as an indicator to determine the region of SIRT1 that harbored the putative phosphoaminoacids. In a primary screen, C-terminal deletions of SIRT1 were expressed as HA-tagged species in 293 cells and the derivative cell extracts were either

treated or not with λ-PPase. The logic behind this experiment was that because of the *de facto* reduced molecular weight of the truncated species, it would be difficult to compare their electrophoretic mobilities to that of the full-length species. If the absence of a protein region abolished the mobility shift, this should be observed when the truncated species is treated with λ-PPase by comprison to its untreated counterpart.

FIGURE 4-13A shows the results of this screen. A clear shift in the mobility of HA-SIRT1 could be observed validating this approach since it showed that exogenous SIRT1 behaves similarly to its endogenous counterpart. Furthermore, mAb12/1 could recognise all C-terminal truncations indicating that its epitope is still intact while it also allowed to monitor endogenous SIRT1 mobility as a control for the efficiency of the λ-PPase treatment.

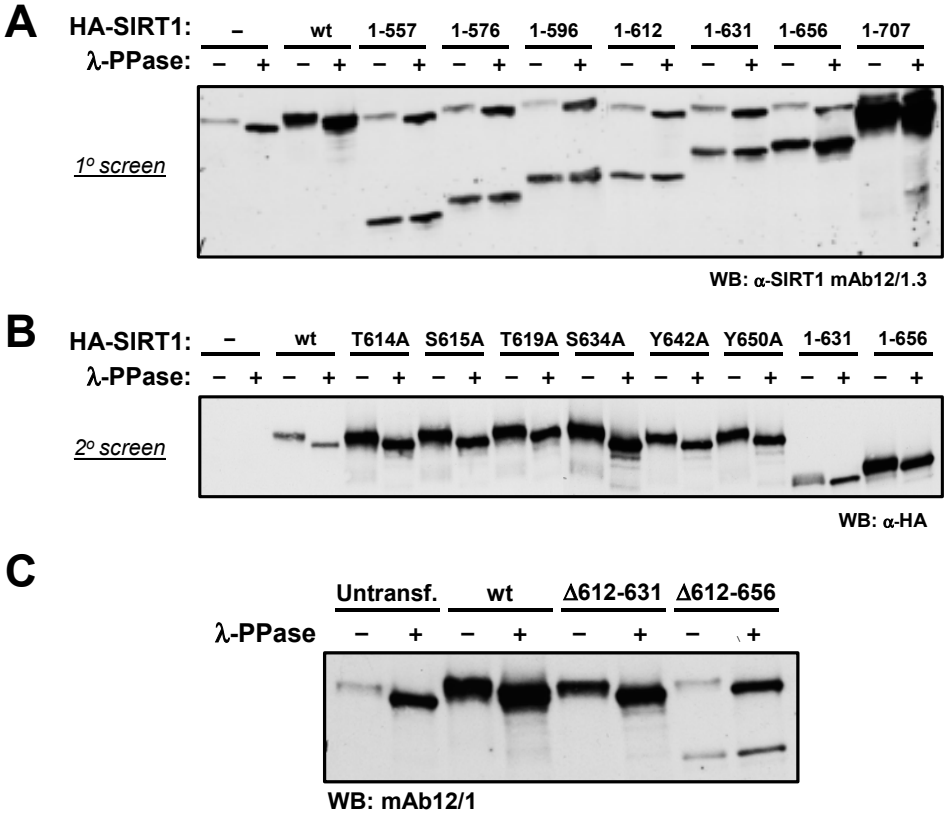


FIGURE 4-13. Screen for the identification of residues involved in phosphorylation-induced electrophoretic mobility retardation of SIRT1. *See* text for details.

A SIRT1 species that lacked the ultimate 134 aminoacids [HA-SIRT1(1-612)] did not exhibit any mobility shift upon λ-PPase treatment suggesting that the "phosphoshift" sites are located within this region. The species HA-SIRT1(1-631) did not show an

obvious shift, yet the intensity of the SIRT1 band in the λ-PPase treated cells was higher, a phenomenon which, based on experience acquired during this project, may arise when multiple protein species are concentrated to a single band. Indeed this proved to be the case when this experiment was repeated (*see* FIGURE 4-13B). Thus based on this experiment it was concluded that phosphorylation of SIRT1 between residues 613 and 656 causes the observed mobility shift of SIRT1 in interphase.

To confirm this, all phosphorylatable aminoacids within this region were mutated to alanines and subjected to the same type of assay (FIGURE 4-13B). None of these individual mutations sufficed to abolish λ-PPase sensitivity suggesting that multiple phosphorylation sites within this region may contribute to the observed shift. Given the fact that any combination of 2 or more of the 6 aminoacids could be phosphorylated, it was considered appropriate to address this issue by creating a species which lacked aminoacids 612-631 and 612-656 henceforth referred to as Δ612-631 and Δ612-656 respectively. As Δ612-631 migrated very similarly to wt SIRT1 it is difficult to conclude whether this mutant still shows a shift. However, it is clear that the Δ612-656 deletion renders SIRT1 insensitive to λ-PPase treatment providing further evidence that the 612-656 region of SIRT1 is phosphorylated in interphase.

An alternative interpretation of these results is that aa 612-656 do not harbor the phosphorylation sites in question but rather they serve as the docking site for the responsible kinase. Despite the transient nature of enzyme-substrate interactions, some kinases have been shown to interact with their substrates stably enough to allow the co-purification of the complex by affinity chromatography. Such an example is cyclin A/Cdk2 with E2F-1 (Krek *et al.*, 1994) and Jnk with c-Jun (Hibi *et al.*, 1993). To investigate the possibility that SIRT1 co-purifies with a kinase, SIRT1 was immunopurified (IP'd) with each of the Ctp, GST-S1 and mAb12/1 antibodies, the buffer of the immunoprecipitates (IPs) was exhanged for a standard kinase buffer supplemmented with ^{32}P-ATP. In the presence of a co-purifying kinase, SIRT1 should be labelled with ^{32}P as visualised by autoradiography.

As seen in Figure 4-14A, despite the presence of IP'd SIRT1 with all antibodies, no signal was present in the mAb12/1 IP whereas a very weak signal may be seen in the immunoprecipitates of the polyclonal antibodies (arrowheads). This suggested either the complete absence of a co-purifying kinase or the unsuitability of the reaction conditions for the succesful outcome of this experiment. It is also possible that the weak signal in the

Ctp and GST IPs reflects a very transient interaction between SIRT1 and the putative kinase.

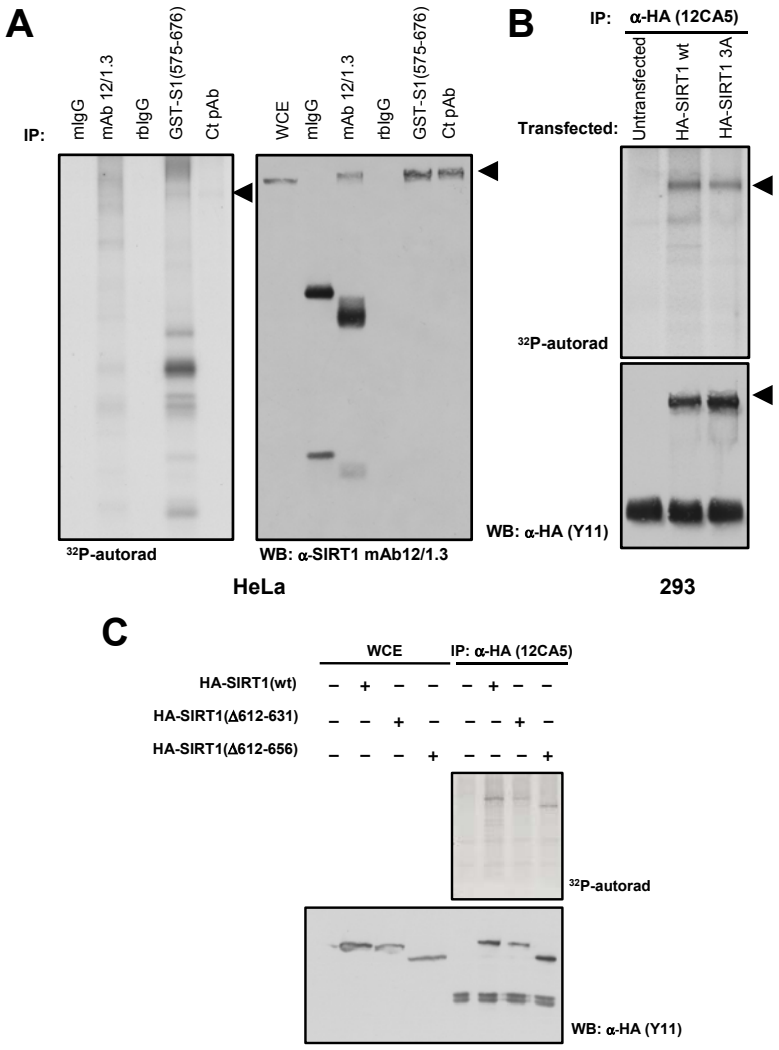


FIGURE 4-14. Phosphorylation of SIRT1 by a stably bound kinase. *See* text for details.

It was thus hypothesised that overexpression of SIRT1 might facilitate the visualisation of this interaction. 293 cells were transfected with HA-SIRT1 and subjected to IP with an α-HA mAb. WB confirmed the presence of the IP'd protein (FIGURE 4-14B) and the autoradiogram showed a strong signal migrating at the same molecular weight as exogenous HA-SIRT1 in the transfected but not the untransfected samples suggesting the presence of a co-purifying kinase.

To test whether this kinase interacted with SIRT1 through the phosphoshift site, the same experiment was repeated with wild-type HA-SIRT1 and the Δ612-631, Δ612-656 mutants. As shown in FIGURE 4-14C, neither deletion abolishes the ^{32}P-labelling of the corresponding IP'd species.

These experiments suggest that there is a kinase that can bind stably to SIRT1 causing its *in vitro* phosphorylation in a region outside 612-656 which was shown to harbour residues responsible for the protein's electrophoretic mobility shift. This does not exclude the possibility that the kinase causing the shift is bound to this region in which case the interaction might be too weak to be detected with this assay especially in the presence of another kinase demonstrated to be present in these experiments. Thus it is necessary to probe further the identity of this kinase and identify the sites affected by its action.

4.1.3.3 *In vitro* phosphorylation of SIRT1 by Casein Kinase 2

Kinases exhibit profound selectivity not only for the residue that they target for phosphorylation but also for the aminoacids that flank it. The availability of information on phosphorylation consensus sites targeted by specific kinases allowed the creation of searchable databases that provide the researcher with the ability to investigate whether their protein of interest harbours any or specified phosphorylation sites. Clearly, though, this approach is limited to previously reported data and cannot indicate whether a proposed phosphorylation site occurs *in vivo* and has physiological relevance.

In search for potential phosphorylation sites on SIRT1 the program Scansite was used (http://scansite.mit.edu/motifscanner/motifscan1.phtml?database=_SWS_). This program allows the input protein sequence to be investigated for harbouring phosphorylation sites within any of 62 motifs comprising kinase consensus target sites as well as known binding motifs for several protein interaction-mediating domains such as SH2 and SH3 domains.

This approach indicated that the C-terminus of SIRT harbours a putative phosphorylation site for casein kinase 2 (CK2) at residue S693. The CK2 consensus site consists of a phosphorylatable serine or threonine residue followed by acidic aminoacids i.e. D or E or phosphorylated S and T, especially at the +3 position (Litchfield, 2003). Manual observation of the sequence revealed two more putative CK2 sites at residues S659 and

S661 (FIGURE 4-15A top). Further support for a potential interaction between SIRT1 and CK2 was provided by the results of a recent large-scale yeast two-hybrid screen aiming at the creation of global protein interaction networks in *Drosophila* (Giot *et al.*, 2003). In this screen, the *Drosophila* homologue of SIRT1, dSir2 was shown to interact with the β subunit of *Drosophila* CK2.

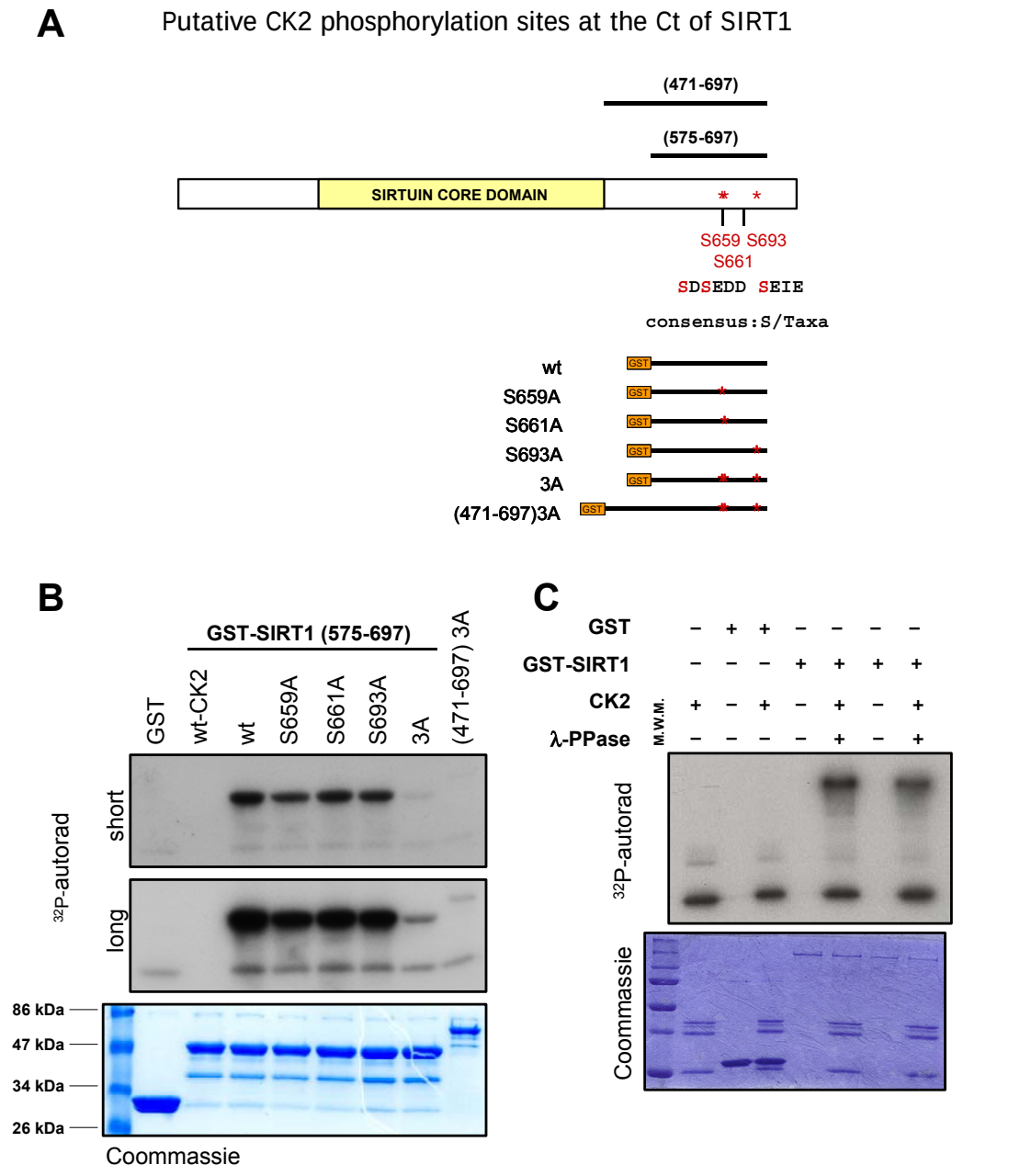


FIGURE 4-15. *In vitro* phosphorylation of SIRT1 by CK2. (A) Putative CK2 phosphorylation sites in the C-terminus of SIRT1 and the SIRT1 C-terminal GST-fusion constructs used in (B); **(B)** *In vitro* phosphorylation of GST-SIRT1 constructs as in (A) by CK2; **(C)** Sf9-expressed GST-SIRT1 is not phosphorylated in CK2 sites.

CK2 is a serine/threonine protein kinase with ubiquitous presence in eucaryotic cells. It comprises three catalytic subunits α, α' and the recently identified α''' and two regulatory β subunits which can combine to form a tetrameric holoenzyme in the following configurations: $\alpha_2\beta_2$, αα' β_2 or $\alpha'_2\beta_2$ (Ahmed *et al.*, 2002). CK2 exhibits broad specificity and has consequently been shown to phosphorylate hundreds of proteins participating in diverse cellular processes (Meggio and Pinna, 2003). Of note is its function in promoting cell survival in agreement with the consistent increase in CK2 protein levels in all human tumours examined, which can be separated from their hyperproliferative behaviour (Ahmed *et al.*, 2002). An interesting mode by which CK2 is thought to promote survival is by phosphorylating caspase target proteins close to their cleavage site precluding the recognition of the site and thus inhibiting their caspase-mediated processing (Desagher *et al.*, 2001; Krippner-Heidenreich *et al.*, 2001). CK2 is required for implementation of the spindle assembly checkpoint in response to microtubule poisons while in the same context it is also required for p53-mediated apoptosis (Sayed *et al.*, 2001). CK2 phosphorylates the protein X-ray cross-complementing gene 1 (XRCC1) mediating its recruitment to sites of DNA damage upon oxidative stress where it is required for the repair of the incurred lesions (Loizou *et al.*, 2004). Furthermore, it regulates transcription through RNAPolI by phosphorylating the initiation factor UBF promoting its transactivation potential (Voit *et al.*, 1992). CK2 has also been shown to phosphorylate different components of the RNAPolIII machinery resulting in either inhibition or activation of transcription depending on the phase of the cell cycle (Hu *et al.*, 2004).

To investigate whether SIRT1 residues S659, S661 ad S693 are indeed phosphorylated by CK2, commercially available recombinant CK2 was used in an *in vitro* kinase assay against bacterially expressed GST-fusions of the SIRT1 C-terminus (aa 575-697) harbouring either the wild-type sequence or mutations of S659, S661 and S693 to alanine which cannot be phosphorylated (FIGURE 4-15A). A mutant where all three residues were mutated to alanine in the same species was also tested. CK2 potently phosphorylated wild-type SIRT1 *in vitro* but not GST alone suggesting that phosphate incorporation occurred within the SIRT1 Ct sequence (FIGURE 4-15B). None of the single mutants abolished this phosphorylation. However, the 3A mutant exhibited only background phosphorylation levels suggesting that all putative CK2 phosphoacceptor sites were abolished. Another 3A mutant, also harbouring aminoacids 471-574 was not significantly phosphorylated indicating that indeed S659, S661 and S693 are the only sites targeted by CK2 *in vitro*. This experiment does not exclude the possibility that the

rest of SIRT1 sequence also harbours CK2 sites, which is desirable to know for an in-depth analysis of CK2 action on the protein. SIRT1 fusions do not express well in *E. coli* DH5α strain tested, thus GST-SIRT1 expressed in Sf9 cells by a recombinant baculovirus was tested. Proteins expressed in Sf9 cells may be phosphorylated by insect kinases in which case the residues in question would not incorporate ^{32}P. To exclude this, an *in vitro* CK2 kinase assay was performed on GST-SIRT1 either mock-treated or treated with λ-PPase (FIGURE 4-8A) which led to the characteristic increased mobility of the protein. As shown in FIGURE 4-15C comparable ^{32}P incorporation was observed for both GST-SIRT1 species suggesting that CK2 does not target SIRT1 in Sf9 cells. Thus Sf9 cells-expressed recombinant SIRT1 would provide a good system to further analyse the effects of CK2 phosphorylation on SIRT1 activity.

These observations raised the possibility that the CK2 phosphorylation sites are responsible for the observed mobility shift of SIRT1 in interphase cell extracts. To test this hypothesis, the full-length cDNA of SIRT1 harbouring S659A, S661A and S693A (henceforth referred to as "3A") substitutions was expressed as an HA-tagged fusion in HeLa cells and its mobility was compared to the mobility of wild-type HA-SIRT1. As a control the lysates from both transfected cell populations were treated with λ-PPase. The migratory behaviour of either SIRT1 species was indistinguishable (FIGURE 4-16) suggesting either that these three serine residues are not phosphorylated *in vivo* or that even if they are phosphorylated they do not alter the mobility of the protein. Interestingly, HA-SIRT1 3A was expressed at much higher levels compared to its wild-type counterpart suggesting that inability to phosphorylate these sites correlates with increased stability of SIRT1 protein.

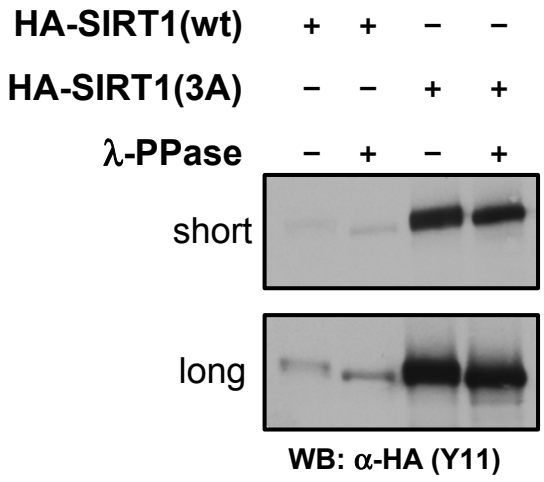


FIGURE 4-16. Alanine substitution of the in vitro CK2 sites does not abolish phosphorylation-induced electrophoretic mobility retardation of SIRT1. Cells were transfected with the indicated HA-tagged SIRT1 constructs, lysed and either treated with λ-PPase or mock-treated. The electrophoretic mobility of the corresponding proteins was analysed by immunoblotting with α-HA antibody.

4.1.3.3.1 SIRT1 in the UV response

The experimental approaches delineated above were based on the widely accepted assumption that CK2 is a constitutively active kinase (Litchfield, 2003). However, several recent reports have proposed novel modes of CK2 action where its activity is inducible by extracellular stimuli. In particular a new emerging role of CK2 in response to UV irradiation is of note.

CK2 is part of a complex with the chromatin transcriptional elongation factor FACT (facilitates chromatin transcription) which comprises hSpt16 and SSRP1 (structure-specific recognition protein 1). In the context of this complex whose assembly is induced upon UV irradiation, CK2 phosphorylates p53 at S392 resulting in enhanced binding of p53 to DNA which in turn correlates with its ability to induce transcription (Keller *et al.*, 2001).

In another report, Kato and co-workers (Kato *et al.*, 2003) showed that CK2 mediates the phosphorylation-driven degradation of IκB upon ultraviolet (UV) irradiation in a pathway distinct from that of IκB kinase (IKK). In response to inflammatory cytokines and ionising radiation IKK phosphorylates the N-terminus of IκB and promotes its degradation via a β-TrCP-based SCF E3 ubiquitin ligase complex (Hayden and Ghosh, 2004). In contrast, upon UV irradiation CK2 phosphorylates six residues in the C-terminus of IκB in a manner dependent on the kinase p38 and leads to the degradation of IκB and subsequent NFκB activation. Furthermore, the CK2 sites reside within a region known as the PEST motif (FIGURE 4-17). The PEST motif is characterised by enrichment in P, E, S and T residues but lacks positively-charged aminoacids. It is found in several proteins including the myc, fos and jun oncogenes and p53 tumour suppressor which it targets for degradation by the ubiquitin-proteasome pathway (Rechsteiner and Rogers, 1996).

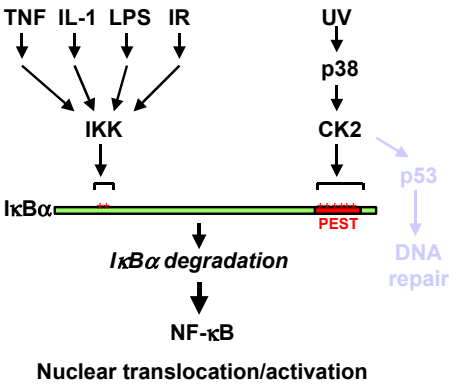


FIGURE 4-17. The CK2 phosphorylation sites of IκB are located within a PEST sequence. *See* text for details.

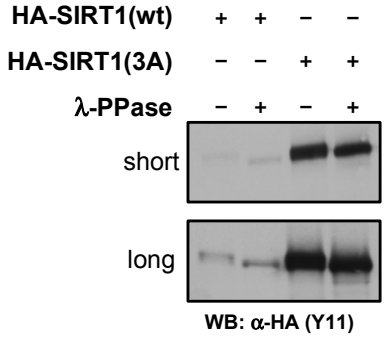
To investigate whether SIRT1 also carries a similar motif and is therefore potentially regulated by PEST-mediated proteolysis, a web-based algorithm that scores for potential PEST motifs in protein sequences was used[1]. A region between aa's 653-711

[1] (https://emb1.bcc.univie.ac.at/toolbox/pestfind/pestfind-analysis-webtool.htm)

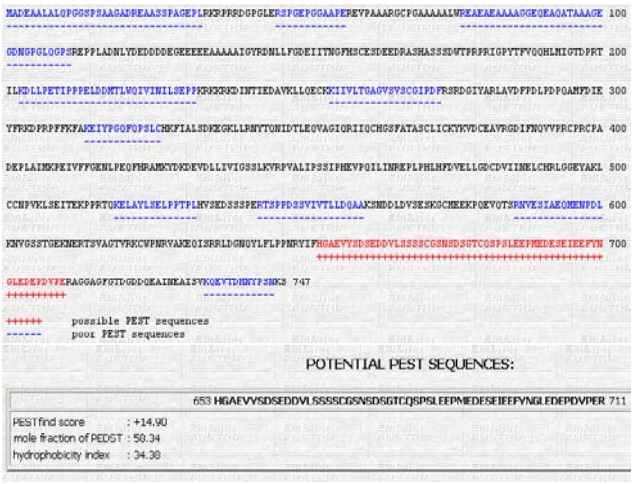
harbours a high-scoring PEST sequence (score: +14.9, FIGURE 4-18A). This is among the best scores of previously identified PEST motifs (FIGURE 4-18B) further validating the argument that SIRT1 harbours a *bona fide* PEST sequence. Moreover, the PEST region encompasses all three putative CK2 phosphorylation sites similar to the PEST motif in IκB.

Interestingly, SIRT1 was also shown to negatively regulate the transcriptional activity of NFκB by deacetylation in response to TNFα (Yeung *et al.*, 2004) and in the work presented here, CK2 is a proposed SIRT1 kinase. Thus, two negative regulators of NFκB activity, IκB and SIRT1 share a common regulatory domain, the PEST motif, which harbours CK2 phosphorylation sites. This raised the possibility that a CK2-mediated regulatory mechanism exists that dictates the activation of NFκB in response to UV irradiation by ensuring the concomitant inactivation of two of its negative regulators. This observation prompted the exploration of the hypothesis that SIRT1 is regulated in response to UV irradiation.

To examine the question whether a UV-inducible kinase associates with and phosphorylates



A



B

Table I. PEST sequences known to function as proteolytic signals

Protein	Sequence	PEST-FIND score
GCN4	KTVLPIPELDDAVVESFFSSSTDSTPMFEYENLEDKSK KEWTSLFDNDIPVTTDDVSLADK KAIKSTEKVKLVPEKLEVETTEFLPTPULKDAK	5.3 1.7 3.5
IκBα	RIQQQLQQLTLENLQMLPESSDEESYDTESEFTFTEDFI- PVSDCVPYDQR	5.9
Fos	KVRQLSPEEEEK KIPDDLGFPEEMSVASLDLTGGLPEVATPESEEAFT- LPLLNDPEPK GSSSNEPSSDSLSSPTLLAL	10.1 5.7 4.6
Ornithine decarboxylase	KEQPGSDDEDESNEQTPMYYVNDGVYGSFNCILYDH HQFPPEVEEQDDGTLPMSCAQESGMDR	3.7 5.2
Cactus	KEFAVPNETSDSGFISGPQSSQIFSEEIVPDSEFQDK KEQPVVLDGIIDEEEDQEEQEK RGAETVTPMGGVTGSSDIEDLDDTK	7.7 10.4 17.9
CLN2	KLTISTPSCSFENSNSTSIPSPASSSQSH	7.0
CLN3	KDSISPPFAPTPTSSSSSPSPPNSPVV KTSSSMTCPDSASH	8.2 10.6
NIMA	HSGQMGSGNEIEGDFPSGTDISQLSLRSPTNK KFEPTLAYSDDEDDCPELPSPTR	12.6 16.4

FIGURE 4-18. Identification of a PEST sequence in the C-terminus of SIRT1. (A) Results screen of the PESTFIND programme indicating regions scoring highly (red) or poorly for the presence of a PEST sequence; **(B)** A table of previously identified protein harbouring PEST sequences and their corresponding PESTFIND scores provided for comparison.
(B) adapted from Rechsteiner and Rogers, 1996.

SIRT, GST or GST-SIRT1 expressed in Sf9 cells were bound to glutathione beads which were subsequently incubated with extracts of HeLa cells irradiated with 20 J/m^2 UV for 30' prior to lysis. The beads were subsequenlty washed, equilibrated with kinase buffer and ^{32}P-γ-ATP was added to the preparation allowing any co-precipitating kinase to label SIRT1 with ^{32}P. No phosphorylation activity was observed against GST alone, however GST-SIRT1 showed significant incorporation of radioactivity (FIGURE 4-19). Yet, this activity was independent of UV treatment suggesting that, consistent with the notion of SIRT1 being stably bound to a kinase (*see* section 1.2.1.2, this chapter), GST-SIRT1 also binds to and gets phosphorylated by a kinase from HeLa cell extracts but this phosphorylation is not inducible by UV irradiation.

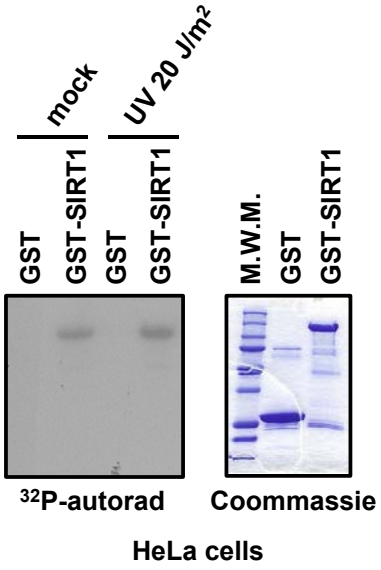


FIGURE 4-19. No evidence for UV-inducible phosphorylation of SIRT1 by a stably-bound kinase. GST or GST-SIRT1 expressed in Sf9 cells was purified using glutathione beads (right panel) and subsequently incubated with extracts of HeLa cells treated with 20 J/m^2 UV or mock-treated. The beads were washed, resuspended in kinase buffer including ^{32}P-γ-ATP and incorporation of ^{32}P by GST-SIRT1 was assessed by autoradiography (left panel).

To test the possibility that due to the presence of the PEST motif, SIRT1 degradation is induced in response to UV irradiation, the degradation kinetics of SIRT1 protein were probed by incubation of cells with the translation inhibitor cycloheximide (CHX). In such experiments, the rate of dissapearence of specific immunoreactive bands correlates with the rate of degradation of the corresponding protein since no more protein is newly synthesised. Thus, cells were either left untreated or treated with 20 J/m^2 UV and cycloheximide was added either 1 h or 8 hours post-treatment to account for potential differences between early- and late-responses (Agami and Bernards, 2000). 1 hour after UV treatment, p53 stabilisation was evident (FIGURE 4-20A). In contrast, a modest but existing increase in the rate of SIRT1 degradation was observed at this time point. This increase in SIRT1 degradation kinetics was also seen even after 0.5 h of UV treatment, in an independent experiment (FIGURE 4-20B). Similarly, at 8 h after UV treatment, a modest loss of SIRT1 stability occured compared to untreated cells (*cf.* 0 and 2 h CHX treatment time-points in UV-treated and untreated samples). Nevertheless, a significant increase in SIRT1 protein levels ensued

UV irradiation in the absence of CHX (CHX 0 h) suggesting that despite the observed increase in SIRT1 turnover, total protein levels increased.

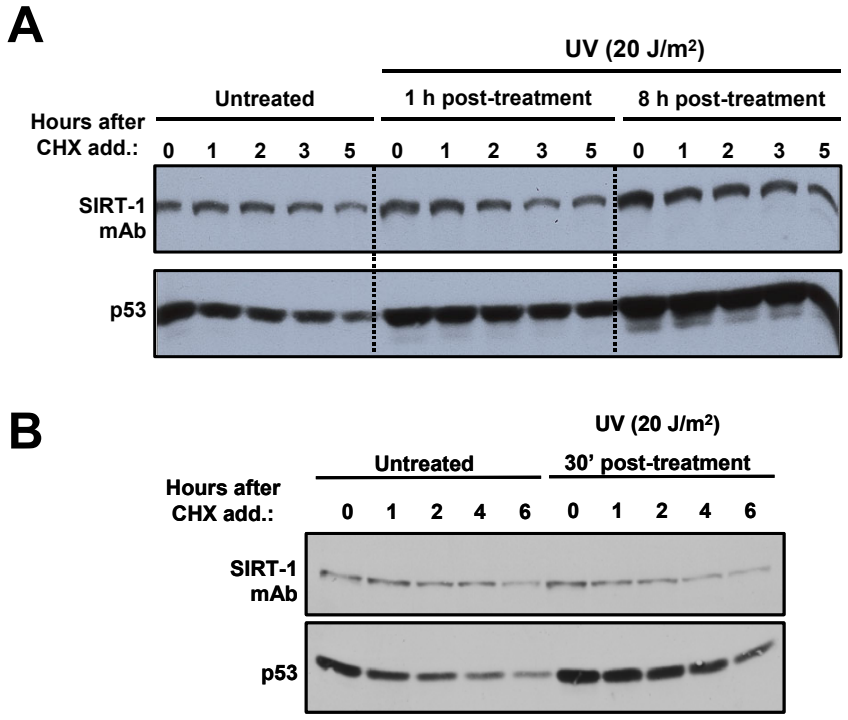


FIGURE 4-20. Effects of UV treatment on endogenous SIRT1 protein stability. (A) U2OS cells were either mock-treated or treated with 20 J/m^2 UV for 1 or 8 h prior to addition of cycloheximide (CHX) for the indicated amounts of time. Cells were harvested and subjected to immunoblotting either with mAb12/1 or p53 antibody as a control; **(B)** The same experiment was performed as in (A) but CHX was added 30' after exposure to UV for the indicated amounts of time.

Initial efforts to examine the effects of downregulating CK2 or p38 activity, using the chemical inhibitors DRB (5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole) or SB203580 respectively, did not provide any conclusive evidence for a role of these kinases in the regulation of SIRT1 turnover (not shown). In an alternative approach, to test whether the putative CK2 phosphorylation sites played a role in UV-induced SIRT1 turnover, pools of HeLa cells (uncloned mass culture) infected with retroviruses encoding either wild-type HA-SIRT1 or HA-SIRT1(3A) were generated. This was important as consistent amounts of protein are necessary to ensure reliable measurements of protein immunoreactivity which is more difficult to attain in transient transfection assays. FIGURE 4-21 shows the comparison of the turnover of exogenous wild-type SIRT1 in the presence or absence of treatment with UV. Consistent with the previous results, low dose of UV irradiation caused a modest increase in SIRT1

turnover. Unfortunately, due to the reduced expression of the 3A mutant, it was not possible to probe the effect of the putative CK2 sites, however this experiment validates the feasibility of the approach and attempts to resolve the differential expression of the mutant are underway.

The above results (FIGURE 4-20) also raised the interesting possibility that SIRT1 total protein levels increase in response to UV irradiation. Consequently, a closer analysis of this phenomenon was undertaken. To examine the kinetics of SIRT1 accumulation in response to UV irradiation, the human osteosarcoma U2OS cell line which has an intact p53 response was treated with increasing amount of UV light for various time-points (FIGURE 4-22A). In parallel, the effects of actinomycin-D, a chemical compound which is known to inhibit RNA polymerase II inducing single-strand breaks that mimic DNA lesions arising from UV-induced damage, was also investigated (FIGURE 4-22B). Low doses of UV induced an accumulation of SIRT1 protein which was apparent already at 2 hours post-treatment. This was also evident upon actinomycin-D treatment. In both cases, total p53 protein levels increased consisten with an induction of the DNA damage response. Interestingly, increasing levels of UV irradiation blunted the increase of SIRT protein levels, a phenomenon also observed for p53 (FIGURE 4-22A). This may indicate that at high doses of DNA damage, the p53-driven DNA repair pathway is inhibited to favour cell-death over lesion repair in face of the insurmountable damage incurred upon the genome. It is also of note that a second, lower molecular weight band, reactive to mAb12/1 appeared in a time- and dose-dependent manner. The identity of this SIRT1 species is the subject of discussion in part 2 of this chapter.

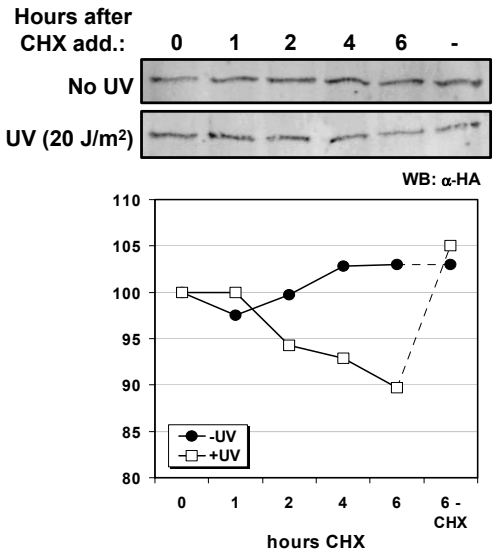


FIGURE 4-21. Effects of UV treatment on exogenous SIRT1 protein stability. HeLa cells were stably infected with a recombinant retrovirus expressing HA-SIRT1 and selected with puromycin. Puromycin-resistant cells were treated with 20 J/m^2 UV or mock-treated (no UV) 30' prior to addition of CHX for the indicated amounts of time. Cell lysates were subjected to immunoblotting, visualised using the Odyssey imaging system (upper pannel) and band intensity was quantified using the accompanying software (lower panel).

Given that SIRT1 abundance paralled that of p53, the possibility that SIRT1 expression is governed by p53 transcription was raised. Indeed, it was recently reported that the SIRT1 gene promoter harbours two p53 binding sites that allow the p53-mediated

transcriptional induction of the gene in response to nutrient deprivation (Nemoto *et al.*, 2004). However, this is unlikely to be the case here in the light of the fact that the transcriptional inhibitor actinomycin-D also elicited the induction of SIRT1 protein levels yet, to confirm this, it would be necessary to investigate the inducibility of the SIRT1 promoter upon UV irradiation. Furthermore, this does not rule out the possibility of a p53-mediated event in the control of SIRT1 mRNA translation.

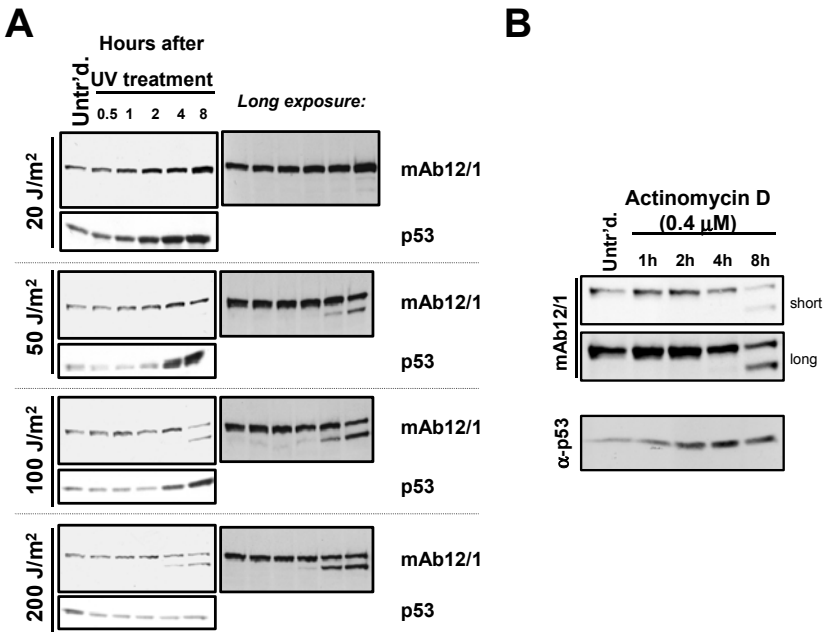


FIGURE 4-22. Effects of DNA damage-inducing agents on endogenous SIRT1 protein abundance. U2OS cells were treated with 0, 20, 50, 100 or 200 J/m^2 UV **(A)** or 0.4 μM actinomycin-D **(B)** and cells were harvested at the indicated times. SIRT1 protein levels were assessed by immunoblotting using mAb12/1. As a control, p53 antibody was used on the same blots.

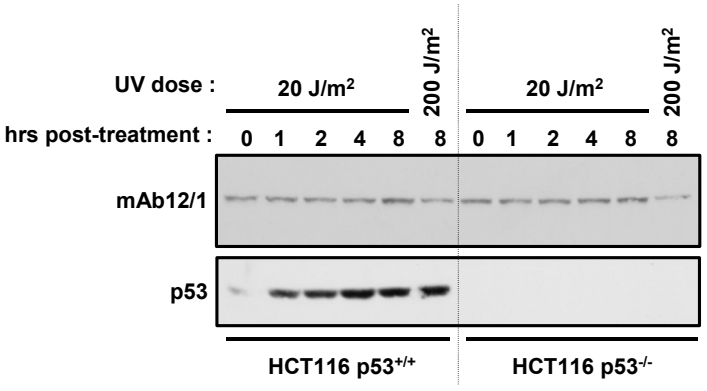


FIGURE 4-23. Effects of p53 on endogenous SIRT1 protein abundance after UV exposure. HCT116 cells (p53$^{+/+}$) or an isogenic derivative lacking both p53 alleles (p53$^{-/-}$) were treated with 20 J/m^2 or 200 J/m^2 UV, cells were harvested at the indicated times and subjected to immunoblotting using mAb12/1 or p53 antibody as a control.

To test this, SIRT1 protein levels in response to UV treatment were examined in two human colon carcinoma cell lines, HCT116 and an isogenic derivative where the p53 locus is deleted by means of homologous recombination (Cahill *et al.*, 1998). Interestingly, SIRT1 protein levels are almost unchangeable in either of these cell lines raising the possibility that the previously observed effects of UV on SIRT1 may be determined by cell-type or cell-line-specific factors. It would thus be important to repeat this experiment in U2OS cells in combination with p53 depletion by e.g. siRNA to answer this question conclusively.

Another possibility explored was that in response to UV, the binding of SIRT1 to other proteins may be affected. p300 is a transcriptional co-activator with acetyltransferase activity which has been proposed to acetylate several SIRT1 deacetylation targets such as NFκB, p53 and FOXO (*see* Chapter 2). To establish the validity of these observations and define the topology of the SIRT1/p300 interaction, the binding of GST-fusion proteins corresponding to fragments of p300 spanning the entire protein were tested for their ability to bind IVT full-length SIRT1. Aminoacids 1197-1673 which harbour the acetyltransferase activity of p300 exhibited strong binding to SIRT1 while a modest degree of binding was shown by the C-terminal fragment of the protein (aa's 1572-2371, FIGURE 4-24A). An attempt to define the region of SIRT1 that binds to p300 was inconclusive since, while the IVTs of N- and C-terminal regions of SIRT1 did not bind GST-p300(HAT), the remaining region corresponding to the sirtuin core domain exhibited profound binding to GST alone. It is thus possible that the sirtuin core domain and the zinc binding module in particular

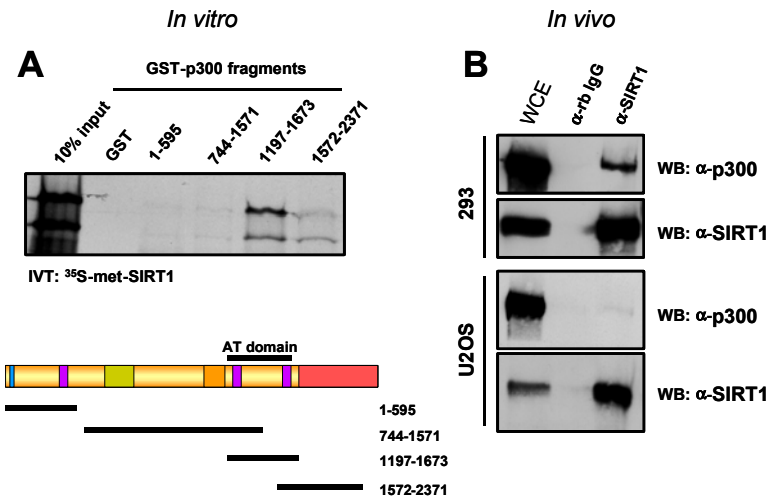


FIGURE 4-24. Interaction of SIRT1 with the acetyltransferase p300. (A) *In vitro* interaction of IVT (*in vitro* translated) SIRT1 with bacterially-expressed GST fused to polypeptides spanning the entire p300 coding region as indicated schematically in the lower panel; **(B)** *In vivo* interaction between SIRT1 and p300 in 293 or U2OS cells. SIRT1 was immunoprecipitated with GST-S1 antibody.

may mediate the interaction with p300 consistent with the role of this domain as a mediator of protein interactions (Finnin *et al.*, 2001).

To investigate whether endogenous SIRT1 and p300 interact, GST-S1 IPs from U2OS and 293 cells were probed with antibodies against p300 (FIGURE 4-24B). In both cases an interaction was observed albeit of different strength with the one in 293 cells being the strongest. 293 cells but not U2OS cells express the transforming E1A viral protein which partially exerts its oncogenic effects by binding to the p300 HAT region similarly to SIRT1. This could indicate that in 293 cells the observed interaction is mediated by E1A. However, the strength of the *in vitro* interaction as well as the observed co-purification of the two proteins in U2OS cells provide evidence against this possibility.

The acetyltransferase activity of p300 has been recently shown to be regulated by acetylation itself (Thompson *et al.*, 2004). To investigate whether the deacetylase activity of SIRT1 actually regulates acetylation of p300, GST-SIRT1 or the catalytically inactive mutant GST-SIRT1(H363Y) expressed in Sf9 cells were incubated with GST-p300(HAT) expressed in bacteria. The latter protein species is able to autoacetylate within the bacterial cells providing a substrate to assay SIRT1 activity *in vitro*. GST-SIRT1 but not its catalytically inactive counterpart exhibited a robust deacetylation activity against GST-p300(HAT) (FIGURE 4-25A). Moreover, this effect was sensitive to inhibition by nicotinamide but not TSA further supporting the concept that it is exclusively mediated by a sirtuin deacetylase rather than a co-purifying HDAC. These results firnly establish SIRT1 as an *in vitro* deacetylase of p300 raising the possibility that it may act as a regulator of p300 activity *in vivo*.

To investigate this possibility, NIH3T3 mouse fibroblasts were infected with retroviruses expressing either SIRT1 or SIRT1(H363Y). As a control cells infected with a virus expressing GFP were also included in the analysis. p300 IP's from these cells were probed with α-acetylated lysine (α-AcK) antibodies to examine the acetylation status of p300. A significant reduction of p300 acetylation was observed in the cells expressing wild-type but not the catalytically inactive SIRT1 confirming that SIRT1 can deacetylate p300 *in vivo* (FIGURE 4-25B). A small reduction in the total levels of p300 was also observed (FIGURE 4-25B, middle panel, lanes 7 and 8). However, the relative reduction of p300 acetylation levels when SIRT1 was expressed was significantly higher (FIGURE 4-25B, upper panel, lanes 7 and 8) compared to that of total p300 protein levels supporting the above conclusions. Also note that the absence of SIRT1 immunoreactivity in lane 1 does not

necessarily indicate lack of endogenous SIRT1 expression. Rather, it stems from the inability of mAb12/1 to recognise the mouse protein (*see* section 1.1, this chapter).

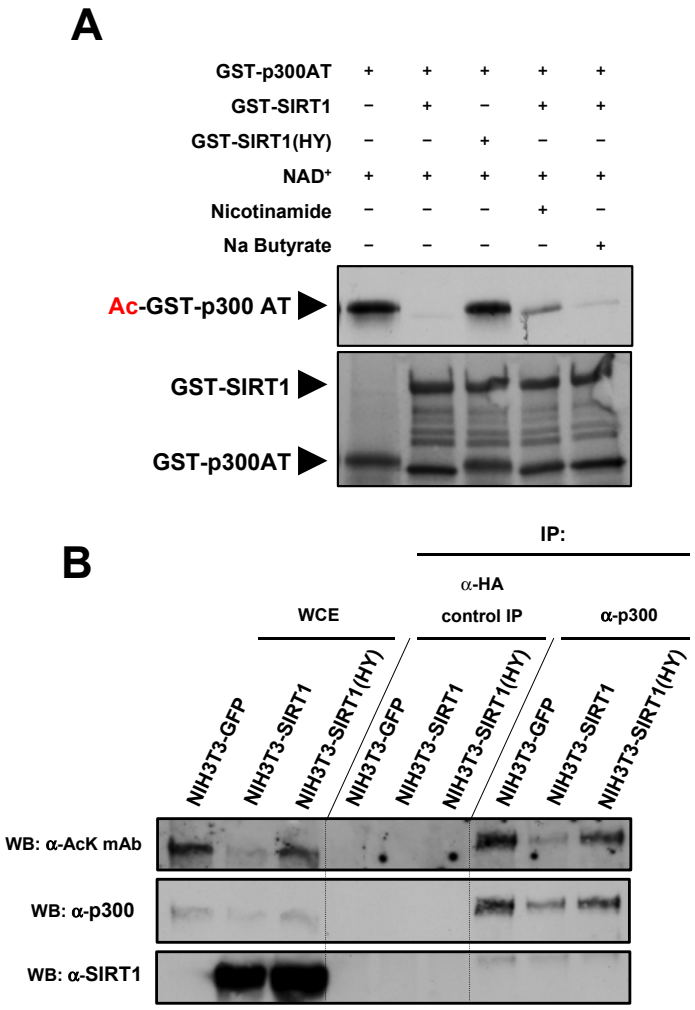


FIGURE 4-25. Deacetylation of p300 by SIRT1 (A) *In vitro* deacetylation assay was performed as in Figure 4.8B except that in addition a catalytically inactive mutant of SIRT1 was also used [GST-SIRT1(HY)] and the sensitivity of SIRT1 deacetylase activity was assayed using nicotinamide and butyrate. GST-p300(HAT) acetylation status was assayed using an α-acetyl-K mAb and presence of the indicated GST-fusion proteins was probed by α-GST polyclonal antibody; **(B)** *In vivo* deacetylation of p300 by SIRT1. NIH3T3 mouse fibroblasts were infected with recombinant retroviruses expressing eGFP as a control, SIRT1 or catalytically inactive SIRT1 [SIRT1(HY)] and selected using puromycin. Puromycin resistant cell lysates were subjected to immunoprecipitation with α-p300 mAb and acetylation levels of p300 were probed as in (A).

Upon UV irradiation p53 mediates the establishement of the DNA repair response and acetylation of p53 by p300 is required for its transcriptional activity (Luo *et al.*, 2004). Thus, it was hypothesised that attenuation of SIRT1 deacetylase activity upon p300 as well as p53 itself would be required for the activation of p53 after UV irradiation. One way to achieve this would be the loss of interaction between p300 and SIRT1 although, strictly speaking, the necessity of the interaction for the deacetylation of p300 by SIRT1 to occur has not been firmly established. To test this possibility, U2OS cells were irradiated with UV, harvested at either 2 or 8 hours following treatment, subjected to IP with the GST-S1 antibody and probed with an α-p300 antibody. p300 was present at equal levels in SIRT1

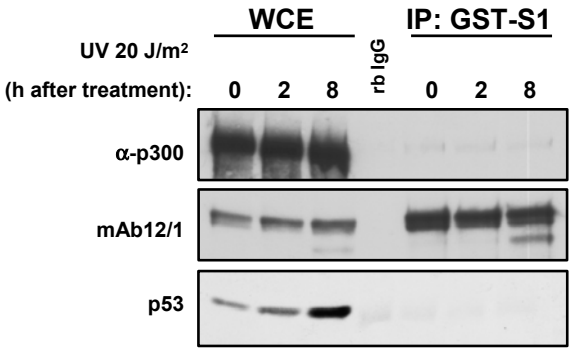
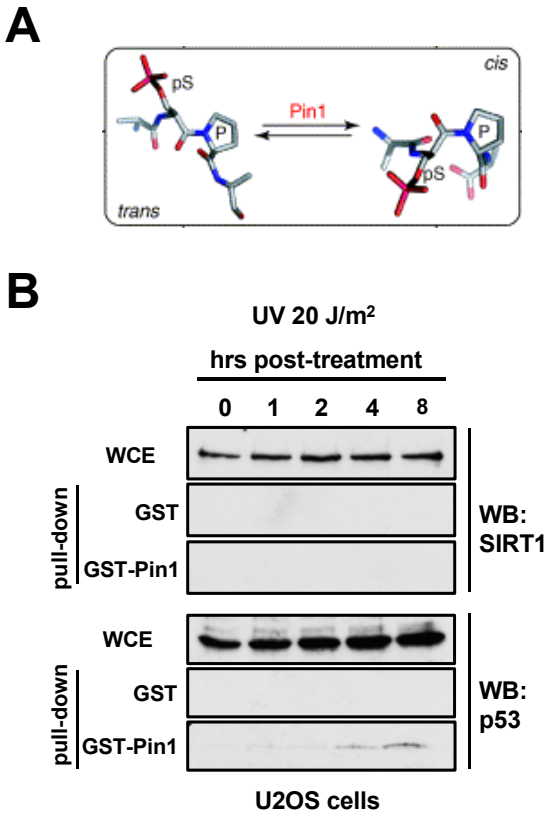


FIGURE 4-26. Interaction of SIRT1 with the acetyltransferase p300 is not abolished by UV treatment. U2OS cells were mock-treated or treated with 20 J/m^2 UV, cells were harvested at the indicated amounts of time, lysed and subjected to immunoprecipitation with either control or GST-S1 antibodies. Interaction between p300 and SIRT1 was probed using the corresponding antibodies. α-p53 antibody was used as control for the UV treatment.



IPs from both UV-treated and untreated cells (FIGURE 4-26) suggesting that the interaction between the two proteins is not subjected to regulation in response to UV treatment under the experimental conditions employed here.

UV irradiation was also shown to promote the association of p53 with the peptidyl-prolyl-*cis*/*trans* isomerase (PPIase) Pin1 in a manner dependent on phosphorylation of S33, T81 and S315 (Zacchi *et al.*, 2002, Zheng *et al.*, 2002). Pin1 catalyses the *cis*/*trans* isomerisation (Lu *et al.*, 2004) of proline residues located directly at the C-terminus of phosphorylated S or T (Yaffe *et al.*,1997; FIGURE 4-27A). This induces a conformational change in the target protein with various poorly understood effects (Lu *et al.*, 2004). Under this attribute Pin1 has been linked to several pathological states including cancer and Alzheimer's disease (Wulf *et al.*, 2005). Upon UV treatment, the interaction of Pin1 with p53 results in a conformational change of the latter which allows its dissociation from its negative regulator Mdm2 leading to the transcriptional activation of target genes required for the DNA damage response (Zacchi *et al.*, 2002). Furthermore, in response to cytokine treatment, Pin1 binds to

FIGURE 4-27. No UV-inducible interaction between Pin1 and SIRT1. (A) Stick model depicting the proline isomerisation reaction catalysed by Pin1 PPIase (adapted from Lu, 2004); **(B)** Bacterially-expressed GST or GST-Pin1 were purified using glutathione beads and incubated with cell lysates derived from U2OS cells mock-treated or treated with 20 J/m^2 UV for the indicated amounts of time. The previously reported interaction of Pin1 with p53 was included as a positive control.

phosphorylated T254 of the NFκB p65/RelA subunit, an event that is proposed to induce a conformational change that leads to reduced binding to the inhibitory IκB, resulting in enhanced activity of NFκB (Ryo *et al.*, 2003). Thus at least two pathways in which a role for SIRT1 has been demonstrated are regulated by Pin1-mediated prolyl isomerisation, raising the possibility that SIRT1 may also be a Pin1 target potentially enabling their activation by promoting its dissociation from these factors.

To explore the possibility of an interaction occuring between SIRT1 and Pin1, U2OS cells were irradiated with UV and harvested at various time-points. Lysates thereof were incubated with glutathione beads bearing bound GST or GST-Pin1, washed and subjected to immunoblotting with either p53 antibody or mAb12/1. UV treatment induced the interaction between p53 and Pin1 (FIGURE 4-27B, lower panels) consistent with the published reports. In contrast, no interaction between SIRT1 and Pin1 was observed in the same assay either before or after UV treatment, thus dismissing the proposed hypothesis.

Regulation of protein function can also be implemented by changes of its subcellular localisation e.g. providing thus access to specific substrates or other regulators. To examine the localisation of SIRT1 in response to UV treatment, U2OS cells were irradiated with 20 or 50 J/m^2 UV, fixed and stained with GST-S1 antibody. Increasing amounts of UV radiation caused a dose-specific change of SIRT1 staining from a diffuse nucleoplasmic pattern to characteristic rings surrounding nuclear regions of poor DAPI staining reminiscent of nucleoli (Figure 4-28). As a control for the specificity of the antibody, irradiated cells incubated without the primary antibody were used (FIGURE 4-28g).

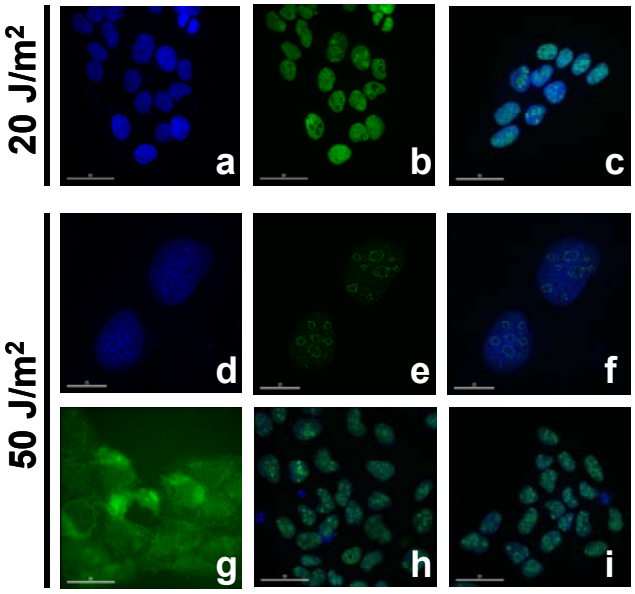


FIGURE 4-28. Effects of UV treatment to SIRT1 subnuclear localisation. U2OS cells were treated with 20 **(a-c)** or 50 J/m^2 **(d-i)** UV and immunostained with GST-S1 antibody (green) or DAPI (blue) except for **g** where the primary (GST-S1) antibody was omitted. Scalebars represent 90 μm (a-c and g) or 15 μm (d-f and h,i).

In addition to the well-established function of nucleoli in the assembly of ribosomes, a role in cellular stress responses is also emerging (Olson, 2004). In particular, nucleoli appear to function as nuclear depots of proteins in a manner depending on their structural integrity. Upon stress stimulation, disruption of nucleolar structure allows the release of such factors partly contributing to the ensuing cellular responses to these stimuli. A more detailed account of this function of nucleoli will be given in Chapter 5. Under the light of these, it would be important to conclusively establish that the distinct localisation of SIRT1 is indeed associated with nucleoli and subsequently investigate the functional significance of this phenomenon.

4.1.4 Regulation of SIRT1 by phosphorylation in mitosis

According to its electrophoretic mobility during SDS-PAGE, SIRT1 was shown to be a phosphoprotein which is subjected to hyper-phosphorylation in cells arrested in mitosis by treatment with the microtubule-destabilising agent nocodazole (FIGURE 4-7). This observation raised the possibility that SIRT1 is phosphorylated in a cell cycle-dependent manner. Mitotic phosphorylation of SIRT1 may be particularly relevant especially given the proposed roles of situins in life-span determination under the light of increasing evidence for a link between components of the mitotic regulatory apparatus and the aging process (e.g. Ly *et al.*, 2000; Baker *et al.*, 2005 and discussion in Chapter 5).

Cell proliferation requires a series of events that are collectively known as the cell cycle. Four distinct phases comprise a cell cycle (FIGURE 4-29A): the S (synthesis) phase where the genome is replicated, the M (mitosis) phase where all components of the mother cell divide to give rise to the daughter cells and two "gap" phases G1 preceding the S phase and G2 preceding the M phase. All phases between two successive mitoses are known as the interphase. Quiescent

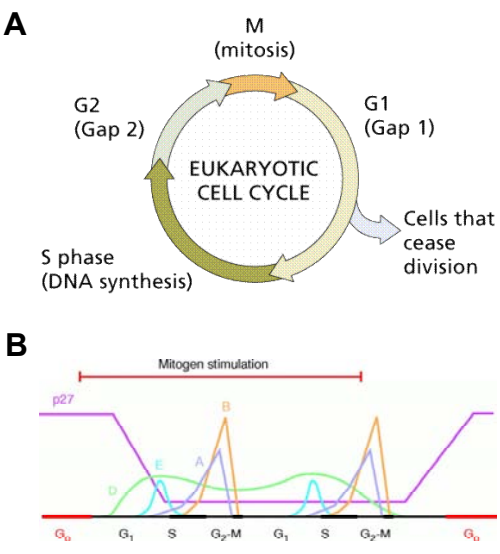


Figure 4-29. Schematic representation of the eucaryotic cell cycle (A) and associated changes in cyclin protein levels (B). (A) adapted from Purves *et al.* and (B) from Sherr, 1996.

cells are said to be in the G0 phase.

A primary feature of cell cycle regulation is the tightly controlled periodic expression of proteins known as cyclins. Cyclins heterodimerise with proteins known as cyclin-dependent kinases (CDKs). CDK protein levels are largely constant throughout the cell cycle, their activity though correlates with the expression levels of cyclins (FIGURE 4-29B). The expression of cyclins, in turn, is regulated by a combination of transcriptional mechanisms as well as ubiquitin-mediated proteolysis so that each step of the cell cycle is unidirectional (Reed, 2003).

CDK1 (a.k.a. cdc2 kinase) with its partner cyclin B1 regulate several key features of mitosis, including nuclear envelope break-down by phosphorylation of nuclear lamins, chromosome condensation by the phosphorylation of condensin as well as the activation of the anaphase promoting complex (APC) by the phosphorylation of its substrate-recognition subunit Cdc20 (Ferrari, 2006; Peters, 2002). Thus, other mitotic kinases are required for proper cell division (Ferrari, 2006) CDK1 plays a central role. To understand the role of the observed mitotic phosphorylation of SIRT1 its primary sequence was examined for the presence of CDK consensus phosphorylation sites (S/T-X-K/R where X is any aminoacid; Brown *et al.*, 1999). One such sequence was located around S540 in the C-terminal region following the sirtuin core domain (FIGURE 4-30A). To test whether this site is phosphorylated by CDK1 *in vitro*, His-CKD1/GST-cyclin B1 were co-expressed in Sf9 cells, purified with glutathione beads and were incubated in the presence of ^{32}P-γ-ATP with GST-SIRT1 also expressed in Sf9 cells. Two examples representative of the results obtained are shown in FIGURE 4-30B. As a positive control, histone H1, a widely used *in vitro* substrate of CDKs was used. A certain degree of GST-SIRT1 phosphorylation was observed in the absence of CDK1/cyclinB1 suggesting the presence of an Sf9-cell derived kinase that co-purifies with the deacetylase. However, ^{32}P incorporation was significantly enhanced in the presence of CDK1/cyclinB1 implying that SIRT1 may be a substrate of this kinase *in vitro*. It is of note that GST alone is phosphorylated by the kinase complex, albeit at a level lower than that of GST-SIRT1. These results raised the possibility that SIRT1 may be a substrate of CDK1 in mitosis.

To test whether *in vitro* phosphorylation of SIRT1 by CDK1 induces the characteristic electrophoretic mobility shift of SIRT1 in mitosis, HA-SIRT1 was *in vitro* transcribed/translated, immunoprecipiated using α-HA antibodies and incubated with CDK1 in the presence of ATP. The same reaction was also performed on IVT-SIRT1

without immunoprecipitation. In neither case did an electrophoretic mobility shift occur (FIGURE 4-30C) suggesting that phosphorylation of SIRT1 by CDK1, if it occurs *in vivo*, is unlikely to account for the hyper-shifting of SIRT1 correlating with mitotic cells.

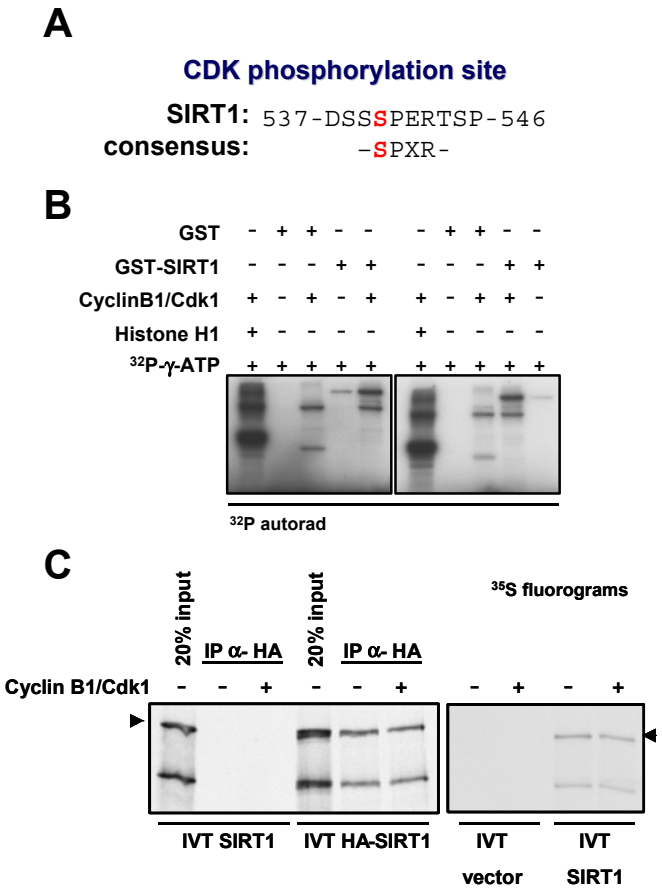


FIGURE 4-30. SIRT1 phosphorylation by CDK1 *in vitro*. (A) CDK1 consensus phosphorylation site flanking S540 in the C-terminus of SIRT1; **(B)** *In* vitro kinase assay using purified components expressed in Sf9 cells; **(C)** *In vitro* phosphorylation of SIRT1 by Cdk1 does not cause electrophoretic mobility shift. IVT SIRT1 was phosphorylated *in vitro* by cyclin B1/Ckd1 complexes expressed in Sf9 cells either directly (right) or following immunoprecipitation of the IVT protein with α-HA antibody (left). Arrowhead indicates full-length IVT (HA-)SIRT1

In an attempt to confirm the generality of the previously described electrophoretic mobility shift of SIRT1 in mitotic cells, mAb12/1 was used to detect SIRT1 in untreated or nocodazole-arrested cells of HCT116 cells. Surprisingly, mAb12/1 immunoreactivity was dramatically decreased in nocodazole-arrested cells in a manner independent of p53 (FIGURE 4-31A)(Vogel *et al.*, 2004; Lanni and Jacks, 1998). MG132 treatment increased mAb12/1 immunoreactivity in logarithmically growing cells in contrast to nocodazole-treated cells where although it did not rescue mAb12/1 immunoreactivity, it induced the appearance of a faster-migrating band in two independent experiments (FIGURE 4-31B). The migration of this band is consistent with a hypo-phosphorylated SIRT1 species raising the possibility that phosphorylation functions as a mechanism preventing the proteasome-mediated degradation of SIRT1. Nevertheless, judging from the combined intensity of the mAb12/1

in the nocodazole- and MG132-treated samples, it was clear that MG132 does not result in complete recovery of the mAb12/1 signal.

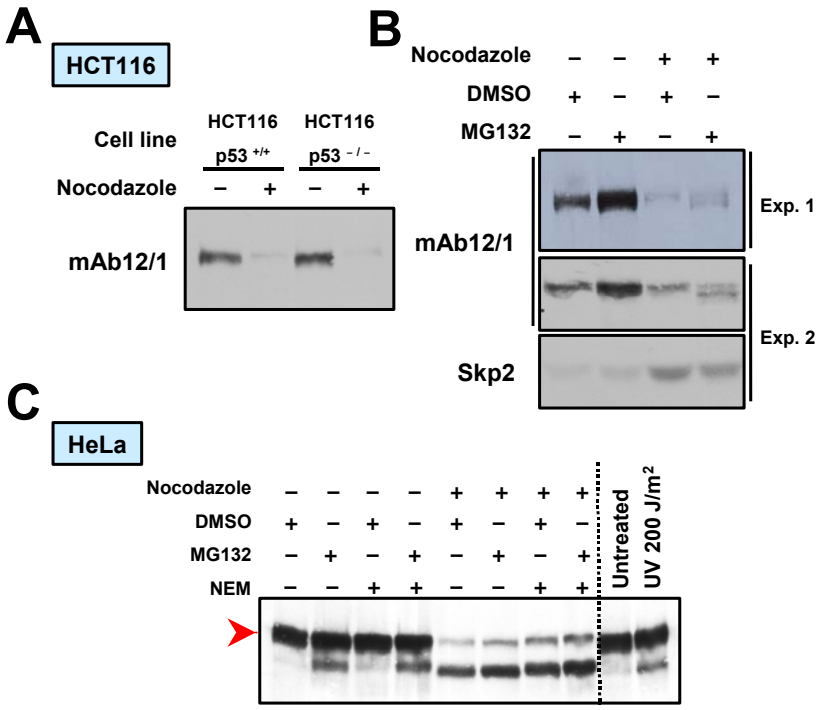


FIGURE 4-31. Loss of mAb12/1 α-SIRT1 immunoreactivity in mitotic cells. (A) HCT116 cells ($p53^{+/+}$) or an isogenic derivative lacking both p53 alleles ($p53^{-/-}$) were treated with nocodazole or mock treated lysed and immunoblotted with mAb12/1; **(B)** HCT116 cells ($p53^{+/+}$) were treated with nocodazole or mock treated in the presence or absense of the proteasomal inhibitor MG132; **(C)** HeLa cells were treated as in (B) and in addition with or without de-ubiquitination/de-sumoylation inhibitor NEM. UV-treated cells were also analysed in parallel to indicate co-migration of the lower band seen in nocodazole-treated cells with that in UV-treated cells. Arrowhead indicates full-length SIRT1.

Given that the initial experiments were performed in HeLa cells, it was possible that these results reflected a peculiarity of the HCT116 cell line. Thus, in a similar experiment extracts of HeLa cells were probed with mAb12/1. In parallel, to examine whether ubiquitination or sumoylation of SIRT1 occurs, logarithmic or nocodazole-arrested HeLa populations were treated with N-ethylmaleimide (NEM) which inhibits the hydrolysis of ubiquitin or SUMO from modified proteins. The results of this experiment recapitulated the ones using HCT116 cells, while indicated no NEM-sensitive SIRT1 species (FIGURE 4-31C). A fast migrating band distinct from that observed in FIGURE 4-31B also appeared in the nocodazole-treated cells. This band co-migrated with the SIRT1 species arising from UV treatment of HeLa cells (FIGURES 4-22, 4-31C). These results suggested the reduction of SIRT1 protein levels specifically in nocodazole-arrested cells.

The best-studied mechanism of proteasome-dependent degradation during mitosis is mediated by the APC ubiquitin E3 ligase. Unlike other E3 ligases whose activity is targeted by their substrates' phosphorylation status, APC activity is dictated by its binding to two distinct substrate-binding modules, Cdc20 and Cdh1 (Zachariae and Nasmyth, 1999). Recognition of substrates for APC/Cdc20-mediated ubiquitination is mediated by a short motif known as the D-box (Glotzer *et al.*, 1991) whereas recognition by APC/Cdh1 occurs through a distinct motif called the KEN box (Pfleger and Kirschner, 2000). APC/Cdc20 complexes are present from early mitosis through the anaphase/telophase transition when APC/Cdh1 mediates Cdc20 degradation along with mitotic cyclins thus controlling mitotic exit and entry into G1 (Kurland and Tansey, 2004). APC/Cdh1 complexes persist throughout interphase. Recent work suggests that at least one of the functions of APC/Cdh1 is to regulate G1 duration as well by degrading the F-box protein Skp2 which targets the CDK inhibitor p27 for ubiquitin-mediated proteolysis thus permiting entry into S phase (Wei *et al.*, 2004, Bashir *et al.*, 2004).

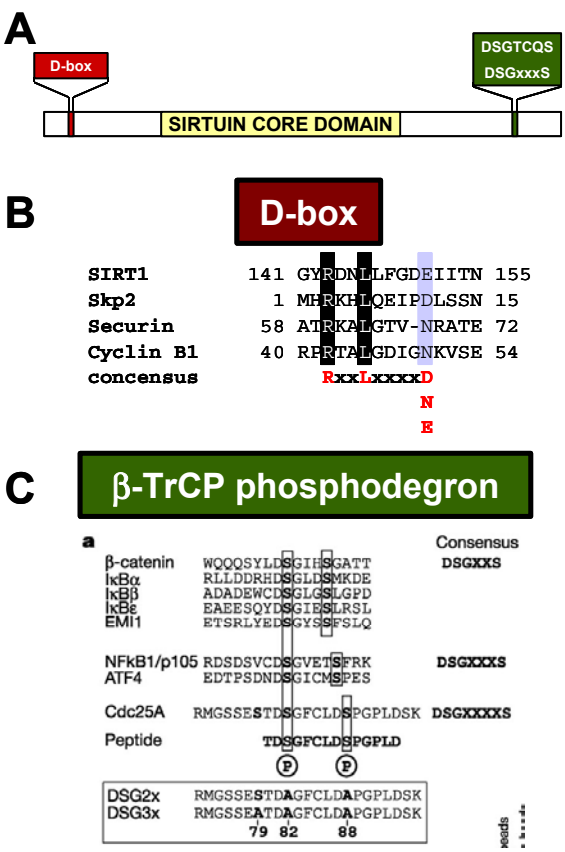


FIGURE 4-32. Sequence motifs putatively mediating mitosis-specific SIRT1 degradation. (A) The locations of the D-box and the β-TrCP phosphodegron are indicated; **(B)** Multiple alignment of the SIRT1 D-box sequence with other *bona fide* D-box sequences found in proteins shown to be degraded in mitosis; **(C)** Multiple alignment of phosphodegron sequences mediating protein targeting for ubiquitination by β-TrCP.
(C) Adapted from Busino *et al.*, 2003

An SCF E3 ubiquitin ligase complex based on the F-box protein β-TrCP has also been shown to play a role in protein turnover during mitosis. β-TrCP substrate recognition depends on a short motif of the general consensus sequence -D-pS-G-$(X)_{2-4}$-pS- where pS is a phosphorylated serine (Cardozo and Pagano, 2004). Thus, this sequence is also known as a "phosphodegron". Phosphorylation of the CDK1 inhibitory kinase Wee1 by Plk1 and CDK1 results in its recognition by $SCF^{\beta\text{-TrCP}}$ which ubiquitinates and targets it for degradation (Watanabe *et al.*, 2004). This event is required for the onset of M phase.

Similarly, phosphorylation by Plk1 targets Emi1 (early mitotic inhibitor 1) for $SCF^{\beta\text{-}TrCP}$-driven degradation (Margottin-Goguet *et al.*, 2003; Guardavaccaro *et al.*, 2003; Moshe *et al.*, 2004). Emi1 prevents APC activation probably by inhibiting its association to Cdc20 and Cdh1 which allows a sufficient degree of stability for cyclins to initiate mitosis. Thus Emi1 degradation is also required for entry into mitosis confirming an important role for $SCF^{\beta\text{-}TrcP}$ in the regulation of mitotic progression.

Observation of the primary sequence of SIRT1 revealed the presence of a D-box in the N-terminus and a potential β-TrCP phosphodegron in the C-terminus of the protein (FIGURE 4-32). This raised the possibility that SIRT1 may be targeted for degradation by either an APC or SCF ubiquitin ligase and prompted experiments to establish the functional significance of these consensus sites.

As SIRT1 expressed in Sf9 cells is phosphorylated (FIGURE 4-8A), it is possible that the proposed C-terminal phosphodegron can be recognised by β-TrCP. To test this, GST-SIRT1 expressed in Sf9 cells was either treated with λ-PPase or mock-treated (FIGURE 4-8A) and incubated with IVT Flag-β-TrCP1. As a control GST alone expressed in Sf9 cells was also incubated with the same amount of IVT. GST-SIRT1 but not GST alone exhibited a strong interaction with IVT Flag-β-TrCP1. λ-PPase-treated GST-SIRT1 was significantly less able to interact with Flag-β-TrCP1 suggesting that a phosphorylation-dependent interacton can occur *in vitro* between the two proteins (FIGURE 4-33A).

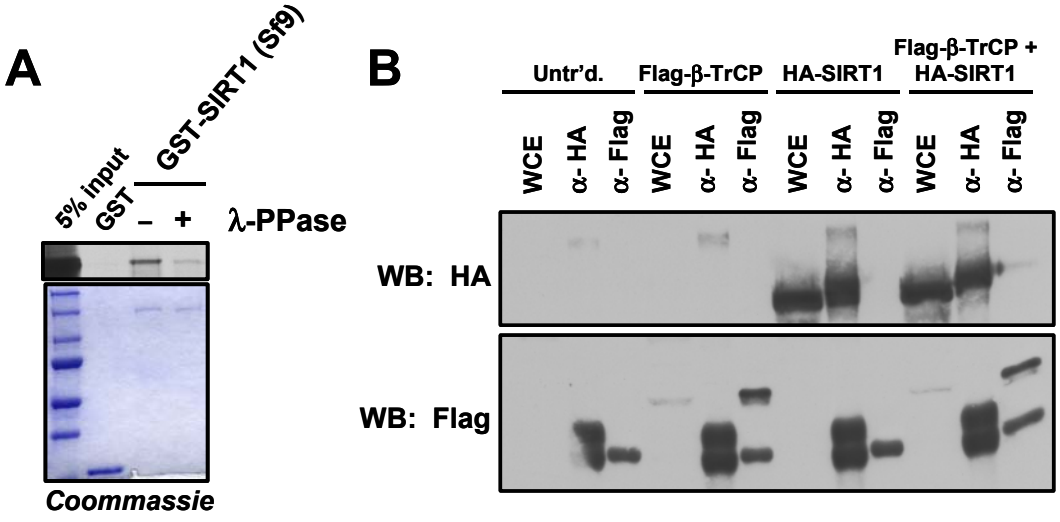


FIGURE 4-33. Investigation of interaction between SIRT1 and β-TrCP. (A) *In vitro* association assay using GST or mock-/λ-PPase-treated GST-SIRT1 expressed in Sf9 cells as a bait and IVT β-TrCP as the prey; **(B)** Weak interaction between co-transfected SIRT1 and β-TrCP in HeLa cells.

To test whether this interaction could also be observed *in vivo*, tagged SIRT1 and β-TrCP were co-transfected in HeLa cells and immunoprecipitated with the corresponding antibodies. A very weak band corresponding to HA-SIRT1 was observed in Flag-β-TrCP1 IPs but not *vice versa* (FIGURE 4-33B). This implied that either the interaction does not occur *in vivo*, that the co-purification conditions are not optimal to preserve the interaction or that it occurs in a very small subfraction of cells, probably in a cell cycle-dependent manner hence explaining the low abundance of the observed complex. It is also possible that the position of the tags is not optimal for the interaction to be detected. Unfortunately, attempts to probe the interaction between the endogenous proteins *in vivo* was unsuccessful due to the poor performance of the β-TrCP antibodies available at the lab at the time of the experiments.

Given the ambiguity of these results and the absence or a good antibody to monitor β-TrCP downregulation by siRNA for genetic studies, the effects of depleting another F-box protein Skp2 (S-phase kinase-associated protein 2) which also functions in the context of SCF complexes (FIGURE 4-34A), were investigated. Skp2 was also a relevant choice since in experiments where nocodazole-arrested cells were released into G1, the

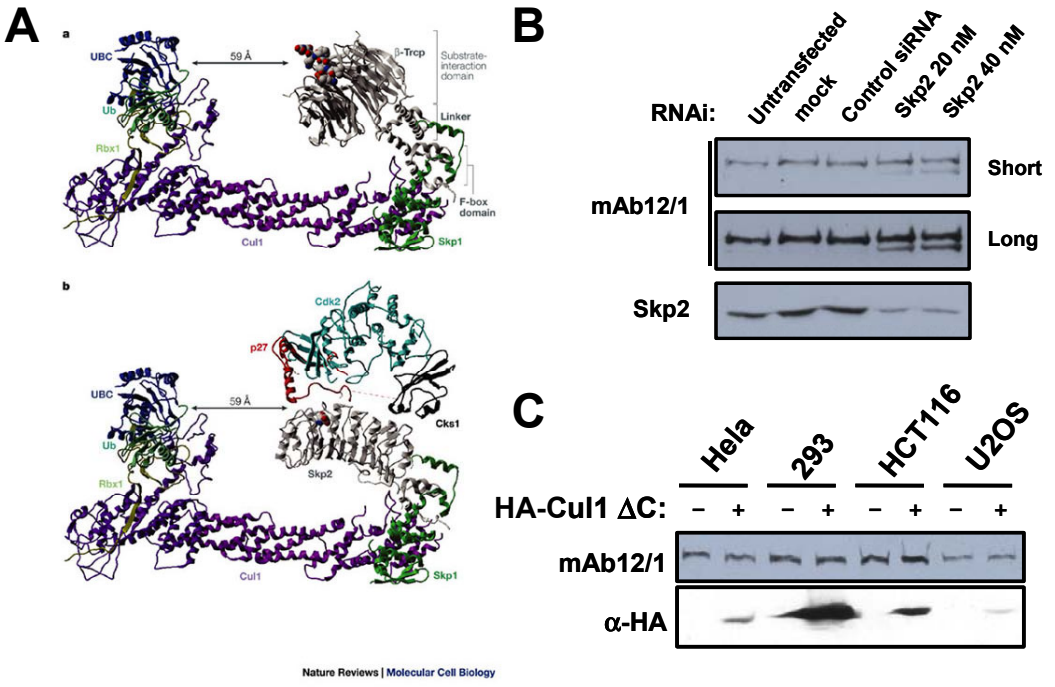


FIGURE 4-34. SIRT1 is not targeted for degradation by Cul1-based E3 ligases. (A) Ribbon representation of the crystal structure of β-TrCP and Skp2-based SCF E3 ligase complexes; **(B)** No SIRT1 accumulation following siRNA-mediated downregulation of Skp2 in HeLa cells; **(C)** No SIRT1 accumulation following transfection of a construct expressing dominant negative Cul1 in various cell lines.
(A) adapted from Cardozo and Pagano, 2004

kinetics of re-appearance of SIRT1 mAb12/1 immunoreactivity was reminiscent of the Skp2 substrate p27 CDK inhibitor (FIGURE 4-39A and Wei *et al.*, 2004). Skp2 protein downregulation by siRNA resulted in no obvious difference in mAb12/1 immunoreactivity suggesting that SIRT1 is not a target of an SCF^{Skp2} E3 ligase complex (FIGURE 4-34B). In a more generic approach, different cell lines were transfected with a Cul1 contruct lacking the C-terminus (HA-Cul1ΔC). The expressed protein can bind endogenous Skp1 but not Rbx1 thus acting as a dominant negative species (Donzelli *et al.*, 2002). Overexpression of HA-Cul1ΔC has no effect on the abundance of SIRT1 (FIGURE 4-34C) suggesting that SIRT1 is not subjected to proteasome-mediated proteolysis driven by Cul1-based SCF complexes.

To investigate the potential involvement of APC in SIRT1 turnover during mitosis the ability of SIRT1 to interact with the APC substrate recognition components Cdc20 and Cdh1 was tested. Despite the absence of a KEN box which is usually required for the recognition of substrates by Cdh1, the latter was included in the analyses firstly as a comparison to Cdc20 but also because of the proposed role of *S. cerevisiae* Sir2p in mitotic exit. Bacterially-expressed GST-Cdh1 could co-purify IVT HA-SIRT1 while GST-SIRT1 expressed in Sf9 cells could interact with either IVT HA-Cdh1 or HA-Cdc20 (FIGURE 4-35). In the latter case, a signal was also detected in the pull-downs containing GST alone albeit at significantly lower levels. Again though, despite the good quality of antibodies in hand, an interaction between SIRT1 and either Cdc20 or Cdh1 could not be established.

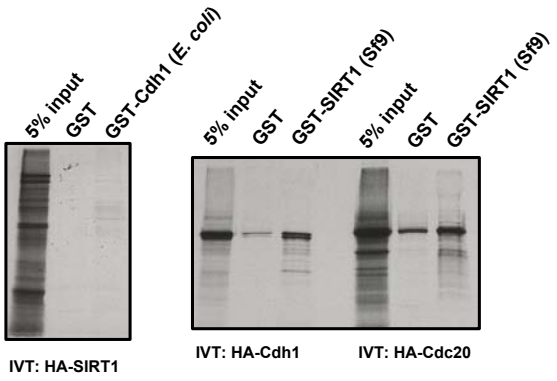


FIGURE 4-35. *In vitro* interaction between SIRT1 and the APC activating subunits Cdh1 and Cdc20. *In vitro* association assay between bacterially-expressed GST or GST-Cdh1 and IVT SIRT1 (left); *In vitro* association assay between GST or GST-SIRT1 expressed in Sf9 cells and IVT HA-Cdh1 or HA-Cdc20.

As it is possible that the inability to detect protein-protein interactions does not necessariy reflect their absence but rather may be due to suboptimal assaying conditions, a genetic link between these E3 ligases and SIRT1 was sought. The protein levels of Cdh1 or Cdc20 were downregulated in HeLa cells by siRNA and the levels of SIRT1 were assayed at various times following siRNA transfection using mAb12/1. Despite efficient downregulation of either Cdh1 and to a lesser extend Cdc20 protein levels, no

accumulation of SIRT1 mAb12/1 immunoreactivity was observed (FIGURE 4-36A). siRNA-mediated downregulation of either Cdh1 or Cdc20 in asynchronous cell populations suffices to induce the accumulation of their established sustrate Cdc25A (Donzelli *et al.* 2002). In agreement to this, Cdh1 depletion resulted in increased levels of Skp2 (FIGURE 4-36A, Wei *et al.*, 2004, Bashir *et al.*, 2004). In order though to exclude the possibility that a potential mitosis-specific effect on SIRT1 is masked in an asynchronous population, HeLa cells treated with siRNAs targeting Cdh1 or Cdc20 were either mock-treated or arrested in prometaphase with nocodazole and assaye for SIRT1 levels with mAb12/1. Similarly to the previous results, no change in SIRT1 protein abundance was observed despite the characteristic loss of immunoreactivity in nocodazole-treated cells (FIGURE 4-36B). These results suggested that APC is not likely to act as an E3 ligase that mediates SIRT1 turnover *in vivo*.

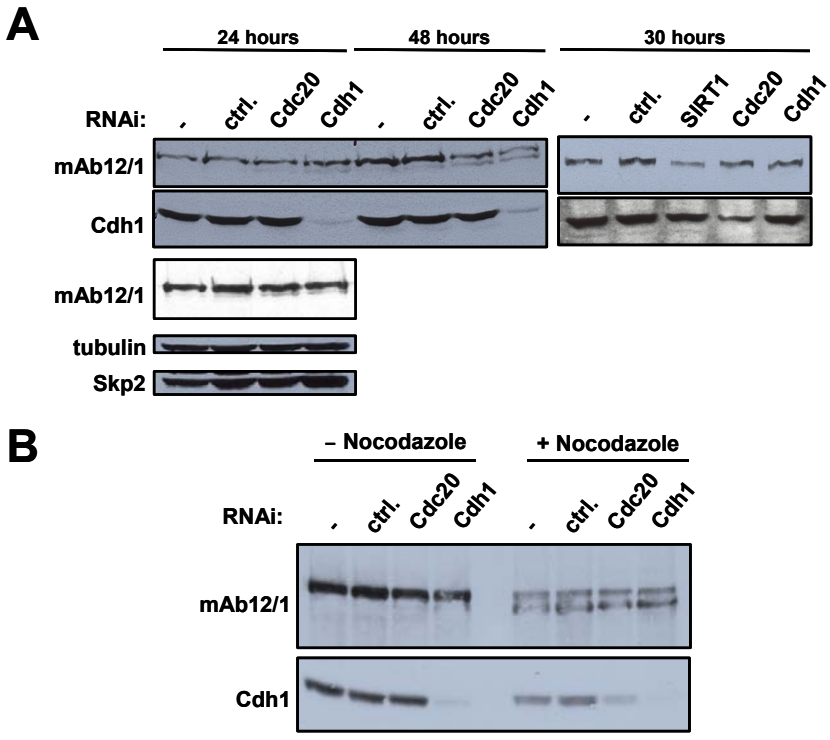


FIGURE 4-36. No effect of Cdh1 or Cdc20 downregulation on SIRT1 abundance. (A) HeLa cells were treated with siRNAs targeting Cdh1, Cdc20 or SIRT1 as a positive transfection control for the indicated times and probed with mAb12/1 to assay SIRT1 levels; **(B)** HeLa cells were treated with siRNAs targeting Cdh1 or Cdc20 following mitotic arrest with nocodazole. Cells were probed for SIRT1 levels with mAb12/1.

As attempts to demonstrate that the observed loss of mAb12/1 immunoreactivity is due to ubiquitin-mediated proteolysis were unsuccessful, we were prompted to re-evaluate the behaviour of SIRT1 during the course of normal progression through the cell cycle in the absence of nocodazole. For this, HeLa or HCT116 cells were arrested at the G1/S boundary by successive treatments with thymidine and aphidicolin and subsequenlty allowed to progress through the cell cycle in a synchronous manner. Cell

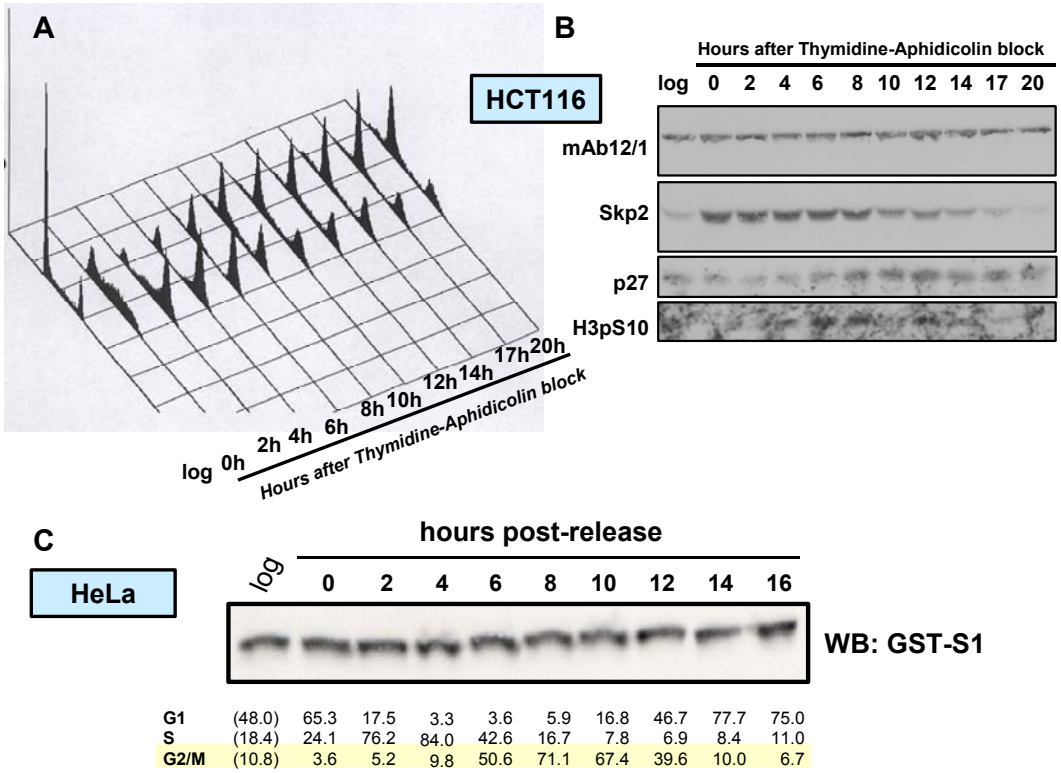


FIGURE 4-37. Investigation of SIRT1 protein abundance during the cell cycle. Thymidine/aphidicolin block-release experiment in HCT116 [**(A)** and **(B)**] or HeLa cells **(C)**. *See* text for further details

cycle progression can be monitored by measuring the DNA content of the cells using fluorescence-activated cell sorting (FACS) and follow molecular markers characteristic of each phase by Western blot. Such an analysis revealed that total protein levels of SIRT1 did not change throughout the cell cycle as assayed using both mAb12/1 and GST-S1 (FIGURE 4-37B and C respectively). At the same time, there was no obvious mobility shift of SIRT1 throughout the experiment again contradicting the data obtained with nocodazole-treated cells.

Unlike mAb12/1, the GST-S1 antibody does not indicate reduction of SIRT1 protein levels in nocodazole-arrested mitotic cells (*cf.* FIGURES 4-31A and 4-7). Given the results excluding that proteolysis can account for this behaviour, it was hypothesised that the mAb12/1-specific effects observed may be accounted for by a modification occuring in the corresponding antigenic epitope. As mAb12/1 immunoreactivity was reduced in mitotic cells where phosphorylation plays a primary role in its orchestration, the possibility that such a modification is actually phosphorylation was invetigated.

HeLa lysates of logarithmically growing and nocodazole-arrested cells were either treated with λ-PPase or mock-treated, loaded in parallel on the same SDS-PAG and

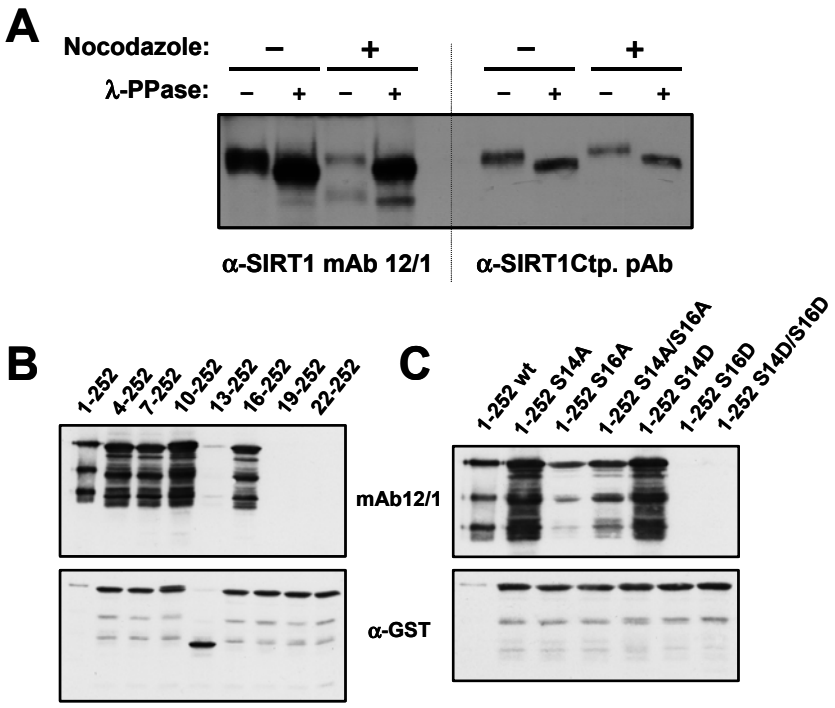


FIGURE 4-38. mAb12/1 reactivity is sensitive to SIRT1 phosphorylation. **(A)** λ-PPase treatment restores mAb12/1 immunoreactivity in nocodazole cell extracts; **(B)** Mapping of mAb12/1 epitope and N-terminal aminoacid sequence of SIRT1 (bottom).

subjected to immunoblotting by either mAb12/1 or GST-S1. Consistent with the previous experiments, mAb12/1 but not GST-S1 antibodies indicated a nocodazole-specific loss of SIRT1 immunoreactivity (FIGURE 4-38A). Surprisingly, mAb12/1 immunoreactivity could be recovered upon treatment with λ-PPase in nocodazole-treated cells and to a certain extend in unsynchronous cell extracts. In contrast, GST-S1 immunoreactivity was largely unaltered by λ-PPase treatment. These results suggested that phosphorylation of SIRT1 in nocodazole-arrested mitotic cells precludes its recognition by the mAb12/1 antibody.

The results above obviated the neccessity to identify the antigenic epitope of mAb21/1. The preliminary epitope mapping pointed to the first 121 aminoacids (FIGURE 4-3C). Thus stepwise 3-aa N-terminal SIRT1 truncations were expressed as GST-fusions in bacteria and subjected to immunoblotting with mAb12/1. Truncation of the first 18 aminoacids of SIRT1 abolished recognition of the corresponding fusion protein while deletion of the first 15 aminoacids did not (FIGURE 4-38B). This indicated that at least aminoacids 16-18 are essential for the recognition of SIRT1 by mAb12/1.

As residue 16 is a serine and the data of FIGURE 4-38A suggest that phosphorylation of the antigenic epitope modulates mAb12/1 binding, the possibility that S16 phosphorylation alters the binding capacity of mAb12/1 was investigated. To this end, S16

in a fragment harbouring SIRT1 aa's 1-252 was mutated to either A or D to mimic a non-phosphorylatable and a constitutively phosphorylated species respectively and expressed as GST-fusions in bacteria. As a control, similar mutations were introduced to the nearby S14 whose absence did not affect recognition by mAb12/1 (FIGURE 4-38B). The S16D mutation completely abolished recognition of the corresponding fragment (FIGURE 4-38C). The S16A mutation did not eliminate mAb12/1 recognition but significantly reduced it. Mutations in the proximal S14 did not affect mAb12/1 binding. These results are consistent with the interpretation that S16 is a residue within the antigenic epitope of SIRT1 required for its recognition by mAb12/1 and that S16 phosphorylation can abolish binding of the antibody.

To test whether *in vivo* mutations of S16 affect binding of mAb12/1, HeLa cells were transfected with HA-SIRT wild-type or harbouring A and D substitutions at positions S14 and S16. Lysates were probed with either mAb12/1 or stripped and re-probed with GST-S1. As shown in FIGURE 4-39A, either A or D mutations of S16 abolished mAb12/1 binding in agreement with the results using bacterially expressed proteins. Interestingly though, the S14A mutant was recognised significantly more efficiently than wild-type SIRT1 while the S14D mutant exhibited the oposite effect. These observations would be consistent with S14 phosphorylation being required for the phosphorylation of S16 *in vivo*.

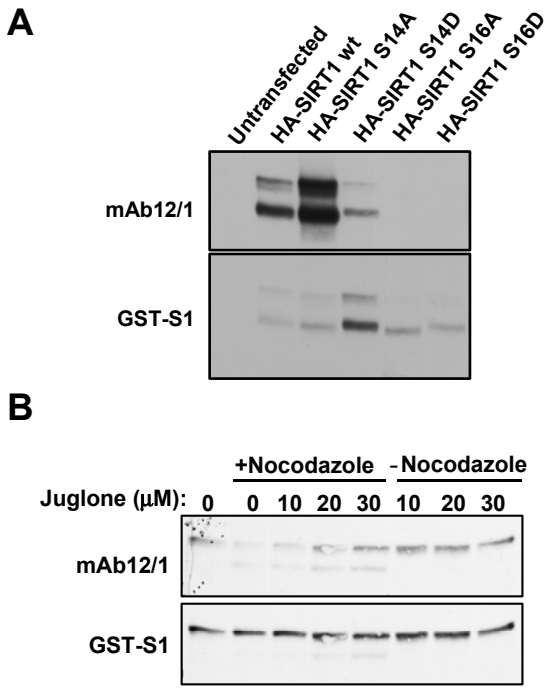


FIGURE 4-39. mAb12/1 reactivity is sensitive to SIRT1 phosphorylation of both S14 and S16.(A) HeLa cells were transfected with the indicated contructs and immunobloted with mAb12/1 and GST-S1 antibodies; **(B)** Dose-dependent inhibition of SIRT1 S16 phosphorylation by the Pin1 prolyl isomerase inhibitor juglone.

The action of some kinases and phosphatases is dictated by the adoption of specific conformations of their substrates. The PPIase Pin1 catalyses the phosphorylation-dependent isomerisation of proline residues and is required to generate the appropriate conformational change on proteins to allow their subsequent targeting by such kinases and phosphatases (Lu,

2004; FIGURE 4-27A). Thus is appeared possible that the interdependence of S16 phosphorylation on S14 phosphorylation is due to a pS14-driven P15 isomerisation which in turn may lead to the recruitment of a S16 kinase or prevent phosphate hydrolysis at S16 by a conformation-specific phosphatase.

To test this, unsynchronised or nocodazole-arrested HeLa cells were in parallel treated with increasing concentrations of Juglone, an inhibitor of the Pin1 family of PPIases but not of the cyclophilin and FKBP families. GST-S1 immunoblotting showed no significant differences in SIRT1 abundance. Conversely, juglone treatment abolished the nocodazole-induced loss of mAb12/1 reactivity in a dose-dependent manner (FIGURE 4-39B). Jugone treatment of asynchronous cell populations had no effect on mAb12/1 reactivity. These data provide support for the hypothesis that Pin1-mediated PPIase activity driven by S14 phosphorylation enhances S16 phosphorylation.

Taken together, the above analyses suggest that the loss of SIRT1 immunoreactivity in nocodazole-treated cells is due to phosphorylation of S16. As the cells in the experiment of FIGURE 4-39A were not treated with nocodazole, it is possible that S14-dependent S16 phosphorylation is not exclusive to nocodazole-arrested cells, at least to a certain extend. This would be consistent with the observation that λ-PPase treatment enhances recognition of SIRT1 by mAb12/1 in asynchronous cells (FIGURE 4-38A).

Under the light of these results the behaviour of SIRT1 in mitotic cells was re-evaluated. If the proposed phosphorylation of S16 is specific to mitotic cells, mAb12/1 immunoreactivity should be recovered as cells re-enter G1. To explore this, HeLa cells were arrested in mitosis with nocodazole then allowed to resume cycling by removing the drug. Cells were harvested at various time-points after release and analysed by immunobloting with either mAb12/1 or GST-S1. The GST-S1 signal was constant throughout the experiment. That of mAb12/1 was dramatically decreased in nocodazole-arrested compared to untreated cells but it gradually recovered in a time-dependent fashion as cells were allowed to enter G1 (FIGURE 4-40A) consistent with the hypothesis that phosphorylation of S16 is specific to nocodazole-arrested cells and can be reversed in the absence of the drug.

As another human SIRT1 homologue, SIRT2 is a tubulin deacetylase, it is possible that in mitosis SIRT1 plays a similar role perhaps participating in a signaling network that regulates or monitors microtubule dynamics. This raises the possibility that the binding of nocodazole to microtubules may elicit a signaling cascade that induces phosphorylation of

SIRT1 at S16 as assayed by mAb12/1. If this were the case, a rapid phosphorylation of SIRT1 would occur in response to nocodazole treatment similar to all kinase signalling cascades without neccessitating the synchronisation of the cell population in mitosis which is achieved after nocodazole treatment of duration equivalent at least to one cell cycle.

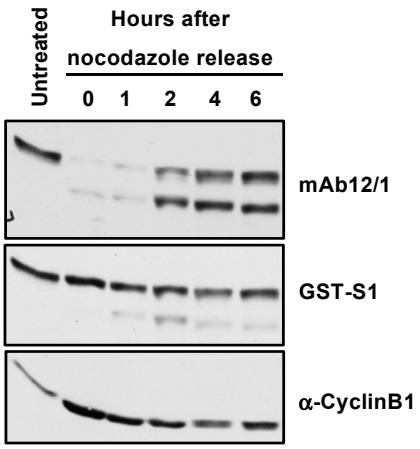


FIGURE 4-40. Restoration of mAb12/1 reactivity following mitotic exit. Cells were arrested in mitosis with nocodazole, the drug was washed away and cells were re-plated for the indicated amounts of time.

To investigate this possibility, HeLa cells were treated with nocodzole and harvested at various time points after initiation of treatment. As a control taxol, a microtubule stabilising agent was also used. Although both taxol and nocodazole elicit the spindle-assembly checkpoint and result in cell cycle arrest, they do so by different mechanisms. Nocodazole prevents the attachment of kinetochores to spindle microtubules whereas taxol prevents microtubule tension to be built up (Bharadwaj and Yu, 2004).

SIRT1 levels remained largely unaltered for the first 12 hours after treatment as assayed by either mAb12/1 or GST-S1 antibodies (FIGURE 4-41). As cells became synchronised in mitosis though by 20 hours, mAb12/1 but no GST-S1 reactivity was reduced. At longer time-points GST-S1 reactivity also droped presumably due to increased cell death occuring as a result of prolonged exposure of cells to the microtubule poisons. In the case of nocodazole, a slight recovery of mAb12/1 signal was observed by 48 hours of treatment, likely to arise because of adaptation of cells to the drug (Rieder and Maiato, 2004).

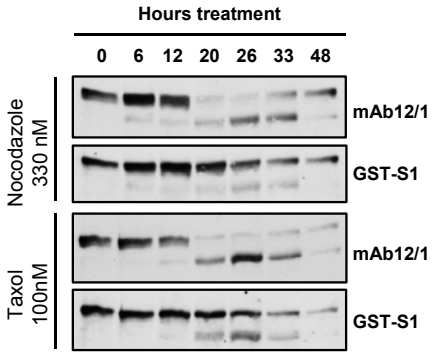


FIGURE 4-41. Time-course investigation of mAb12/1 immunoreactivity in response to nocodazole and taxol treatment. Cells were treated with either nocodazole or taxol, harvested at the indicated times and subjected to immunoblotting with either mAb12/1 or GST-S1 antibodies.

It is possible that a rapid phosphorylation within the first 6 h of treatment occurs which could not be observed in this experiment. However, the kinetics of mAb12/1 activity loss correlate with kinetics of cell accumulation in mitosis suggesting that rather than being elicited by microtubule destabilisation, SIRT1 S16 phosphorylation is the result of a mitosis-specific

phosphorylation event. Interestingly, in a similar experiment, loss of mAb12/1 reactivity follows the kinetics of activation of BubR1, a protein kinase involved in the implementation of the spindle assembly checkpoint (Shin *et al.*, 2003). As mAb12/1 loss of reactivity is specific to nocodazole-treated cells but does not occur during progression through normal mitosis (FIGURE 4-37) this raised the possibility that SIRT1 is a target of a kinase activated upon engagement of the spindle assembly checkpoint.

The spindle assembly checkpoint (SAC) also known as the mitotic checkpoint ensures that all kinetochores are attached to the mitotic spindle in a bipolar manner before anaphase is allowed to occur. This is important to maintain genomic stability as improper chromosome segregation leads to aneuploidy and cancer (Kops *et al.*, 2005).

The SAC is elicited by unattached kinetochores upon entry into mitosis (FIGURE 4-42). Several highly conserved proteins participate in SAC signalling including Mad1, Mad2, BubR1, Bub1, Bub3 and Mps1 (Kops *et al.*, 2005). Initiation of the SAC involves the recruitment of Bub1 to the unattached kinetochore in a Bub3-dependent manner to promote the binding of other checkpoint proteins. BubR1, which harbours protein kinase activity is similarly recruited to kinetochores. The enzymatic activity of BubR1 is induced upon binding of the kinetochore-associated microtubule motor protein CENP-E. BubR1 kinase activity is in turn required for the recruitment of a Mad1-Mad2 heterodimer to the kinetochore resulting in the adoption of an active conformation by Mad2 which allows it to bind to the APC substrate recognition subunit Cdc20. Dissociation of Mad2 from kinetochore-bound Mad1 may be promoted by phosphorylation of Mad1 by Bub1 (Bharadwaj and Yu, 2004). Mad2 and Cdc20 associate with BubR1 and Bub3 to form a soluble complex known as the mitotic checkpoint complex (MCC) which is able to inhibit APC activity. Consequently, anaphase cannot occur until all kinetochores are attached in which case formation of the MCC is prevented.

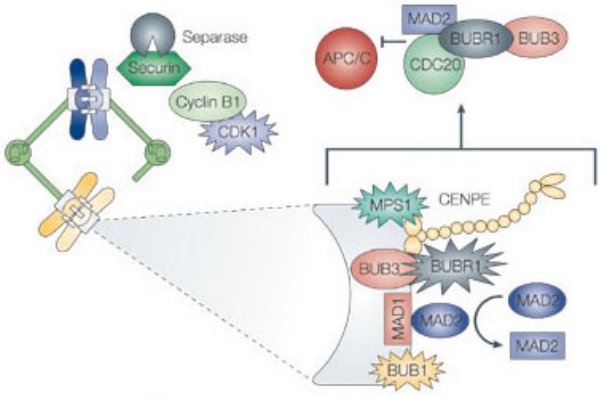


FIGURE 4-42. Schematic description of the mammalian spindle assembly checkpoint.
Figure adapted from Kops *et al.*, 2005

Genomic instability is a prime contributing factor to the occurrence of neoplasia (Hanahan and Weinberg, 2000). It may arise from malfunction of DNA repair pathways that

lead to microsatellite instability (MIN) but also several human cancers carry mutations in genes encoding for SAC protein components leading to chromosomal instability (CIN) which in turn results in abnormal chromosome numbers, a phenomenon also known as aneuploidy (Cahill *et al.*, 1998; Kops *et al.*, 2005). Stable cell lines derived from cancers with either CIN or MIN have been established. A major difference between these cell lines is the inability of those carrying CIN mutations to arrest in response to nocodazole treatment, because of a disfunctional SAC.

To test the possibility that SAC kinases phosphorylate SIRT1 at S16, the CIN colon cancer cell line SW480 and the MIN colon cancer cell line HCT116 along with an isogenic derivative thereof lacking p53 by targeted homologous recombination were used. These cell lines were treated with nocodazole for 18 hours or mock-treated with carrier (DMSO) and probed with either mAb12/1 or GST-S1. GST-S1 immunoreactivity was unchanged, exhibiting the characteristic mobility shift in both HCT116 cell-lines treated with nocodazole. This paralleled a decrease in mAb12/1 reactivity (FIGURE 4-43). Conversely, the relative mAb12/1 reativity decrease elicited by nocodazole in SW480 cells was considerably reduced while no apparent mobility shift was detected with GST-S1. This experiment suggested that intact checkpoint function is required for the phosphorylation of SIRT1 on S16 in response to nocodazole.

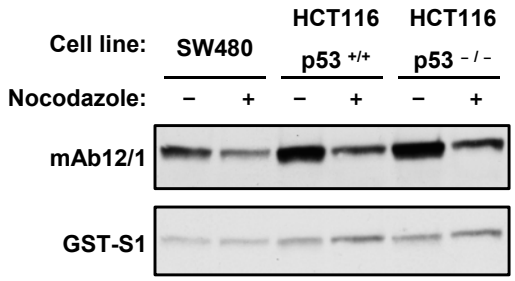


FIGURE 4-43. Comparison of mAb12/1 immunoreactivity in colon cancer cell lines with or without defects in the spindle assembly checkpoint. mAb12/1 immunoreactivity in logarithmically growing or nocodazole-arrested cells was compared in the SW480 cell line that exhibits chromosomal instability (CIN) due to a defect in the mitotic checkpoin apparatus and two isogenic HCT116 cell lines that exhibit microsatellite instability (MIN) (Cahill *et al.*, 1998).

To investigate this further, the impact of siRNA-mediated depletion of Bub1, BubR1 or Mps1 on SIRT1 S16 phosphorylation was tested. siRNA depletion of these three SAC kinases suffices to abolish checkpoint function in response to nocodazole (Stucke *et al.*, 2002; Meraldi *et al.*, 2004; Meraldi and Sorger, 2005). Depletion of any of these kinases had no effect on mAb12/1 immunoreactivity in untreated cells (FIGURE 4-44A). However, BubR1 but not Bub1 or Mps1 siRNA resulted in the rescue of mab12/1 recognition of SIRT1 in HeLa cells arrested in mitosis with nocodazole. These data suggest that BubR1 may specifically phosphorylate SIRT1 S16 in

response to SAC activation. This was confirmed in U2OS cells and further investigated by titrating increasing amounts of BubR1 siRNA in HeLa cells (Figure 4-44B).

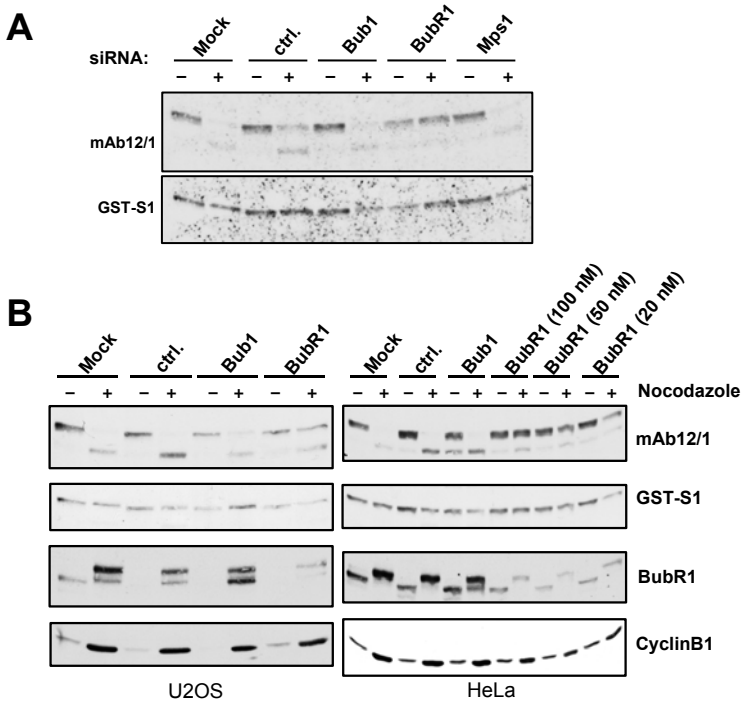


FIGURE 4-44. Investigation of SIRT1 S16 phosphorylation by mitotic checkpoint kinases. See text for details

Subsequently, an effort to demonstrate direct phosphorylation of SIRT1 by BubR1 was undertaken. In an initial approach, BubR1 from untreated or nocodazole-arrested cells was immunoprecipitated, washed and tested for its ability to phosphorylate Sf9-expressed GST-SIRT1 *in vitro*, as assayed by mAb12/1 reactivity. The expected decrease of mAb12/1 recognition of GST-SIRT1 was not observed (Figure 4-45A). CENP-E was shown to be required for efficient catalysis by BubR1 *in vitro* (Mao *et al.*, 2003) raising the possibility that inefficient BubR1 kinase activity was present in the IPs resulting in significantly substoichiometric phosphorylation of SIRT1 which could not be detected by the loss of mAb12/1 signal. To achieve higher sensitivity in this assay, the same experiment was repeated using ^{32}P-γ-ATP and GST-SIRT1(1-252) expressed in bacteria as a substrate. Significant background phosphorylation was present in this assay, yet no obvious fluctuation in the levels of ^{32}P incorporation was observed despite the fact that significant autophosphorylation activity of the IP'd BubR1 was evident (Figure 4-45B).

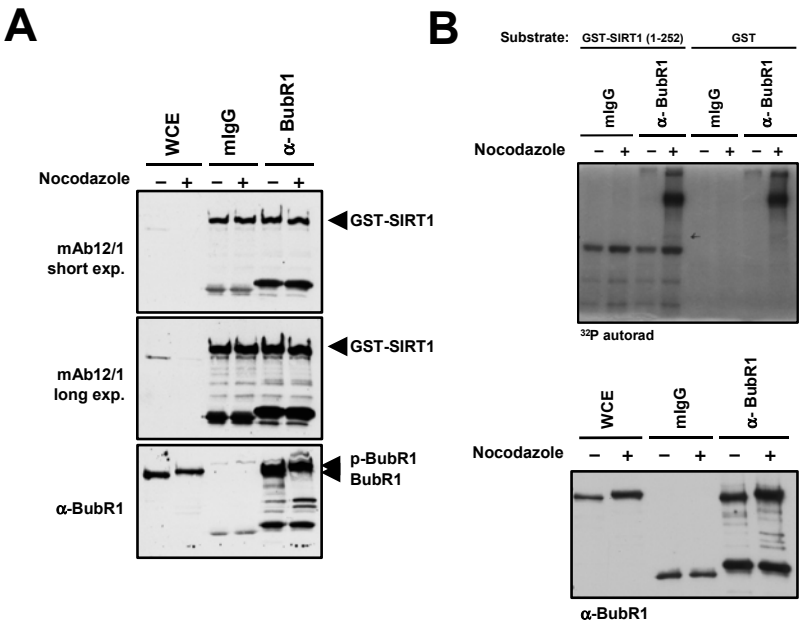


FIGURE 4-45. BubR1 *in vitro* kinase assays. BubR1 was immunoprecipitated and used for *in vitro* immuno-complex kinase assays using either Sf9 cell-expressed substrates **(A)**, or bacterially-expressed substrates **(B)** as indicated.

Despite the clarity of the results presented in FIGURE 4-44, the inability to demonstrate *in vitro* SIRT1 phosphorylation by BubR1 prompted a deeper evaluation of the effects of nocodazole on SIRT1 S16 phosphorylation.

In the experiment of FIGURE 4-37, cells that reach the G2/M phase of the cell cycle are harvested as a whole population and subsequent flow cytometric and immunoblotting analyses do not distinguish between the G2 and M populations. To compare the immunoractivity of mAb12/1 in pure mitotic cells and nocodazole-arrested cells, HeLa cells were sunchronised in G1/S phase by successive thymidine-aphidicolin treatments and allowed to progress through the cell cycle for 12 hours at which point previous analyses showed that they enter G2/M. At this point the mitotic population was separated by mitotic shake-off from the G2 cells which remained attached to the culture dish and harvested separately. In parallel, identically treated cells were supplemented with MG132 to prevent progression beyond metaphase (Meraldi and Sorger, 2005 and *see* below) or nocodazole to activate the SAC and prevent progression beyong prometaphase. For comparison, untreated or nocodazole-treated cells were also prepared. Immunobloting analysis with mAb12/1 revealed that mitotic cells contain SIRT1 essentially stoichiometrically phosphorylated at S16 in contrast to the related G2 population which shows significant mAb12/1 reactivity (FIGURE 4-46A). This is the case also for MG132-arrested metaphase cells. Addition of nocodazole to the synchronised G2/M cells resulted

in a further decrease in the SIRT1 signal suggesting an additive effect of mitotic and checkpoint kinase contribution to SIRT1 S16 phosphorylation.

To further elaborate these results, in a separate experiment, HeLa cells were arrested in mitosis with nocodazole and released for 4 hours in the presence or absence of the proteasome inhibitor MG132. In this setting cells are expected to remain arrested in metaphase independently of the spindle-assembly checkpoint, because the degradation of proteins (e.g. securin) required for the transition from metaphase to anaphase is prevented (Meraldi and Sorger, 2005). In the absence of MG132 mAb12/1 reactivity is recovered four hours after removal of nocodazole, whereas in the presence of MG132, where cells are expected to remain in metaphase, mAb12/1 reactivity remains low (FIGURE 4-46B). This suggests that SAC activation is dispensable for S16 phosphorylation to occur and that anaphase-to-metaphase transition is required for the dephosphorylation of this residue.

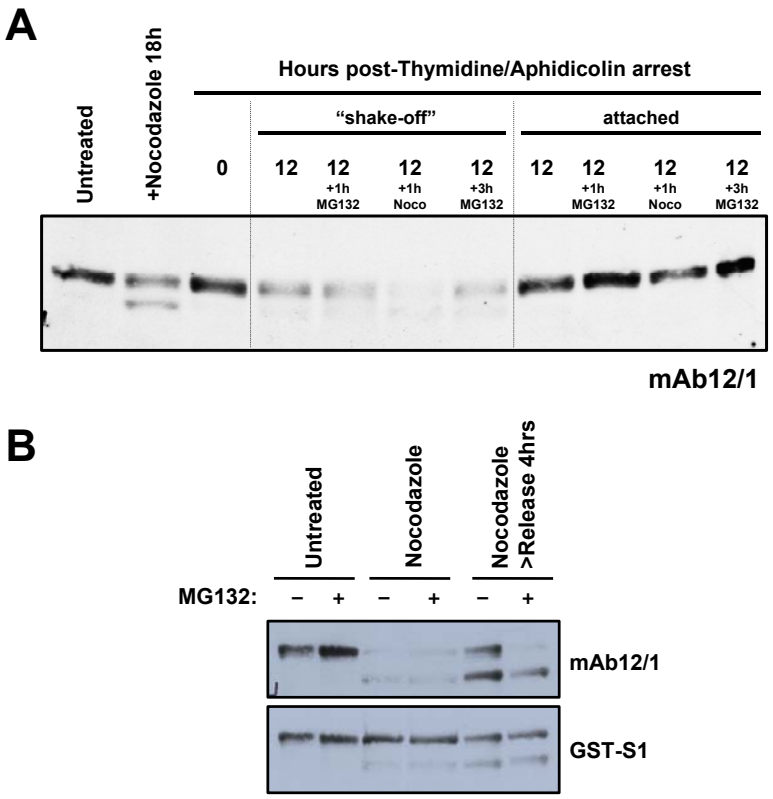


FIGURE 4-46. SIRT1 is phosphorylated at S16 in normal mitosis. *See* text for details

These experiments demonstrated that contrary to the initial belief that SIRT1 S16 phosphorylation requires the activation of the mitotic checkpoint, this phosphorylation can also occur in normal mitosis.

To understand the functional role of SIRT1 in mitosis, SIRT1 protein levels were reduced by siRNA treatment in HeLa cells and the ability of cells to arrest in mitosis in response to nocodazole treatment was investigated. Judging from the extend of accumulation of cyclinB1 and histone H3 S10 phosphorylation, two *bona fide* markers of

mitosis, no obvious impairment in the ability of cells to arrest in mitosis was observed with either of two siRNAs targeting distinct regions of the SIRT1 mRNA (FIGURE 4-47A).

Similar results were obtained in HCT116 cells and their isogenic derivative lacking p53 alleles (FIGURE 4-47B). Furthermore, SIRT1 depletion did not affect the levels of the CDK inhibitors p21 and p27.

Protein downregulation by siRNA can have variable effects depending on its efficiency as residual amounts of the targeted protein may suffice to carry out its task in the cell precluding the emergence of any obvious phenotypes. To address this issue, mouse embryonic fibroblasts (MEFs) derived from mice with targeted deletion of the *SIRT1* locus were prepared and their cell cycle profiles in response to nocodazole treatment were assayed. Compared to their wild-type counterparts, cells heterozygous or homozygous null for the *SIRT1* gene exhibited an approx. 15% reduction in G2/M arrest following 18 hours of nocodazole treatment, a difference that persisted for at least another 22 hours (FIGURE 4-48). This indicated that SIRT1 may be required for entering mitosis or for proper implementation of the spindle assembly checkpoint in mouse fibroblasts.

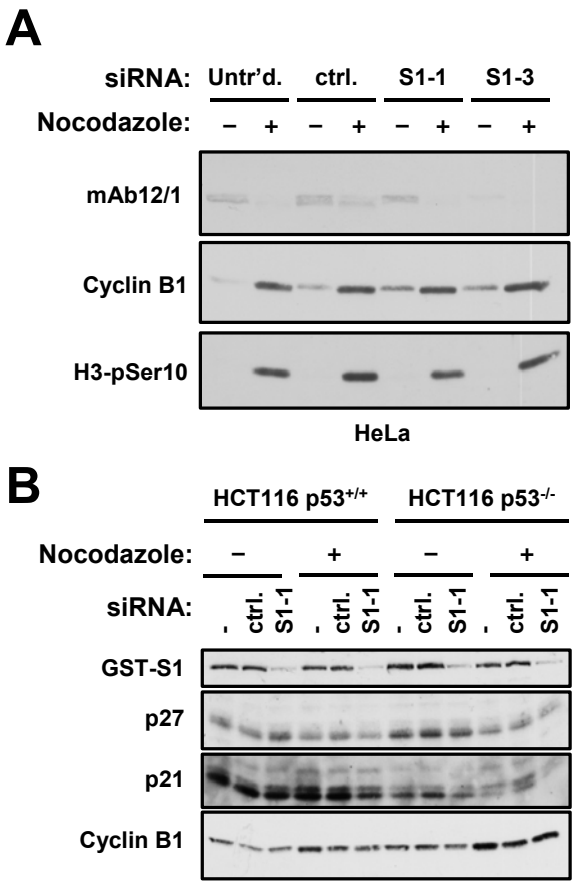


FIGURE 4-47. Effects of siRNA-mediated downregulation of SIRT1 on mitotic markers. *See* text for details.

During mitosis the DNA undergoes extensive condensation which leads to the formation of compact chromosomes suitable for segregation minimising the possibilities of chromosomal breaks which would compromise genomic integrity. Treatment of cells with topoisomerase inhibitors that prevent DNA decatenation without introducing DNA breaks delays entry into mitosis. This led to the proposal that during the G2 phase a "decatenation checkpoint" is in operation to ensure that the newly replicated DNA is sufficently untangled prior to progression to mitosis (Downes *et al.*, 1994).

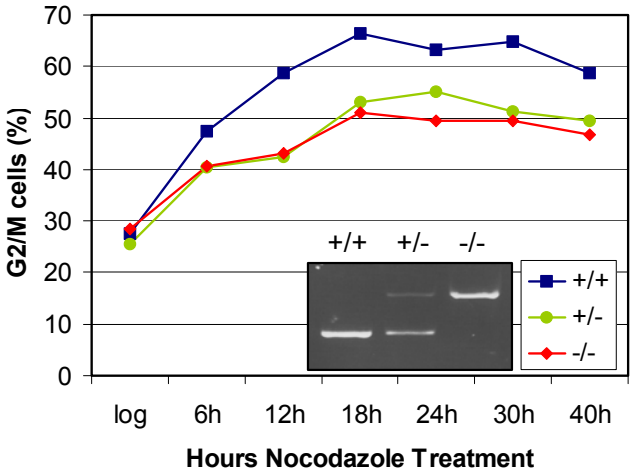


FIGURE 4-48. Impaired nocodazole-induced mitotic arrest in SIRT1$^{-/-}$ MEFs. *See* text for details.

In an early experiment during the course of these studies, a large-scale immunoprecipitation using the Ctp antibody was performed in HeLa cell lysates and mass spectrometry was subsequently employed to identify proteins co-purifying with SIRT1. One of these proteins was DNA topoisomerase IIβ. This interaction was subseqeunlty confrimed in independent experiments using the Ctp antibody but could not be observed with neither the GST-S1 antibody nor mAb12/1 (FIGURE 4-49 and not shown). Interestingly, a further link between sirtuin activity and DNA decatenation was provided from studies in *S. cerevisiae*. Cioci *et al.* reported that deletion of *top1* causes increased histone acetylation at the rDNA locus similar to *Sir2* deletions (Cioci *et al.*, 2002). Mutations of either gene lead to increased accessibility of the rDNA locus assayed by nuclease sensitivity suggesting that Sir2-

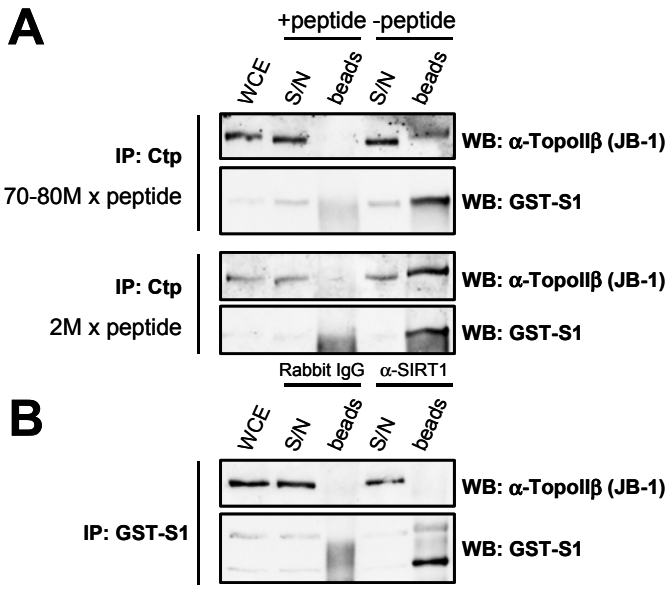


FIGURE 4-49. In vivo interaction of SIRT1 with topoisomerase IIβ. SIRT1 was immunoprecipitated with the indicated antibodies from HeLa cells and probed for the presence of topoisomerase IIβ with JB-1 polyclonal antibody.

mediated modification of the nucleosome and topological changes in DNA structure contribute to gene silencing in a similar manner.

These observations raise the possibility that a role for SIRT1 in mitosis may be to act in conjunction with topoisomerase IIβ as a regulatory complex that implements the necessary structural alterations to prepare the newly replicated DNA to undergo segregation in a faithful manner.

Overall, these results provide evidence for a mitosis-specific post-translational modification of SIRT1, namely phosphorylation of S16. Prior phosphorylation of S14 which mediates a predicted Pin1-dependent conformational change is required for S16 phosphorylation. Preliminary evidence in mouse firbroblasts indicates a potential function of SIRT1 in mitosis indicating that S16 phosphorylation may constitute a molecular mechanism underlying the regulation of SIRT1 in this context.

4.2 REGULATION OF SIRT1 BY CASPASE-MEDIATED CLEAVAGE

On several occassions during the course of these studies, a novel protein species highly immunoreactive to mAb12/1 and to a lesser extend to GST-S1 was observed (FIGURES 4-7B, 4-22, 4-40, 4-41, 4-46). The appearance of this band, which is approx. 10 kDa smaller than full-length SIRT1 (henceforth $p110^{SIRT1}$), correlated with the occurrence of apoptotic figures in the corresponding cell populations as observed by light microscopy. This raised the possibility of a causal link between cell death and the appearance of this putative novel SIRT1 species.

To confirm this correlative link, HeLa and U2OS cells were subjected to treatments with various stress stimuli to induce cell death and lysates thereof were probed with SIRT1 antibodies. UV irradiation as well as actinomycin-D treatment both of which elicit cellular apoptotic responses by causing DNA damage induce the appearance of $p110^{SIRT1}$ (FIGURE 4-49A). This band co-migrates with a similar SIRT1 band appearing in nocodazole-treated cells. Anicomycin is a potent inducer of Jun N-terminal kinase (Jnk) which is a positive mediator of cellular apoptotic pathways (Davis, 2000). Anicomycin treatment also induces the appearance of $p110^{SIRT1}$ (FIGURE 4-50A lower panel). Another apoptosis inducer in the inflammatory cytokine TNFα. In cell culture experiments TNFα alone is not sufficient to elicit apoptosis because activated TNF receptors induce the NFκB survival pathway that mediates the transcription of genes which prevent activation of Jnk and its pro-apoptotic

function (Kamata *et al.*, 2005; De Smaele *et al.*, 2001; Tang *et al.*, 2001). Inhibition of NFκB target gene expression by actinomycin-D to block transcription or cycloheximide (CHX) to block translation enables TNFα to induce apoptosis. CHX alone can also induce apoptosis. As shown in FIGURE 4-50B CHX alone and to a greater extend the combination of TNFα and CHX, but not TNFα alone lead to the induction of $p110^{SIRT1}$. Thus multiple stress stimuli that induce cell death elicit the appearance of a novel SIRT1 species, $p110^{SIRT1}$.

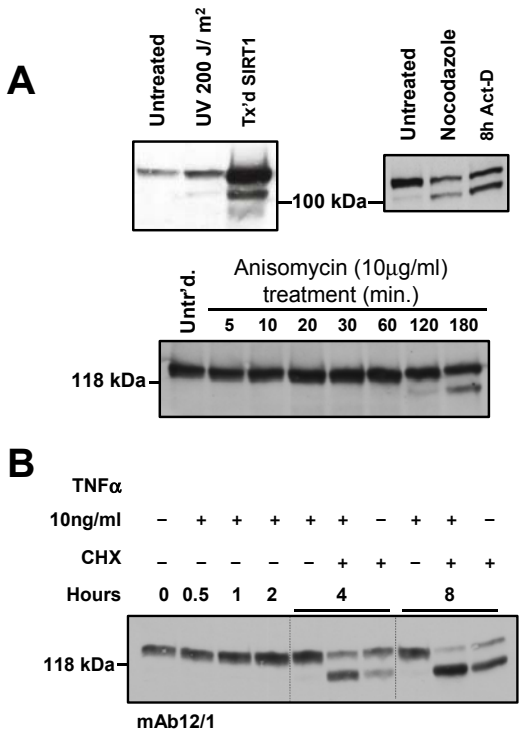


FIGURE 4-50. Various stress stimuli induce SIRT1 processing. Cells in **(A)** were U2OS and in **(B)** HeLa. *See* text for details.

This observation prompted the investigation of the molecular mechanism that results in the appearance of $p110^{SIRT1}$. DNA database sequence searches indicated that $p110^{SIRT1}$ is unlilkely to arise by expression of a *SIRT1* gene alternative transcript or alternative splicing. This was further supported by the fact that neither actinomycin-D nor CHX prevented formation of this band (FIGURE 4-50). Furthermore, alternative translation initiation was also excluded since two potential methionine residues that could account for this give rise to SIRT1 species which are smaller than $p110^{SIRT1}$ (*see* FIGURE 4-10). Apart from its role in protein degradation, the proteolytic activity of the proteasome is also implicated in regulated protein processing (e.g. Orian *et al.*, 2000; Hoiby *et al.*, 2004). Proteolytic processing by the proteasome is also unlikely since MG132 treatment of cells does not prevent the occurrence of $p110^{SIRT1}$ (FIGURE 4-46B).

A specific class of cysteine proteases known as caspases are essential mediators of the apoptotic programme (Danial and Korsmeyer, 2004). In unstimulated cells, caspases are present as inactive zymogens (Riedl and Shi, 2004). When the apoptotic pathway is activated, limited proteolytic events result in the cleavage of the zymogens to yield active caspases which in turn catalyse the cleavage of effector proteins (Danial and Korsmeyer, 2004).

To test whether $p110^{SIRT1}$ formation is sensitive to caspase inhibition, U2OS cells were induced to undergo apoptosis by either UV or actinomycin-D treatment in the presence or absence of a general caspase inhibitor. This inhibitor prevented the appearance of $p110^{SIRT1}$ suggesting that this SIRT1 species may arise by caspase-mediated cleavage (FIGURE 4-51A). Similarly, titration of increasing concentrations of a caspase-8 inhibitor prevented $p110^{SIRT1}$ formation in a dose-dependent manner (FIGURE 4-51B).

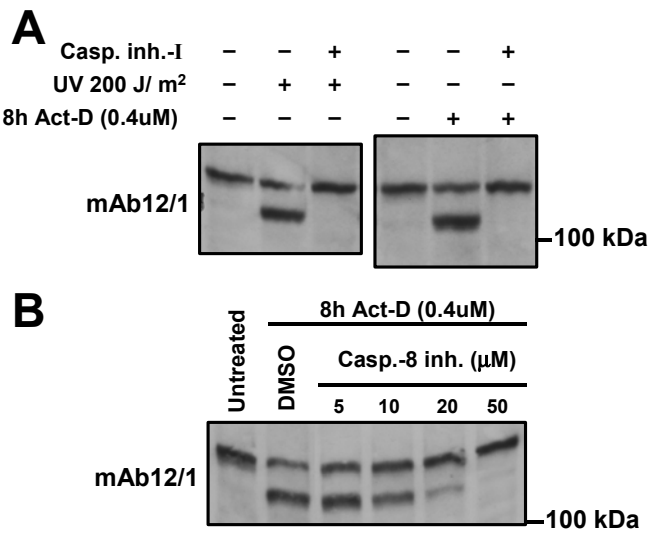


FIGURE 4-51. Caspase inhibitors prevent stress-induced SIRT1 cleavage. *See* text for details.

As $p110^{SIRT1}$ is detected by mAb12/1 which maps at the N-terminus of SIRT1 (FIGURE 4-38B) it was expected that this novel SIRT1 species arises by cleavage of the C-terminus of the protein. To confirm this, U2OS cells were induced to undergo apoptosis with various treatments and subjected to immunoblotting with mAb12/1 a well as GST-S1 and Ctp which were raised against epitopes in the C-terminus of the protein. mAb12/1 is significantly more reactive to $p110^{SIRT1}$ compared to GST-S1 which can recognise the band too (FIGURE 4-52). $p110^{SIRT1}$ is not recognised by Ctp corroborating the prediction that caspase-mediated SIRT1 processing occurs in the C-terminus of the protein.

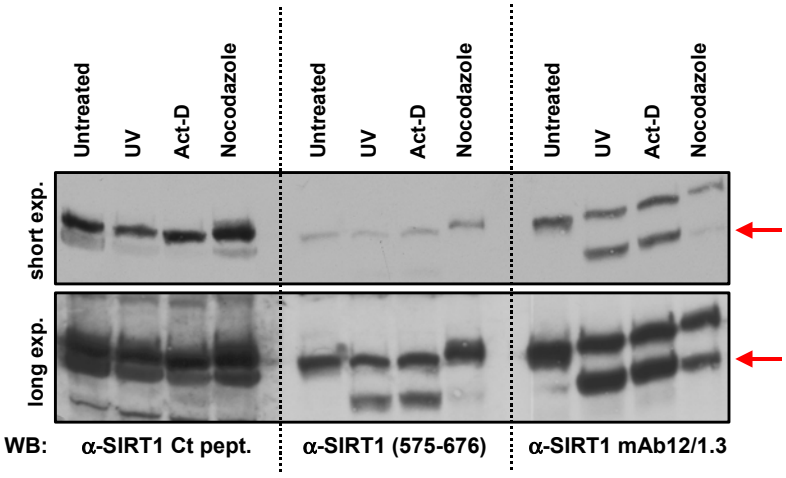


FIGURE 4-52. Stress-induced SIRT1 processing occurs in the C-terminus of the protein. Cells were treated with the indicated stress stimuli lysed and equal amounts of each lysate were loaded in triplicate on the same gel followed by immunoblotting with Ctp, GAT-S1 or mAb12/1 α-SIRT1 antibodies. The processed SIRT1 species is indicated with red arrows.

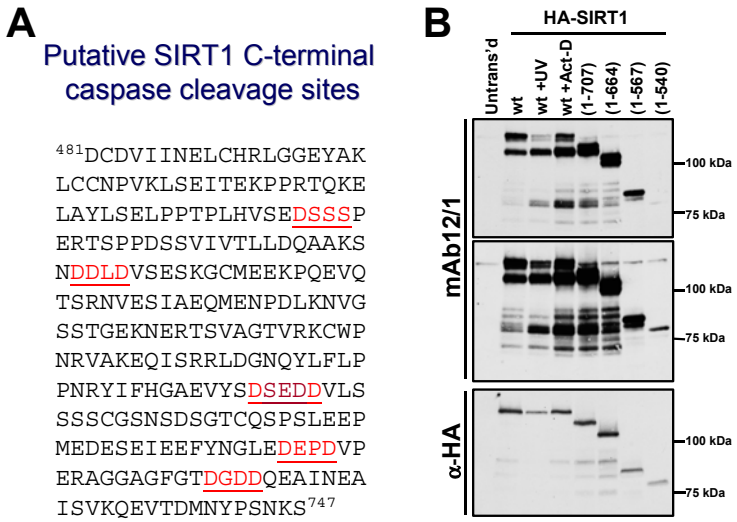


FIGURE 4-53. Identification of the SIRT1 caspase cleavage site. (A) Putative caspase cleavage sites in the C-terminus of SIRT1 are highlighted in red; **(B)** Mapping of the putative SIRT1 caspase cleavage sites by comparison of the electrophoretic mobilities of truncated SIRT1 species with processed SIRT1 following stress.

To precisely map the caspase cleavage site of SIRT1 was examined for the presence of putative caspase consensus target sequences. Several such sites were identified in the C-terminus of the protein (FIGURE 4-53A). Caspases show specificity for the peptide bond following aspartic acid and in particular target sites with the consensus -D-X-X-D*- where X is any aminoacid and * marks the cleavage site. It was also considered possible that phosphorylation of S or T residues can drive caspase-mediated cleavage probably because the negative charge of the phosphate mimics the acidic side-chain of D. It is of note, however, the report of Tozser *et al.* 2003 who showed that such phosphorylations actually decrease susceptibility to caspases (Tozser *et al.*, 2003). Also interestingly, substitution of D with E abolishes caspase cleavage indicating that caspase targeting is not only determined by the charge but also by steric factors (Riedl and Shi, 2004).

To find which of the putative sites is targeted by caspases *in vivo*, several SIRT1 C-terminal truncation mutants were expressed in U2OS cells as HA-tagged species and analysed by immunobloting with either mAb12/1 or α-HA antibody. Among these species HA-SIRT1(1-707) co-migrated with the HA-p110^{SIRT1} band derived from the cleavage of wild-type HA-SIRT1 in response to UV or actinomycin-D treatment (FIGURE 4-53B). A second less adundant cleavage product co-migrated with HA-SIRT1(1-540). Interestingly, similar bands also appeared in untreated transfected cells, probably arising due to apoptosis induced by the calcium phosphate tranfection method. It should also be noted that the full-length HA-SIRT1 as well as the C-terminal truncations appear as doublets with the mAb12/1 but not with the α-HA antibody. Supeimposition of the films from the two blots revealed that it is the upper band that is recognised by the α-HA antibody suggesting that

the lower band arises due to loss of the HA tag. Indeed, examination of the HA tag sequence revealed the presence of a caspase consensus site providing an explanation as to the origin of this phenomenon.

To confirm that SIRT1(1-707) is the caspase cleavage product, D707 was mutated to E which is expected to hinder proteolytic processing by caspases. This mutation (D707E) abolished stress-induced cleavage as well as the cleavage observed in untreated cells confirming that this D707 is a major caspase proccessing site on SIRT1 (FIGURE 4-54). In the same samples, HA tag loss occurred equally in all samples.

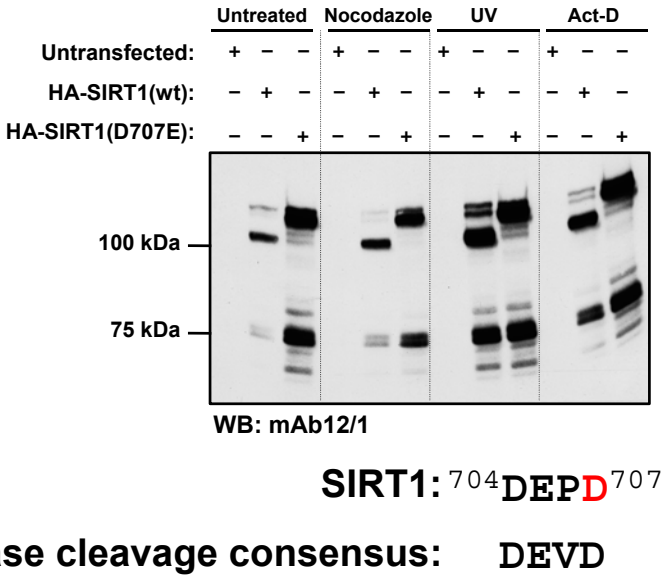


FIGURE 4-54. D707 is the major SIRT1 caspase cleavage site. *See* text for details.

As a SIRT1 ragment corresponding to residues 1-540 appeared to co-migrate with a full-length SIRT1 proteolytic product (FIGURE 4-53B), the effects of S540 modification were examined. S540 was mutated to A or D to mimic a non-phosphorylatable and a constitutively phosphorylated species and expressed in U2OS cells. The proteolytic cleavage pattern of the S540 mutants was indistinguishable from that of wild-type protein (FIGURE 4-55) independently of the presence or absence of apoptotic stimulus (UV), suggesting that even if the region flanking S540 is susceptible to proteolytic processing by caspases, S540 modification is not likely to modulate that.

Despite the evidence that the stress-induced processing of SIRT1 is prevented by caspase inhibitors and that the identified cleavage site conforms with the caspase consensus recognition site, it is possible that the influence of caspase inhibitors on SIRT1 processing (FIGURE 4-51) is a secondary effect arising due to the prevention of apoptosis

thus abolishing an apoptosis-linked but caspase-independent proteolytic activity. To test this, an *in vitro* caspase assay was employed. In this assay cytosolic S100 extracts of unstimulated 293 cells are treated with the nucleotide dATP which is sufficient to elicit an apoptosis-like programme which results in an accute activation of caspases (Liu *et al.*, 1996). Wild-type SIRT1, SIRT1(1-707) and the D707E mutant were *in vitro* transcribed-translated and incubated in the presence of S100 extracts which had been either mock treated or treated with dATP. No cleavage of SIRT1 was observed in the absence of dATP whereas a proteolytic product which co-migrated with the SIRT1(1-707) truncation mutant appeared even after 15' of incubation with dATP-treated extracts (FIGURE 4-56A). In contrast, no proteolytic product arose from the D707E mutant under identical incubation conditions. In the 30' of incubation in this experiment, no further cleavage of the 1-707 mutant was observed.

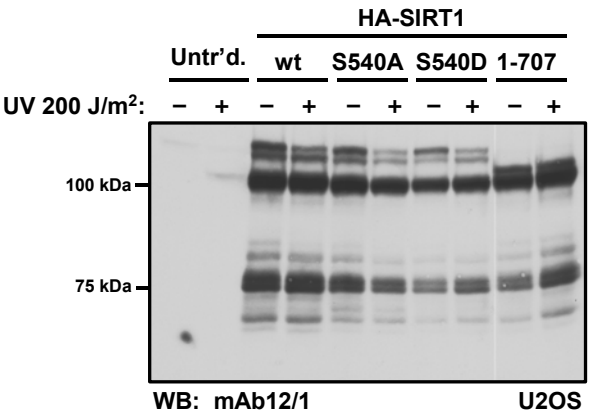


FIGURE 4-55. S540 mutation does not affect SIRT1 processing. *See* text for details.

To confirm that the observed effect is due to caspase activation, SIRT1, SIRT1(1-707) and SIRT1(D707E) were incubated with dATP-activated extracts for 90' and a generic caspase inhibitor was added to the reaction of wild-type SIRT1. Under these conditions, SIRT1 was undetectable in the activted extracts and this could be inhibited by the caspase inhibitor (FIGURE 4-56B). Interestingly, SIRT1(1-707) was also rendered undetectable in activated extracts suggesting that it can undergo further proteolysis. Although it is not clear based on these experiments if this is also a caspase-catalysed processing

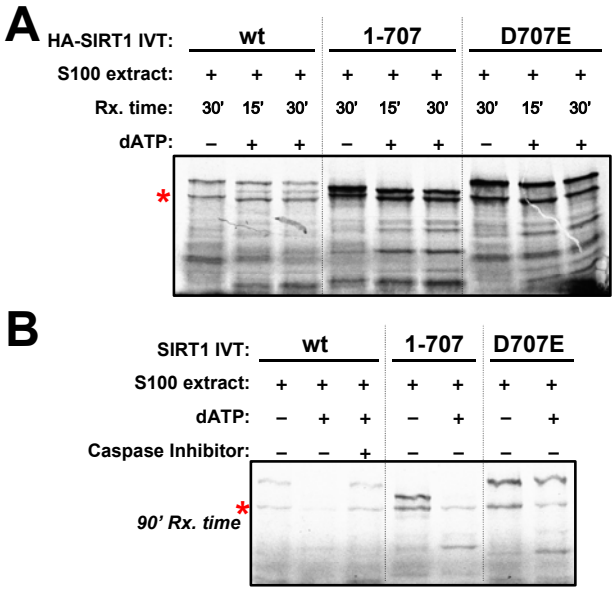


FIGURE 4-56. *In vitro* caspase cleavage assay. *See* text for details.

event, this is a plausible scenario. In contrast, SIRT1(D707E) was completely resistant to proteolysis.

To investigate this point further, U2OS cells were treated with 50 J/m^2 UV light for 24 hours in the presence of the proteasome inhibitor MG132 or carrier solvents for 6 hours prior to harvesting. MG132 treatment had little effect on full-length SIRT1, however it induced a marked increase in the abundance of the caspase cleavage product (FIGURE 4-57). These results suggest that SIRT1 is cleaved by caspases at residue D707 and that caspase cleavage renders the protein susceptible to further proteolytic processing.

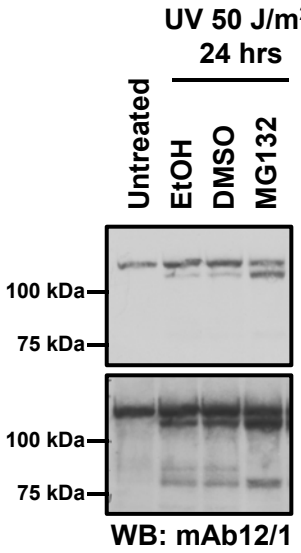


FIGURE 4-57. SIRT1 caspase cleavage product is susceptible to increased proteasomal degradation. U2OS cells were either mock treated or treated with 50 J/m^2 UV for 24 h and with either carrier or 50 μM MG132 for 6h prior to harvesting.

Prolonged arrest of cells in mitosis due to activation of the SAC can lead to apoptotic cell death (Rieder and Maiato, 2004). As cleavage of SIRT1 was also frequently observed in nocodazole-treated cells (e.g. FIGURE 4-50A), to verify that this is also due to activation of caspases, cells arrested in mitosis with nocodazole were treated with a generic caspase inhibitor or caspase-8 inhibitor and immunoblotted with either mAb12/1 or GST-S1. Caspase inhibition prevented the formation of the proteolytic band confirming that its presence can be attributed to nocodazole-induced apoptosis (FIGURE 4-58). Interestingly, caspase-8 inhibition did not abolish completely the processing of SIRT1 revealing also the presence of a slightly faster migrating band with a minimal apparent size difference compared to full-length SIRT1. Unless this reflects an inhibitor dose effect, it can be proposed that multiple caspase

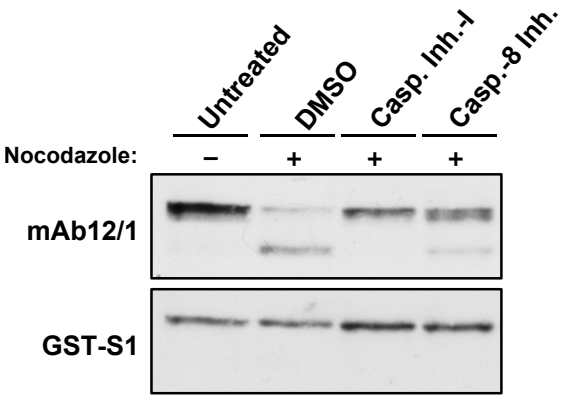


FIGURE 4-58. Nocodazole-induced SIRT1 processing is sensitive to caspase inhibitors. HeLa cells were treated with nocodazole and the indicated caspase inhibitors for 16 h prior to harvesting.

species contribute to SIRT1 processing during apoptosis and that additional subtle proteolytic effects may also occur.

As a first step towards elucidating the functional significance of caspase-mediated SIRT1 cleavage, the subcellular localisation of SIRT1(1-707) was investigated. HeLa or U2OS cells were transfected with HA-tagged constructs of either wild-type SIRT1 or SIRT1 (1-707) and their localisation was probed by indirect immunofluorescence. Both wild-type and the truncated mutant were consistently detected in the nucleus in a manner undistinguishable from each other (FIGURE 4-59). Thus truncation of the last 40 aminoacids of SIRT1 does not have an effect on its subcellular distribution.

The crystal structure of the yeast sirtuin Hst2p revealed that the C-terminus of the protein forms extensive contacts with the catalytic cleft implying a potential autoregulatory mechanism for sirtuins (Zhao *et al.*, 2003). As SIRT1 also shares an extensive C-terminal region it is possible that caspase-mediated truncation of the last 40 aminoacids may influence potential intramolecular contacts which in turn could affect the deacetylase activity of the protein. To this end, HA-SIRT1, HA-SIRT1(1-707) and the catalytically inactive mutant H363Y as a control were transfected into 293 cells, immunoprecipitated with α-HA antibody and their activity was assayed using a fluorescence-based peptide deacetylation assay. Accounting for expression differences, the catalytic activities of wild-type and the 1-707 mutant SIRT1 species were comparable (FIGURE 4-60). It is however possible that SIRT1 truncation results in only subtle differences in its catalytic

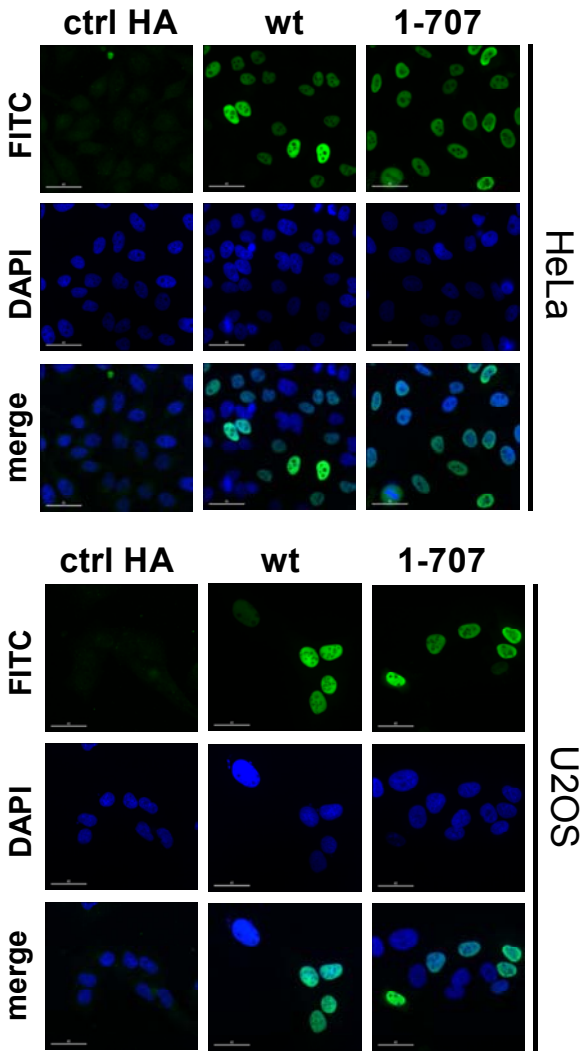


FIGURE 4-59. SIRT1(1-707) does not show altered subcellular distribution. HeLa or U2OS cells were transfected with the indicated constructs and processed for indirect immunofluorescence analysis. Green: SIRT1 species, Blue: DAPI. Scalebars represent 40 μm

properties which would not be revealed in such an end-point assay. Indeed, truncation of the C-terminus of Hst2p leaves the k_{cat} of the enzyme unaffected while it reduces its K_m for NAD^+ 3.6-fold (Zhao *et al.*, 2003). The type of assay used in this experiment, though, is not easily amenable to extensive quantitative analysis since parameters which are difficult to control precisely such as the IP bead volume, can hinter the accuracy of the measurements.

To address this issue, GST-SIRT1(1-707) was cloned in a baculoviral expression vector and expressed in Sf9 cells. Unfortunately, despite good expression levels, this protein species was virtualy impossible to elute from the glutathione beads used to purify it. Further work to resolve this problem is underway. Succesful purification of this protein will provide a major tool for a detailed analysis of the potential effects of caspase-mediated cleavage in the enzymatic activity of SIRT1.

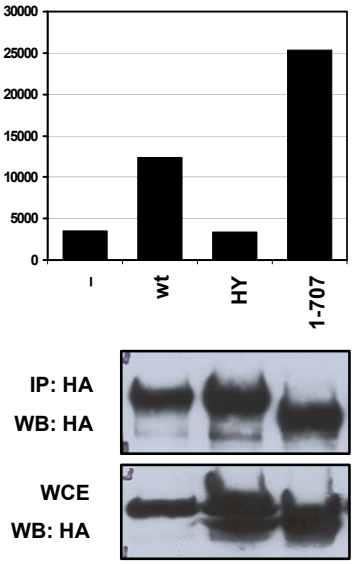


FIGURE 4-60. SIRT1(1-707) does not exhibit altered enzymatic activity. 293 cells were transfected with the indicated constructs and SIRT1 species were subsequently immunoprecipitated and used for *in vitro* SIRT1 deacetylase activity assays as described in Chapter 3.

In view of several reports implicating SIRT1 in the regulation of transcription factor activity, the possibility that caspase-mediated cleavage may affect this function was tested. In response to environmental simuli, signalling and transcriptional responses undergo dynamic changes that underlie cellular adaptability. As SIRT1 was shown to be a negative regulator of NFκB and p53 transcription factors, it is plausible that this function is blunted upon genotoxic stresses, allowing the efficient activation of gene expression programmes driven by these transcription factors. Caspase cleavage could potentially play such a role, either as a modulator of SIRT1 deacetylase activity (described above) or by regulating protein complex formation that requires the C-terminal region of SIRT1.

Towards this end, the effects of wild-type SIRT1 and the 1-707 mutant upon NFκB-driven reporter gene activity were assayed. A luciferase gene driven by a constitutive promoter under the control of three NFκB consensus DNA binding sites (3xκB) was contransfected along with increasing amounts of the SIRT1 contructs. To induce NFκB activity, cells were treated with TNFα and the ability of SIRT1 to suppress this activation was assayed by measuring luciferase activity (compared to identically-transfected

uninduced cells for each reaction). All luciferase measurements were normalised for transfection efficiency by assaying for β-galactosidase activity which was co-expressed in each reaction. FIGURE 4-61 shows to representative examples of results obtained from such experiments. Low amounts of HA-SIRT1(1-707) appeared to be more efficient in suppressing TNFα-induced NFκB reporter activity although at higher SIRT1 concentrations, this effect was more subtle (FIGURE 4-61A). Repetition of this experiment using untagged SIRT1 contructs failed to reproduce this result as it showed equal capacities of both SIRT1 species to repress inducible reporter gene activity (FIGURE 4-61B). Thus, to this point little evidence exist supporting a role for caspase-mediated SIRT1 cleavage in regulating the transcriptional activity of the latter.

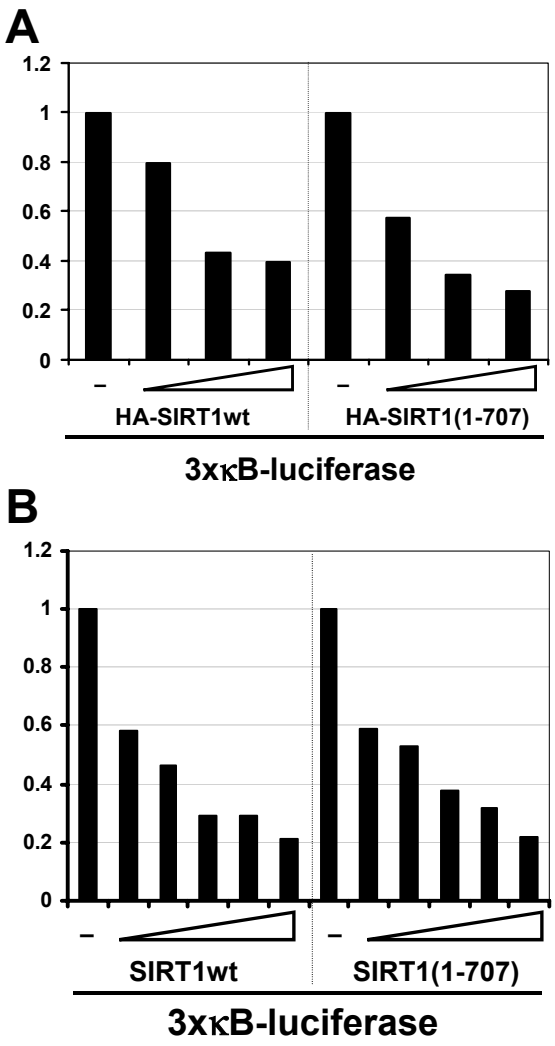


FIGURE 4-61. SIRT1(1-707) does not exhibit altered transcriptional repression activity. *See* text for details.

In conclusion, SIRT1 is subjected to a caspase-catalysed proteolytic cleavage in response to apoptotic stimuli. The major cleavage site was mapped in a capsase consensu target site in the C-terminus of SIRT1 at residue D707. Assessment of the transcriptional respression activity of SIRT1 on gene reporter assays revealed little evidence for an effect attributable to SIRT1 cleavage. However, the cleaved SIRT1 species shows increased proteolytic turnover which can be inhibited by MG132 pointing to a potential role of cleavage at D707 in protein stability.

4.3 OTHER FEATURES OF THE SIRT1 PRIMARY SEQUENCE

During the course of these studies, other features of the primary sequence of SIRT1 were discovered, yet time limitations did not allow the experimental assessment of their functional significance.

Of note among these is a TOR signaling (TOS) motif comprising residues 474-478 immediately following the sirtuin core domain (FIGURE 4-62). The TOS motif was discovered as a protein sequence region required for the activation of S6 kinase, a well-established mTOR effector (Schalm and Blenis, 2002). 4E-BP1, another mTOR target also harbours a TOS motif which was subsequently shown to mediate the interaction of 4E-BP1 with the mTOR binding partner raptor (Schalm *et al.*, 2003) providing a molecular explanation of its functional significance. Indeed, expression of 4E-BP1 with mutated TOS motif results in decreased cell size consistent with its neccessity for TOR signaling. The TOR signaling pathway is a key mediator of cellular responses to nutrient availability and recent evidence suggests that TOR itself may regulate organismal life-span in yeast (Kaeberlein *et al.*, 2005). Thus it would be of interest to investigate whether similar to other TOS-bearing proteins, SIRT1 is also a target of mTOR signaling or indeed a substrate of the latter's kinase activity.

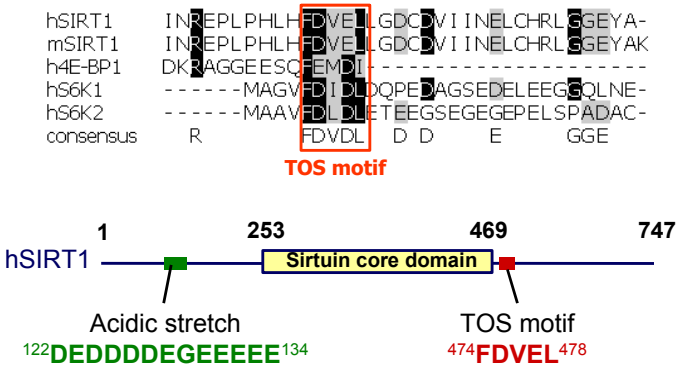


FIGURE 4-62. Novel SIRT1 sequence motifs with potential function in signaling. *See* text for details.

Another characteristic feature of SIRT1 is an extensive stretch of acidic residues comprising residues 122-134 found in the N-terminus of the protein (FIGURE 4-62 lower panel). This feature is well conserved between human, mouse and *Drosophila* SIRT1 but not in lower organisms. Acidic stretches are not an unknown feature and are particularly present in transcriptional regulators possibly reflecting a tendency to associate with histones which are basic.

The importance of these motifs is unclear, yet their identification may provide a useful guide for future structure-function analyses of SIRT1.

CHAPTER 5

DISCUSSION AND FUTURE PERSPECTIVES

Elucidating signaling circuitries that underlie cellular and organismal physiology has allowed a significant understanding of the homeostatic mechanisms employed by living organisms to adapt to environmental stimuli. Such mechanisms have been a major driving force in the evolution process and continue to be important in the way pathogenic organisms as well as aberrantly functioning cells withstand ever more elaborate pharmacological interventions treating disease. Thus, in-depth comprehension of adaptive signaling systems and their interlinks allows to track down the molecular causes of particular disease states leading to the development of novel knowledge-based therapeutic strategies.

5.1 Common pathways underlying homeostatic cellular processes are disregulated in diverse diseases

It has been increasingly evident that molecular players of pathways involved in basic homeostatic processes go frequently awry in diseases apparently as diverse as cancer and the metabolic syndrome. In particular several components of the insulin, TOR and oxygen signaling pathways have been directly implicated in such diseases (Wullschleger *et al.*, 2006; Plas and Thompson, 2005; Barry and Krek, 2004). Aberrant function of these circuitries underlies

dramatic changes in the proliferative, growth and metabolic cellular capacity, which are closely related to the disease phenotype(s).

The fact that these signaling pathways co-ordinate such basic cellular functions provides a first level of explanation as to why their molecular components comprise the common denominator of different diseases. However, the mechanistic details of this phenomenon remain largely elusive. Recent evidence has shed more light into the intriguing cross-talk at play which co-ordinates diverse cellular activities and underlies the complex disease phenotypes associated with mutations of these central homeostatic pathways.

5.1.1 Functional interconnection between the PKB, TOR and oxygen signaling pathways in health and disease

The insulin/IGF pathway involves a highly conserved set of signaling molecules that coordinate cellular growth, proliferation and metabolism (Plas and Thompson, 2005; Brazil *et al.*, 2004). A central orchestrator of these effects is the kinase PKB. PKB phosphorylates a diverse array of proteins to promote cell cycle progression, survival and sustain the metabolic capacity at the levels required for proliferation (Plas and Thompson, 2005).

An independent pathway that yet is under the positive influence of growth factor signaling through the insulin receptor is mediated by the TOR kinase. TOR is also activated by aminoacids through a largely unknown mechanism and is a central regulator of cellular protein biosynthetic capacity by dictating ribosomal biogenesis (Wullschleger *et al.*, 2006). At the same time, cellular energy status, modulated e.g. by glucose, also determines the activity of TOR *via* the AMPK kinase cascade (Hardie, 2005).

The VHL-HIF signaling axis allows cells to adapt their metabolic programme to the oxygen tension experienced at any given time and to elicit appropriate adaptive changes in their environment such as the formation of new blood vessels in response to hypoxic conditions (Barry and Krek, 2004; Kaelin, 2002).

5.1.1.1 Representative mechanisms employed by hypoxia to inhibit growth and proliferation

At least two mechanisms have been shown to implement suppression of cellular growth in response to hypoxia. The first requires a HIF target gene known as REDD1, which is also a p53 target. REDD1 signals *via* the TSC1/TSC2 tumour suppressor complex to inhibit TOR activation (Brugarolas *et al.*, 2004). The second mechanism implicates that reduction in cellular energy due to low oxygen tension results in the activation of the AMPK pathway which in turn inhibits components of the translational apparatus (e.g.eIF2α and eEF2) as well as TOR to suppress translation (Liu *et al.*, 2006).

Similarly, energy availability expressed as the ratio of AMP/ATP depends on another tumour suppressor, p53 to elicit cell cycle arrest under conditions of nutrient limitation. This requires the phosphorylation and subsequent activation of p53 by the AMPK kinase (Jones *et al.*, 2005). Interestingly, p53 is also stabilised by HIF, however no direct experimental evidence exists to support that p53 is required for the cell cycle arrest in response to hypoxia (Dang and Semenza, 1999). An alternative mechanism possibly at play postulates that HIF represses the activity of the myc oncoprotein which itself is a negative regulator of p21 and p27 transcription (Gartel and Shchors, 2003).

5.1.1.2 Reciprocal relation between metabolic enzymes and proteins regulating proliferation

Cancer cells exhibit profoundly altered metabolic characteristics, predominantly increased glucose uptake and enhanced anaerobic glycolysis even at normoxic conditions (Dang and Semenza, 1999). This phenomenon is known as the Warburg effect. Several oncogenes as well as tumour suppressors have been shown to mediate the gene expression changes associated with such a phenotype.

The transcriptional activity of HIF accounts for a large part of such changes as it induces the transcription of essentially all key enzymes in the glycolytic pathway (Dang and Semenza, 1999). In addition, the tumour suppressor p53 and the oncogene myc also regulate respectively the expression of hexokinase, the rate-limiting step in glycolysis and lactate

dehydrogenase which promotes the anaerobic utilisation of pyruvate (Kim *et al.*, 2004; Dang and Semenza, 1999). At the same time, myc promotes mitochondrial biogenesis, and the resulting increased mitochondrial activity has been proposed to contribute to augmented levels of ROS that in turn enhance genomic instability (Dang *et al.*, 2005).

Furthermore, PKB-mediated recruitment of hexokinase to mitochondria has an anti-apoptotic effect (Majewsky *et al.*, 2004) while PKB also promotes survival by contributing to the phosphorylation and thus inactivation of Bad along with S6K and glucokinase (Brazil *et al.*, 2004; Danial *et al.*, 2003). Interestingly, though, recent data suggest that PKB also enhances aerobic glycolysis in cancer cells which renders them more susceptible to apoptosis induced by glucose withdrawal (Elstrom *et al.*, 2004).

Interestingly, other glycolytic enzymes appear to have evolved additional functions parallel to their classic ones in the glycolytic pathway (Kim and Dang, 2005) raising the exciting possibility that their transcriptional regulation by the HIF and other cancer pathways elicits a cellular response that extends far beyond metabolic adaptation. Thus, in addition to the aforementioned hexokinase, GAPDH was shown to participate in the transcriptional activation of histone H2B genes (FIGURE 5-1) (Zheng *et al.*, 2003) while it also appears to have a role in the regulation of apoptosis, albeit an incompletely understood one (Kim and Dange, 2005).

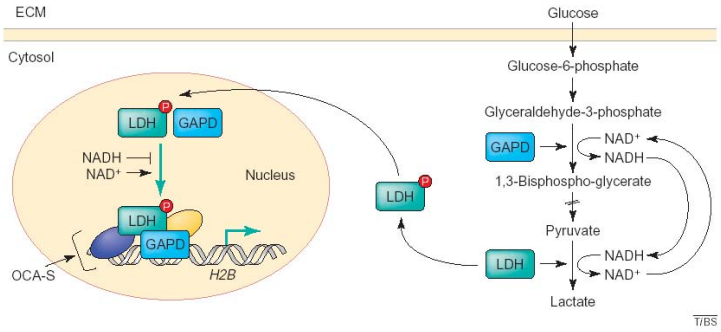


FIGURE 5-1. GAPD and LDH as transcriptional regulators. Both GAPD and LDH are involved in the glycolytic steps that are coupled with the oxidation or reduction reaction of NADH to NAD^+ and vice versa. The phosphorylated form of LDH is exclusively localized in the nucleus. The molecular mechanism responsible for nuclear translocation of GAPD and phosphorylated LDH is not yet known. Along with LDH, nuclear GAPD forms an Oct-1 transcriptional coactivator complex, OCA-S, which is an S-phase-dependent transactivator of the gene encoding histone H2B. The DNA-binding property of LDH and GAPD is increased by a low NADH:NAD^+ ratio, such that OCA-S activity and H2B expression are increased. Yellow and blue ovals represent other components of the OCA-S complex.

Figure and legends adapted from Kim and Dang, 2005

It is possible that transcriptional as well as indirect metabolic effects upon the glycolytic or indeed other metabolic pathways alter the flux through an enzymatic cascade and eventually skew the balance of individual pools charged with specific tasks.

Further to the apparent dependence of cancer cells to glycolysis, mitochondrial function also plays an important role in cancer biology. To begin with, mitochondrial genes participating in the tricarboxylic acid (TCA) cycle such as succinate dehydrogenase and fumarate hydratase were shown to be mutated in different cancers including renal cell carcinoma which is frequently associated with mutations on VHL, the negative regulator of HIF (Barry and Krek, 2004; Gottlieb and Tomlinson, 2005 and references therein). In particular, accumulation of succinate due to succinate dehydrogenase malfunction leads to inhibition of HIF prolyl hydroxylases and thus stabilisation of HIF under normoxic conditions, a phenomenon called pseudohypoxia, providing a possible rationale behind the tumourigenic effects arising from mutations in metabolic enzymes (*see* Chapter 1).

Interestingly, members of the E2F protein family, which are transcription factors with cardinal roles in the regulation of cell cycle progression, have been shown to regulate mitochondrial gene expression. In particular, E2F4 specifically co-operates with the pocket protein p130 to mediate the gene expression programme in response to growth arrest stimuli such as contact inhibition, growth factor withdrawal and forced expression of the $p16^{INK4A}$ CDK inhibitor (Cam *et al.*, 2004). A remarkable enrichment of nuclear respiratory factor-1 (NRF-1) binding sites is exhibited within the set of E2F4 promoters especially of genes required for mitochondrial biogenesis. This work provided a hint of a molecular mechanism linking proliferation and the metabolic status of the cell.

Mitochondrial dysfunction is also linked to the formation of reactive oxygen species (ROS) which promote genomic instability possibly by enhancing DNA mutation rates (Balban *et al.*, 2005). As ROS is a by-product of basic mitochondrial functions, the existence of cellular mechanisms that ensure their prompt elimination is of great importance. MnSOD is one of the enzymes that catalyse ROS removal and is under the transcriptional control of FOXO in response to oxidative stress stimuli. This is of particular interest as the hyperactivation of PKB in tumours would lead not only to increased mitochondrial metabolism but also to enhanced FOXO suppression and thus impaired MnSOD activity. Consequently, mutations that result in PKB hyperactivation such as in the *PTEN* gene constitute a double-edged sword against tumour suppression.

Conversely, aspects of cellular proliferation can also have an impact on organismal metabolism. Abblation of the *p27* gene locus in mice leads to increased pancreatic islet mass

due to enhanced proliferation of β-cells. Consequently, these mice have an improved response to diabetes-associated hyperglycaemia (Uchida *et al.*, 2005). Furthermore, glucose intolerant diabetic patients have remarkably reduced mRNA levels of the HIF heterodimerisation partner ARNT in pancreatic islets (Gunton *et al.*, 2005). This is recapitulated in a mouse model bearing β-cell specific deletion of *ARNT* as well as tissue culture experiments where ARNT loss results in impaired glucose-stimulated insulin release.

In a macroscopic view of these observations, a stricking correlation between diet and the incidence of disease exists. Although in the case of metabolic diseases the causative role of diet quality is more straight-forward, although incompletely understood, the link to cancer is less clear. Excluding the influence of dietary substances that have a tumourigenic effect on their own, there is increasing evidence that the metabolic state of an organism may influence tumour formation propensity independently of genetic background.

In particular, obesity, which frequently results in insulin resistance and consequently to major imbalances in metabolic homeostasis, has been directly linked to some types of cancer occuring in the colon, endometrium, kidney and oesophagus (Calle and Thun, 2004). Some preliminary glimpses of the molecular mechanisms underlying this phenomenon have been recently produced.

Inflammation seems to be a common denominator in both cancer and obesity-associated disorders. For example, in a mouse model of colitis, which is characterised by a persistent inflammatory response, genetic ablation of macrophage-mediated inflammation results in attenuation of tumour incidence and size (Greten *et al.*, 2004). Similarly, ablation of macrophage-mediated inflammation results in enhanced insulin sensitivity in obese mice (Arkan *et al.*, 2005; Wellen and Hotamisligil, 2005).

Furthermore, in the hyperglycaemic diabetic mouse, S6K1, which is under the positive influence of TOR kinase, phosphorylates IRS in an inhibitory site resulting in reduced insulin receptor signaling and thus insulin resistance. Interestingly, inactivating mutations of the genes encoding for the TSC tumour suppressor complex lead to hyperactivation of the TOR signaling pathway and tumour development (Astrinidis and Henske, 2005).

Conspicuously, the incidence of both cancer and diabetes increase with age (Hasty *et al.*, 2003; Moller *et al.*, 2003), and besides its roles in these diseases the IGF/insulin signaling pathway is a well established regulator of the ageing process in a manner remarkably similar

between different species. Genetic ablation of IGF signaling pathway components results in extension of lifespan in *Drosophila*, *C. elegans* and in mice (Partridge and Gems, 2002). This extended longevity phenotype depends on the activity of the forkhead family of transcription factors (Guarente and Kenyon, 2000). For example, genetic studies have revealed that the activity of the forkhead target gene MnSOD leads to increased resistance to oxidative stress-elicited damage and promotes organismal life-span (Kenyon, 2005). Intriguingly, ROS-induced oxidative damage is also a proposed causal factor for cancer, further underlining the commonalities of phenotypes associated with ageing and associated diseases.

In addition to dietary quality and its influence on disease occurrence (McCullough and Giovannucci, 2004), dietary restriction has a major impact on ageing, an effect closely associated with its ability to suppress the IGF signaling pathway (Guarente and Picard, 2005). In lower organisms, the beneficial effects of caloric restriction on organismal life-span have been partially attributed to the sirtuin family of protein deacetylases (Guarente and Picard, 2005). Thus, a great deal of experimental effort has been invested into investigating the role of sirtuins in this process.

5.2 The sirtuin family of protein deacetylases

Further to its well-established role in the regulation of gene expression through alternation of transcription factor activity and chromatin structure, acetylation is being increasingly appreciated as a modification with a broad spectrum of effects (Kouzarides, 2000). Proteins are acetylated by acetyltransferases by transfer of the acetyl-group from acetyl-CoA to either the ε-amino group of lysine residues or the α-amino group of protein amino-terminal residues. While N-terminal acetylation occurs co-translationaly in about 85% or eucaryotic proteins, lysine acetylation occurs post-translationaly (Polevoda-Sherman 2002). Similar to phosphorylation the reversible nature of acetylation is a paramount feature allowing rapid adaptation to a dynamic environment. Thus deacetylases are likely to play key roles in processes regulated by acetylation other than chromatin structure modification.

Sirtuins are a recently identified protein family of deacetylases, often referred to as class III deacetylases, which are distinct from classical HDACs due to the dependence of their activity on NAD^+ and their insensitivity to TSA (Denu, 2003). They are highly conserved

throughout the phylogenetic tree yet they have been shown to regulate metabolic and gene regulatory processes not shared by all species.

The bacterial sirtuin CobB functions in the cobalamin biosynthesis pathway which is absent in higher eucaryotic cells (Tsang and Escalante-Semerena, 1998; Blander and Guarente, 2004). In *S. cerevisiae*, Sir2 has a well-established role in gene silencing at mating-type, rDNA and telomeric loci where its deacetylase activity is required for the establishment and spreading of heterochromatin (Grewal and Moazed, 2003; Guarente, 1999). Several enzymatic targets of mammalian sirtuins have been identified to-date (FIGURE 5-2), primarily transcriptional regulators yet no evidence exists supporting a broader role in global gene silencing and heterochromatin formation similar to yeast (Blander and Guarente, 2004).

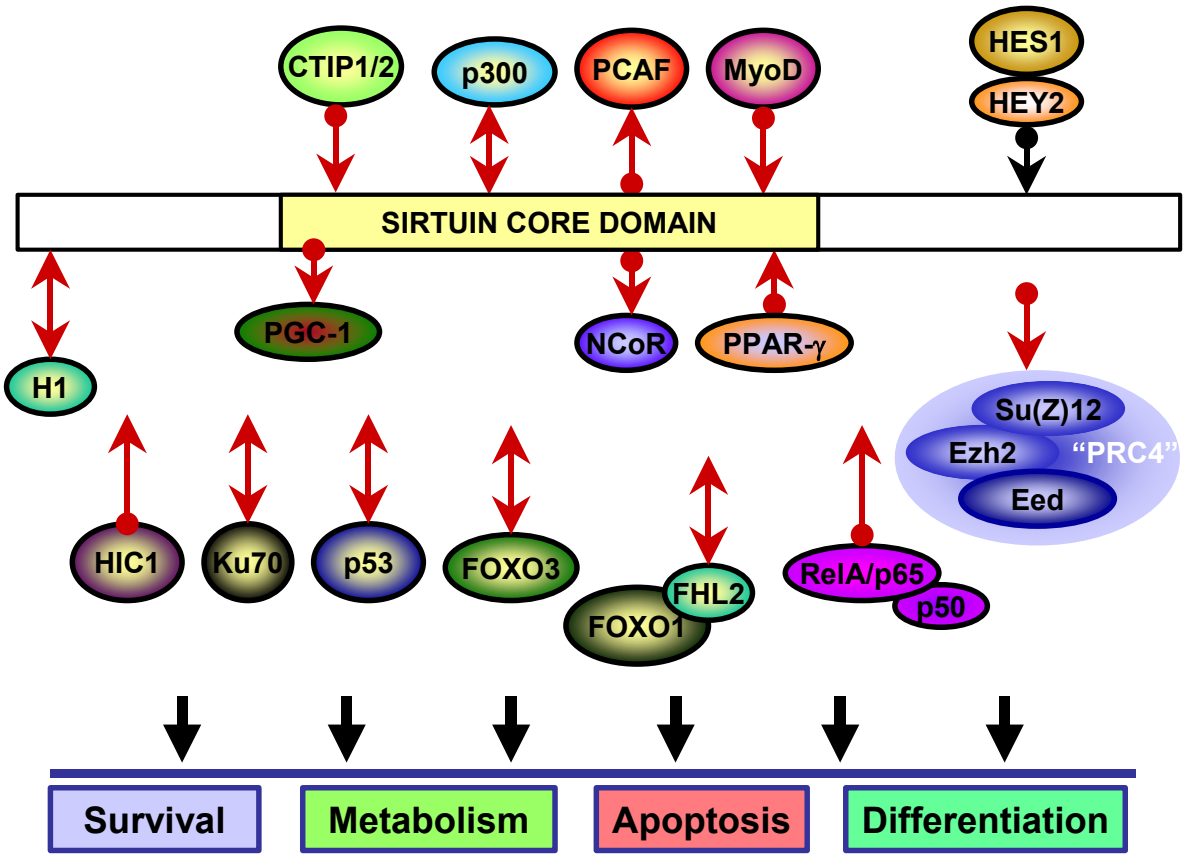


FIGURE 5-2. Schematic summary of reported SIRT1 interacting partners. Directionality of arrows indicates which protein has been used as a bait and which was isolated as pray (arrowhead) in interaction studies. Associations that have been shown to occur directly (by *in vitro* binding assays) are indicated by direct contact of the arrow to SIRT1 and their positioning in relation to the protein domains represents the mapped interaction region in approximation.

5.2.1 Sirtuins and the regulation of organismal life-span

Sir2 has been proposed to be a factor determining longevity in *S. cerevisiae* as well as *C. elegans*. Furthermore, Sir2 mediates life-span extension in response to nutrient limitation. This is based on experimental evidence demonstrating that the presence of Sir2 is required for life-span extension in calorically restricted yeast and worm while overexpression of the protein suffices to increase life-span in a manner not-additive to caloric restriction itself (Guarente 2005; Tissenbaum and Guarente, 2001; Lin *et al.*, 2000).

Various theories have been put forward to explain the role of sirtuins in longevity. Initially it was proposed that the suppression of recombination at the rDNA locus by Sir2p attenuates the formation of ERCs (extrachromosomal rDNA circles) which normally accumulate in mother cells and correlate with yeast ageing but also are suppressed in calorically restricted cells (Lin *et al.*, 2000; Sinclair, 1998; Sinclair and Guarente. 1997). Interestingly, increased recombination rates are observed in ageing yeast cells suggesting a link between genomic stability and life-span which extends beyond the rDNA locus (McMurray and Gottschling, 2003). In addition, preferential segragation of oxidatively damaged proteins in mother cells has also been implicated in yeast ageing and shown to depend on Sir2p (Aguilaniu *et al.* 2003).

Increased respiration and changes in cellular NADH or nicotinamide levels have all been proposed to influence Sir2 activity and contribute to its involvement in caloric restriction (Lin *et al.*, 2004; Anderson *et al.*, 2003; Lin *et al.*, 2002; Anderson *et al.*, 2002). Recent evidence, however has challenged the exclusive role of Sir2 contribution in longevity showing that *TOR1* and *SCH9* deletions can increase yeast life-span in the absence of *Sir2* (Kaeberlein *et al.*, 2005; Kaeberlein *et al.*, 2005). Moreover, caloric restriction fails to increase life-span in yeast strains carrying *TOR1* and *SCH9* deletions indicating a major contribution of these proteins in this process (Kaeberlein *et al.*, 2005). This clearly demonstrated that, at least in yeast, there are Sir2-independent pathways that mediate the effects of nutrient limitation on longevity. These pathways involve the kinases Tor1, a well-established sensor of nutrient availability in yeast and mammals (Jacinto and Hall, 2003) and the yeast orthologue of mammalian PKB kinase SCH9.

Interestingly, TOR regulates ribosomal biogenesis through the PKA pathway. TOR modulates PKA subcellular localisation to maintain the donwstream kinase YAK1 inactive.

TOR inhibition leads to activation of YAK1 which in turn phosphorylates the transcriptional co-repressor CRF1. CRF1 then transclocates to the nucleus and competes with the co-activator IFH1 for binding to the ribosomal gene transcription factor FHL1 thus repressing ribosomal gene transcription (Martin *et al.*, 2004). In addition TOR mediates ribosomal biogenesis by regulation of rDNA transcription through the transcription factors UBF and TIF1A (Mayer *et al.*, 2004).

PKA is also involved in the glucose sensing pathway and PKA mutations mimic caloric restriction to extend life-span in a manner dependent on Sir2p (Lin *et al.*, 2002). Furthermore, SIRT1-mediated deacetylation of the RNAPolI transcription factor subunit TAF_I68 represses ribosomal biogenesis. Thus, TOR and Sir2, both of which are proposed to function as nutrient sensors, regulate life-span and ribosomal biogenesis in opposite ways and are genetically linked to PKA which also responds to nutrient availability. It would hence be of interest to investigate whether TOR and Sir2 genetically interact to regulate ribosomal biogenesis and whether the later process is an active determinant of life-span or simply correlates to proliferation rates associated with ageing.

In *C. elegans* mutations in the IGF signaling pathway regulate life-span in a manner dependent on the forkhead transcription factor daf-16 (Tissenbaum and Guarente, 2002). A genetic interaction between Sir-2.1 and the IGF-signaling pathway has been reported. In particular, life-span extension induced by increased Sir-2.1 expression dependents on the forkhead transcription factor daf-16 which is under the negative regulation of the IGF signaling pathway (Wang and Tissenbaum, 2006; Tissenbaum and Guarente, 2001). This provided evidence that sirtuins may mediate organismal longevity in multicellular organisms albeit by a different, or as-yet unidentified common, mechanism than in yeast. Consequently, the quest to identify a similar function in human sirtuins was pursued.

5.2.2 Mammalian sirtuin function in diseases associated with ageing

5.2.2.1 Molecular pathways involved in ageing are modulated by SIRT1

SIRT1 is considered to be the functional homologue of Sir2p since the core domain of SIRT1 could substitute for that of Sir2p in silencing functions (Sherman *et al.*, 1999). Furthermore, SIRT1 shares characteristic N- and C-terminal extensions flanking the sirtuin core domain with Sir2p. These protein regions are almost absent in other mammalian family members (FIGURES 2-3 & 2-19; Frye, 2000).

Oxidative stress induced by standard cell culture conditions is thought to be the underlying cause of replicative senescence in cultured primary mouse fibroblasts (Parrinello *et al.*, 2003). Unexpectedly, under these conditions SIRT1 regulates replicative life-span by controlling the levels of the tumour suppressor $p19^{ARF}$ which functions to activate p53 leading to senescence (FIGURE 5-3) (Chua *et al.*, 2005; Sherr, 1998). Importantly, this function is distinct from oncogene-induced senescence since activated Ras can induce $p19^{ARF}$ in the absence of SIRT1 (Chua *et al.*, 2005).

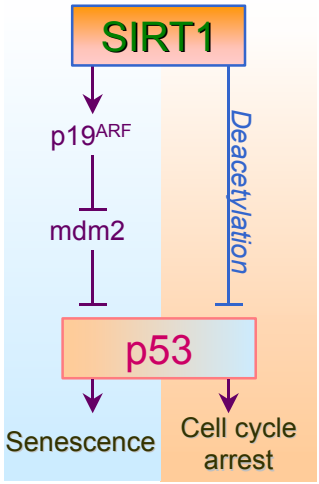


FIGURE 5-3. Dual mode of p53 regulation by SIRT1. *See* text for details.

Until recently, only correlative evidence suggested a link between cellular senescence and organismal life-span. In particular the levels of the p16 tumour suppressor, a positive regulator of the retinoblastoma (pRb) protein, and senescence-associated β-galactosidase (SA-β-gal) were found to be increased during normal ageing (Campisi, 2005). Recent work in mice has provided experimental support to this notion. In a conditional mouse model, Keyes *et al.* showed that abblation of the p53-related protein p63 caused a premature ageing phenotype in mice while at the same time primary keratinocytes lacking p63 show increased senescence in culture (Keyes *et al.*, 2005).

This evidence would imply that the observed effect of SIRT1 in mouse fibroblasts may reflect a similar role at the organismal level, i.e. of SIRT1 as a promoter of ageing. In agreement to this, there is no epidemiological support for a role in *SIRT1* gene variations and increased human longevity (Flachsbart *et al.*, 2006). The perinatal lethality of $SIRT1^{-/-}$ precludes

their use as a model to probe the contribution of SIRT1 on ageing. Interestingly, adipose-specific ablation of IGF receptor results in a ~18% increase in life-span in mice suggesting that organismal life-span can be influenced by the function of certain pathways in specific tissues (Bluher *et al.*, 2003). Thus, conditional deletion of SIRT1 would provide an important tool for resolving its roles in mammalian biology and in particular ageing.

5.2.2.2 Molecular basis of SIRT1 function in ageing phenotypes

In our view of SIRT1 as a regulator of mammalian life-span, there are two ideas to be taken into consideration. Firstly, the evolutionary theories of ageing would suggest that there need not be a single gene or a common mechanism of life-span determination throughout the species (Kipling *et al.*, 2004; Kirkwood, 1996). Secondly, the multiplicity of mutations giving rise to progeroid (premature ageing) syndromes and the wide spectrum of associated phenotypes would support the notion that multiple factors contribute to the ageing process which ultimately determines life-span (Kipling *et al.*, 2004).

In elderly people as well as progeroid syndrome patients and mouse models of aging common phenotypic manifestations include reduced body weight, reduced fecudity, higher cancer incidence, lordokyphosis (curvature of the spine), decreased bone density, decreased organ mass, cellular senescence, alopecia (balding), increased skin ulcerations and atrophy, decreased dermal and adipose thickness, and sebaceous gland hyperplasia (Lombard *et al.*, 2005; Keyes *et al.*, 2005). Werner syndrome (WS), a human autosomal genetic disorder provides a characteristic example of the above. Werner syndrome patients suffer from abnormal stature, atherosclerosis, graying of the hair, type II diabetes, cataracts and osteoporosis, all of which are diseases more prevalent with ageing (Kipling *et al.*, 2004).

Based on the above, in our analysis of SIRT1 as a modulator of mammalian life-span it would be more meaningful to consider its potential functional role in alleviating age-related phenotypes rather than view it as an intrinsic genetic determinant of longevity. This is also a relevant approach when it comes to exploiting sirtuins as drug targets to treat ageing-related symptoms with the aim to ameliorate the life of the elderly.

5.2.2.2.1 Neuroprotective and cardioprotective roles of SIRT1

Immunohistochemical studies have identifed the heart and central nervous system as sites of high murine SIRT1 expression during emryogenesis and in adult animals (Sakamoto *et al.*, 2004). Furthermore, mice with genetic ablation of the *SIRT1* locus exhibit multiple developmental defects some of which are consistent with this localisation (Cheng *et al.*, 2003). Experimental evidence suggests that SIRT1 has a protective role against neuronal and cardiac damage.

In the Wallerian degeneration slow (w*ld*^{s}) mice increased nuclear NAD^{+} underlies the protection exhibited in the neurons of these mice against neurodegenerative agents. Importantly, the neuroprotective effects of NAD^{+} require SIRT1. Moreover, sirtinol and resveratrol, two compounds that inhibit and activate SIRT1 respectively affect this process in a manner consistent with the proposed involvement of SIRT1 (Araki *et al.* 2004). Similar effects of sirtinol and resveratrol were also observed in an independent *in vitro* model of cerebral ischaemia (Raval *et al.*, 2005).

These observations also extend to the heart. In isolated neonatal rat cardiomyocytes sirtuin inhibition by either nicotinamide or sirtinol induced cell death in a p53-dependent manner. SIRT1 overexpression also caused an increase in cardiomyocyte size and protected cells from apoptosis following serum starvation (Alcendor *et al.*, 2004). Importantly, SIRT1 levels were dramatically increased in a dog model of heart failure possibly a result of a failed attempt to prevent cell death (Crow, 2004).

Risk of neurodegenerative and cardiovascular pathological conditions such as Alzheimer's and ischaemic heart disease respectively increase dramatically with age and together account for the vast majority of death rates (Hadley *et al.*, 2005; Kirkwood, 1996). A causative role for the devastating outcomes of ischaemic conditions is also connected to how promptly they are treated so that to minimise tissue damage. This, in combination with life-style factors such as diet, which is proposed to affect sirtuin function, render sirtuins an important potential target for preventive treatments in the context of these diseases.

5.2.2.2.2 SIRT1 and muscle mass maintenance

Multiple factors contribute to muscle mass reduction during ageing and disease states such as cancer or muscular dystrophy. These include oxidative and inflammatory damage due to neutrophil and macrophage recruitment as well as muscle wasting due to increased protein catabolism (Mourkioti and Rosenthal, 2005; McKinnell and Rudnicki, 2004).

Balanced protein turnover is paramount for the maintenance of proper muscle mass (McKinnell and Rudnicki, 2004). Increased protein catabolism associated with cachexia is attributed to the action of specific E3 ubiquitin ligases that target proteins for proteasome-mediated proteolysis. Two such proteins have been identified, MuRF1 (for muscle RING finger 1) and MAFbx/atrogin-1 (for muscle atrophy F-box) which are transcriptionaly controlled by the NFκB and FOXO pathways respectively (Glass, 2005).

IGF-1 has a pleiotropic role in protecting muscle cells from degeneration and contributing to muscle repair (Mourkioti and Rosenthal, 2005). This includes inhibition of FOXO transcription factors to prevent MAFbx expression and protein degradation (FIGURE 5-4). Conversely, inflammatory cytokines such as TNFα induce the NFκB pathway to cause the opposite effect (Glass, 2005).

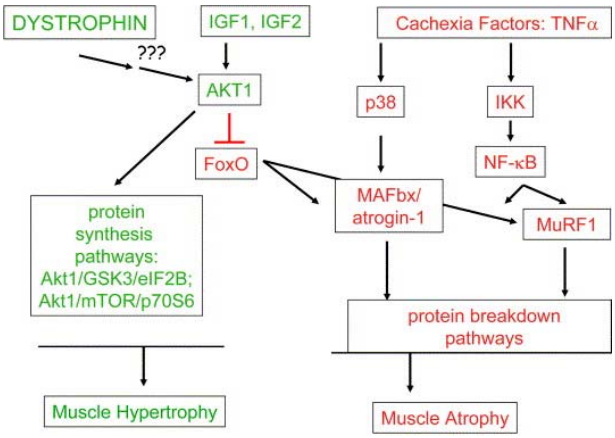


FIGURE 5-4. Signaling diagram illustrating skeletal muscle hypertrophy and atrophy signaling pathways. Modulators that increase skeletal muscle mass are in green; proteins that mediate the loss of muscle mass and that are activated during atrophy are in red. *See* text for further details.

Adapted from Glass, 2005

SIRT1 has been shown to act as a negative regulator of both TNFα-induced NFκB activity and of several forkhead family transcription factors including FOXO1, FOXO3a and FOXO4 (Yang *et al.*, 2005; Yeung *et al.*, 2004; Motta *et al.*, 2004; Brunet *et al.*, 2004; Daitoku *et al.*, 2004; van der Horst *et al.*, 2004). The transcriptional activities of FOXO target genes are heterogeneous including both activation and repression through an N-terminal domain (Yang *et al.*, 2005; Ramaswamy *et al.*, 2002). SIRT1 is thought to enhance the expression of FOXO target genes involved in stress responses and cell cycle arrest such as *MnSOD*, *GADD45* and *p27* (Brunet *et al.*, 2004; Daitoku *et al.*, 2004; van der

Horst *et al.*, 2004) while it suppresses transcription of some IRS (insulin response element)-driven genes (*IGFBP1* and *PEPCK*) (Motta *et al.*, 2004). Similarly, SIRT1 deacetylates the p65 subunit of NFκB to inhibit its transactivation potential and SIRT1 activation by resvertrol inhibits NFκB-mediated antiapoptotic gene expression leading to enhanced cell death upon TNFα treatment (Yeung *et al.*, 2004).

Moreover, SIRT1 deacetylase activity negatively modulates the transcription factor MyoD which is one of the key executors of the muscle differentiation programme. SIRT1 activity in turn is dictated by the progressively decreasing levels of NAD^+ during muscle differentiation thus alleviating SIRT1-mediated MyoD suppression and allowing differentiation (Fulco *et al.*, 2003). Interestingly, in response to TNFα, *MyoD* gene expression is suppressed due to NFκB activation while MyoD is also proposed to be a substrate of the MAFbx E3 ligase (Glass, 2005).

Based on the above an attractive model could be proposed where SIRT1 represses MAFbx and MuRF expression by deacetylating and inhibiting FOXO and NFκB activity respectively to prevent muscle mass loss (FIGURE 5-5). This scenario is seemingly contradictory to the finding that SIRT1 behaves as an inhibitor of myogenesis in the context of *in vitro* muscle differentiation models, yet this need not be the case.

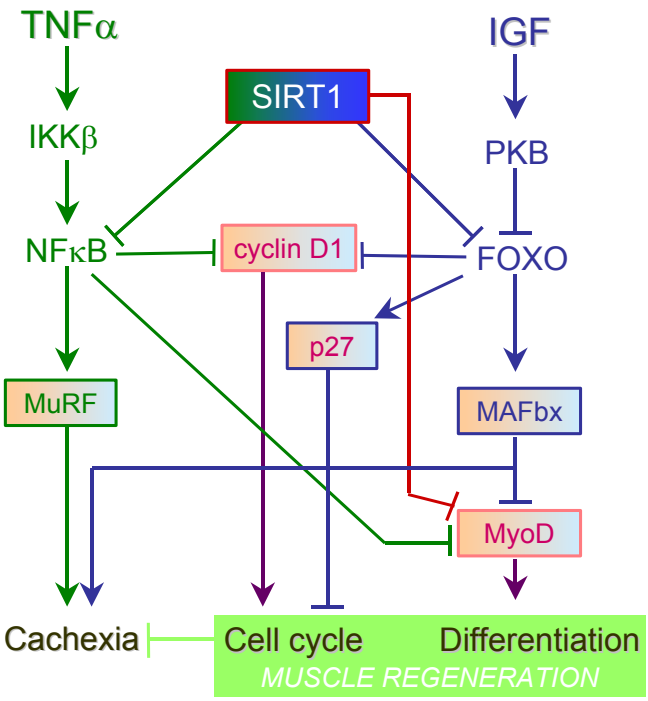


FIGURE 5-5. Model of SIRT1 influence on muscle homeostasis. *See* text for details

Following injury, muscle regeneration involves proliferation of satellite cells prior to their terminal differentiation (Mourkioti and Rosenthal, 2005). Inhibition of MyoD-mediated differentiation by SIRT1 may serve to promote proliferation in this context until sufficient numbers of cells have been generated to replenish the ones lost by injury. This may be assisted by concomitant alleviation of cell cycle inhibition by FOXO (*see* FIGURE 5-4). Alternatively, SIRT1

may promote MyoD mRNA and protein stability through inhibition of FOXO and NFκB as delineated above. Furthermore, NFκB inhibition by SIRT1 may also limit inflammatory cytokine production in macrophages thus mitigating the effects of inflammation-induced damage in the muscle.

Evidently, these interconnections are derived from SIRT1 interactions observed in a variety of heterologous experimental settings and is not clear whether it has a physiological function in the animal. However, they would provide a reasonable starting model for further investigation of exploiting SIRT1 as a therapeutic target for ageing and cancer-associated muscle atrophy.

5.2.2.2.3 SIRT1 functions in metabolic regulation

SIRT1 in homeostatic organ functions regulating glucose metabolism

Ageing is associated with several pathological conditions affecting metabolic functions of the body. Prevalent among them is type 2 diabetes which shows almost an exponential incidence rate increase after the age of 20-30 years (Moller *et al.*, 2003). Type 2 diabetes is associated with decreased insulin secretion or the development of insulin resistance which in turn is a major risk factor for cardiovascular disease and a series of other medical conditions collecively known as the metabolic syndrome (de Luka and Olefsky, 2006).

In response to elevated glucose levels e.g. following a meal, insulin is secreted by the β-cells found in the pancreatic islets of Langerhans to promote glucose uptake and catabolism in peripheral tissues. β-cell function deteriorates with ageing (Moller *et al.*, 2003).

SIRT1 is expressed in the pancreatic β-cells as well as the glucagon-producing α-cells (Moynihan *et al.*, 2005; Bordone *et al.*, 2005). In β-cells SIRT1 enhances insulin secretion by suppressing the expression of uncoupling protein 2 (UCP2). UCP's uncouple metabolic fuel oxidation from ATP production thus leading to a decrease of ATP which is required for the secretion of insulin (Lowell and Spiegelman, 2000). Consistent with a physiological role of SIRT1 in insulin secretion, mice overexpressing SIRT1 in pancreatic β-cells exhibited an enhanced response to glucose challenge attributed to higher blood insulin, while conversely, *SIRT1*$^{-/-}$ animals had lower levels of circulating insulin (Moynihan *et al.*, 2005; Bordone *et al.*, 2005).

Furthermore, SIRT1 appears to have a protective role against glucose-induced cytotoxicity in pancreatic β-cells, an underlying cause of β-cell degeneration seen in diabetic patients whose plasma glucose levels are high. The cytotoxic effects of increased glucose conentrations are attributed to elevated mitochondrial oxidation rates which lead to increased ROS production. Under these conditions, SIRT1 was shown to be required in sustaining FOXO1-mediated transcription of *MafA* and *NeuroD*, two transcription factors required for the expression of the *Insulin 2* gene to prevent apoptosis (Kitamura *et al.*, 2005).

Another site of SIRT1 function in glucose metabolism is the liver. SIRT1 interacts with the co-activator PGC-1α to induce gluconeogenesis in response to fasting (Rodgers *et al.*, 2005). This is rather the opposite role compared to the one it plays in β-cells where SIRT1 essentially functions to promote glucose utilisation. However, the strong expression of the protein in the α-cells may also be similar to its hepatic function, although nothing is known about that yet and it is possible that the different functions can solely be attributed to the proteins SIRT1 partners with to perform the respective functions.

The above evidence suggests a multiple impact of SIRT1 function on body glucose homeostasis through its role in insulin secretion and contribution to survival in the context of pancreatic β-cells and gluconeogenesis in the liver. Interestingly, SIRT3, a mitochondrial sirtuin, was shown to be downregulated in the skeletal muscle of a mouse model of diabetes (Yechoor *et al.*, 2004). Whether this underlies a concerted function of sirtuins in metabolic regulation (*see* section 5.7) or, indeed, the function of SIRT3 in mitochondira, remain to be elucidated.

Thus the activity of SIRT1 and possibly other sirtuins may also be a relevant therapeutic target for diabetes, where aberrant glucose homeostasis and β-cell disfunction are key manifestations of the disease.

SIRT1 function in lipid metabolism

Another tissue of interest in metabolic regulation is the adipose tissue. The adipose tissue is the major site of triglyceride storage, an important energy source when glucose availability is limited. During fasting and starvation, adipose triglyceride (TG) stores are mobilised to give rise to free fatty acids (FFA) which can be utilised by other tissues for

energy production (Rosen *et al.*, 2002). Furthermore, the metabolic activities of adipose tissue may have an impact on organismal longevity since adipose-specific ablation of IGF receptor results in a ~18% increase in life-span in mice.

SIRT1 was shown to suppress adipocyte differentiation by inhibiting the adipogenic factor PPARγ through the transcriptional co-repressor NCoR (Picard *et al.*, 2004). Furthermore, it was suggested that SIRT1 is required for TG mobilisation as *SIRT1*$^{+/-}$ animals exhibited low levels of blood FFAs following fasting or β-adrenergic stimulation.

Interestingly, the bacterial sirtuin orthologue CobB deacetylates and activates the enzyme acetyl-CoA synthase (ACS) which catalyses the synthesis of acetyl-CoA, a key molecule in mitochondrial oxidation and lipid synthesis (Starai *et al.*, 2004; Starai *et al.*, 2002). Thus it is possible that in higher organisms too SIRT1 or another sirtuins may affect lipid metabolism by mediating intracellular acetyl-CoA levels.

Dyslipidemia is a common feature of the metabolic syndrome-associated disorders including cardiovascular disease and atherosclerosis. Thus it would be of interest to investigate whether modulation of SIRT1 activity can be considered as a possible target for treating these conditions or symptoms thereof.

5.2.2.2.4 Reproduction

There is an inverse correlation between age and fecundity in that as animals age their ability to produce viable offspring decreases (Partridge and Gems, 2002). Experimental evidence in model organisms suggests that a hormonal cue from the reproductive system regulates life-span in both the nematode and the mouse.

In *C. elegans*, ablation of germline precursors leads to an approx. 60% life-span extension. Interestingly this is associated with an apparent loss/reduction of IGF signaling in the intestine and adipose tissue since in these animals daf-16 translocates to the nucleus (Kenyon, 2005). In the mouse, transplantation of ovaries derived from young animals to old animals results in 40-60% increase in life-span. As the transplantation process kills the germ cells, these animals have only a somatic gonad, a situation that parallels that in worms described above (Kenyon, 2005).

Despite this apparent evolutionary conservation, there are examples of animals that do not comply with this relationship between reproductive capacity and life-span. This is the case for the honey bee (*Apis mellifera*). Honey bee populations have a long-lived queen cast with a reproductive role and a short-lived cast of sterile workers. Both queens and the long-lived worker bees that remain in the hive to tend the larvae express higher levels of the vitellogenin protein compared to the animals that leave the collony to collect honey (Seehuus *et al.*, 2006). Vitellogenin is controlled by the IGF signaling pathway in *C. elegans* and, consistent with that, reduction of vitellogenin protein levels results in increased life-span in the worm. In contrast, in the honey bee, despite its beneficial role in reproduction by equiping the yolk with nutrients, the protein confers resistance to oxidative stress thus extending life-span (Seehuus *et al.*, 2006).

SIRT1 is highly expressed in the developing spermatocytes and deletion of the *SIRT1* gene leads to severe sperm abnormalities and sterility (McBurney *et al.*, 2003). In this context SIRT1 would apper to be important to the reproductive capacity of the animal thus defying its role in longevity based on the above. Interestingly, reduction of IGF signaling starting at the time of hatching in *C. elegans* extends life-span and delays reproduction whereas IGF signaling reduction in the adult increases life-span to the same extend without affecting reproduction (Kenyon, 2005). Thus it is possible that SIRT1 holds a role in the adult reproductive system that extends beyond embryonic development, possibly by controlling IGF signaling through its documented roles in regulating forkhead factor activity.

5.2.2.2.5 SIRT1 and cancer

Cancer is a term coined to describe a vastly heterogeneous set of diseases that are characterised by aberrant proliferation at the expense to physiological body functions, which, when untreated, leads to death. Thus, although malignansies occur in younger individuals too, sporadic cancer incidence shows a striking correlation with age (DePinho, 2000).

Several genetic and epigenetic processes have been linked to age-induced cancer and mouse model studies have provided extensive evidence that genes involved in the maintenance of genomic stability and cancer are also intimately linked to the development of ageing phenotypes (Lombard *et al.*, 2005; DePinho, 2000).

Perhaps one of the most striking recent molecular links between cancer and ageing was the discovery of an allele of the p53 tumour suppressor which correlates with high p53 activity and confers resistence to tumourigenesis but induces the onset of premature ageing due to increased IGF signaling (Levine *et al.*, 2006; Campisi, 2004; Maier *et al.*, 2004; Tyner *et al.*, 2002). This and other evidence provided support to the notion that ageing is a by-product of an organism's mechanisms for tumour suppression (Ferbeyre and Lowe, 2002).

Furthermore, recent work has identified interlinks between nutrient signaling and pathways involved in cancer development such as the activation of p53 by the AMPK kinase to co-ordinate cell cycle progression with the energy status of the cell (Jones *et al.*, 2005). Both the AMPK and IGF signaling pathways are upstream regulators of the mTOR pathway which plays a paramount role in regulating cell growth and mutations in components of these pathways are frequently found in cancers (Wullshleger *et al.*, 2006 and section 5.1).

Whether SIRT1 has a causative role in tumour development is not known as no studies reporting SIRT1 mutations in cancer have emerged to-date. However, several lines of evidence indicate that SIRT1 may regulate various signaling pathways known to be involved in cancer.

SIRT1 and epigenetic changes occuring in cancer

SIRT1 has been shown to have multiple effects on histone modifications. siRNA-mediated downregulation of SIRT1 leads to H4-K16 hyperacetylation, and reduction in H3-Tri-MeK9 and H4-MeK20 in mammalian cells while *in vitro* SIRT1 preferentially deacetylates H4-K16 (Vaquero *et al.*, 2004; Imai *et al.*, 2000). Interestingly, Fraga *et al.* reported a consistent loss of H4-K16 and H4-Tri-MeK20 in various tumours and tumour-derived cell lines suggesting that these modifications consistute epigenetic hallmarks of cancer (Fraga *et al.*, 2005).

SIRT1 was also identified as a component of the polycomb repressive complex 4 (PRC4) which harbours the SET domain histone methyltransferase Ezh2 (Kuzmichev *et al.*, 2005). Four distinct PRC complexes have been identified to-date that differ in their composition and sustrate specificity. In a mouse model of prostate cancer, the protein levels of all PRC4 components tested, including SIRT1, were upregulated. Concomitantly,

expression of PRC4 target genes was accordingly modified in these tissues. Although there is no evidence confirming a causal role for PRC4 in cancer initiation, it is possible that PRC4-mediated histone modifications contribute to cancer-spicific epigenetic changes.

In addition, in the context of the PRC4 complex, SIRT1 deacetylates histone H1-K26 and promotes heterochromatin formation through spreading hypometylated histone H3-K79 (Kuzmichev *et al.*, 2005; Vaquero *et al.*, 2004). Histone H1 is a linker histone which has primarily a structural role in maintaining chromatin structure and through this regulates genomic stability and ageing (Harvey and Downs, 2004). Furthermore, linker histone H1.2 has also been implicated as a signal transmitter of apoptosis from the nucleus to the cytoplasm (Konishi *et al.*, 2003). Elucidation of the functional significance of SIRT1 in histone modifications is likely to provide further insights into how it may contribute to cancer phenotypes.

A focused effort to develop HDAC inhibitors has been long underway following the ralisation that many cancers and in particular leucemias, exhibit aberrant acetylation (Marks *et al.* 2001). In this respect, SIRT1 joins its kins as a potential target for cancer therapy, although clearly, more has to be known about its exact function in the disease to validate this prospect.

Non-histone targets of SIRT1 in tumour development

Acetylation of transcription factors correlates, in general, with increased transcriptional activity mainly due to enhanced binding to DNA (Kouzarides, 2000). SIRT1 deacetylates several transcription factors involved in the regulation of cell cycle progression and apoptosis consistent with a role in the fundamental processes underlying cancer.

SIRT1 deacetylates the tumour suppressor p53 to inhibit its transcriptional acivity resulting in reduced apoptosis in response to various genotoxic stimuli (Luo *et al.*, 2001; Vaziri *et al.*, 2001). Furthermore, SIRT1 localises to PML bodies to attenutate p53-dependent cellular senescence induced by PML-IV overexpression (Langley *et al.*, 2002).

SIRT1 associates with the tumour suppressor HIC1 which has been shown to act synergistically with p53 in tumourigenesis. Both SIRT1 and HIC1 can bind the SIRT1 promoter to repress gene transcription and thus allow p53 acetylation and activation (Chen *et al.*, 2005). *HIC1*$^{-/-}$ cells exhibit elevated levels of SIRT1, hypoacetylated p53 and enhanced

resistance to DNA-damage induced apoptosis which can be reversed by expression of dominant-negative SIRT1. Interestingly, *HIC1* expression is progressively reduced during ageing due to promoter methylation which would lead to higher SIRT1 levels, reduced p53 activity and prolonged life-span. However, such a model would also predict a higher propensity to form tumours due to higher SIRT1 activity consistent with the inverse relationship between longevity and tumour suppression discussed above.

SIRT1 deacetylates and inactivates another transcription factor NFκB leading to enhanced cell death in response to the inflammatory cytokine TNFα (Yeung *et al.*, 2004). NFκB is required for the transcription of growth factors and cytokines involved in inflammation which has been linked to several diseases including type-2 diabetes and cancer (Karin *et al.*, 2006). Other NFκB target genes include antiapoptotic factors such as cIAP and selective members of the Bcl2 family and the antioxidant MnSOD which protects cells from TNFα-induced apoptosis.

MnSOD is also a target of forkhead transcription factors yet SIRT1 enhances its expression in a FOXO dependent-manner (Giannakou and Partridge, 2004). Forkhead factors regulate genes that are involved in cell cycle arrest and survival and due to their negative regulation by the PKB pathway which is frequently hyperactivated in cancers they are thought to contribute to tumour suppressor. Interestingly, FOXO is under the negative control of IKKβ too, which targets it for ubiquitin-mediated proteolysis (Hu *et al.*, 2004). IKKβ is also required for NFκB activation implying a cross-talk between these two pathways. How SIRT1 co-ordinates the activities of these two factors in a coherent manner remains to be investigated. This will be particularly informative with respect to cancer development as the effects of SIRT1 through these two transcription factors can be seemingly conflicting as in the case for MnSOD and cell survival.

The recent discovery that Ku70 interaction with the pro-apoptotic protein Bax is regulated by acetylation can provide an alternative route by which SIRT1 promotes cancer cell survival. SIRT1-mediated deacetylation of Ku70 preserves its association with Bax which is inhibited by CBP-driven Ku70 acetylation, thus preventing the translocation of Bax to mitochondria to initiate apoptosis (Cohen *et al.*, 2004).

In relation to another theme in cancer biology, SIRT1 was identified in an enhanced retroviral mutagenesis (ERM) screen for negative regulators of telomerase gene expression

(Lin and Elledge, 2003). High telomerase activity and reduced telomere shortening is one of the hallmarks of cancer thus tumour supressor pathways are thought to actively participate in telomerase gene suppression (Hanahan and Weinberg, 2000). However, subsequent experiments provided conflicting evidence as to the exact role of SIRT1 in telomerase regulation. Yet, this work is noteworthy given the unbiased approach followed to implicate SIRT1 in this fundamental for cancer development process.

Cancer drug resistance is a major hindrance in the effectiveness of cancer therapies. Chu *et al.* observed a positive correlation between SIRT1 protein levels and the expression of P-glycoprotein, a drug efflux pump implicated in cancer multidrug resistance further expanding the proposed ways SIRT1 regulates cancer cell biology (Chu *et al.* 2005).

In addition to these experimentally documented roles of SIRT1 involvement in cancer, it would be possible to predict at least another mode of action (Caron *et al.*, 2005). In the context of pancreatic β-cells SIRT1 leads to FOXO deacetylation and increased transcriptional activity yet the stability of the protein decreases (Kitamura *et al.*, 2005). A correlation between acetylation and stability has also been reported for p53 and HIF transcription factors albeit with different end-effects in each case (Bode and Dong, 2004; Jeong *et al.*, 2002). This may arise from the fact that deacetylation allows the lysine residue involved to be targeted by ubiquitination or other modification as is the case with the transcription factor MEF2 where deacetylation by SIRT1 allows the targeting of the relevant lysine by sumoylation resulting in inhibition of its transcriptional activity (Zhao *et al.*, 2005).

Proteasome-mediated degradation of FOXO is primed by PKB- or IKKβ-dependent phosphorylation. In the case of PKB-mediated phosphorylation, Skp2 is the relevant E3 ligase ubiquitinating FOXO. Skp2 is an oncoprotein and frequently overexpressed in human cancers (Gstaiger *et al.*, 2001). Thus it is potentially credible that SIRT1-mediated deacetylation promotes FOXO ubiquitination by Skp2, contributing to its instability in particular conditions.

5.3 Reported roles of other sirtuins in the determination of life-span

In addition to SIRT1, recent reports suggest that other sirtuins may play a role in the determination of life-span in higher eucaryotes. Epidemiological evidence identified polymorphisms in the SIRT3 gene and gene promoter that occur at higher frequency in long-

lived individuals (Bellizzi *et al.*, 2005; Rose *et al.*, 2003). Given the prominent role of mitochondria in ageing processes (Balaban *et al.*, 2005), a possible role of SIRT3 in this context would not be surprising.

Mouse SIRT6 which was shown to harbour an ADP-ribosyltransferase activity (Liszt *et al.*, 2005), is required for DNA repair by the base-excision repair (BER) pathway thus contributing to genomic stability. Deletion of both *SIRT6* alleles in the mouse results in viable animals which however die soon after birth following the onset of multiple degenerative processes reminiscent of ageing symptoms (Mostoslavsky *et al.*, 2006). Consequently, by supporting genomic stability, SIRT6 may contribute to organismal survival and fitness.

Thus, based on current evidence it appears that multiple sirtuins may contribute to various aspects of organismal physiology to regulate fitness. Consistent with this is the proposal put forward here, namely that, in relation to sirtuin function, ageing should be viewed as a set of phenotypes various aspects of which individual sirtuins may contribute to or indeed alleviate.

5.4 Role of NAD in transcriptional regulation and disease

Changes of NAD^+ intracellular dynamics in disease

Under certain conditions, SIRT1 activity may be regulated by controlling SIRT1 protein levels as is the case in the liver following fasting (Rodgers *et al.*, 2005; Kuzmichev *et al.*, 2005) or in prostate cancer. However, a characteristic feature of sirtuins is the dependence of their enzymatic activity on NAD^+ and their proposed regulation by NADH and/or nicotinamide. Consequently, conditions where cellular NAD^+ levels change may lead to modulation of sirtuin activity.

Changes in intracellular NAD^+/NADH concentrations have been implicated in various conditions such as diabetes-associated retinopathy (Wahlberg *et al.*, 2002; Salceda *et al.*, 1998), cardiovascular disease (Liu *et al.*, 2005), fatty liver disease (You and Crabb, 2004) and neurodegenerative diseases such as Alzheimer's (Jackson *et al.*, 1994). Furthermore, NAD^+/NADH has been shown to decrease during muscle differentiation (Fulco *et al.*, 2003).

A key target of such changes has been thought to be the nuclear enzyme poly-ADP ribose polymerase (PARP). The enzymatic activity of PARP shows stricking similarities with that of sirtuins (FIGURE 5-6). They both modify histones in an NAD^+-dependent manner. Unlike redox enzymes that use NAD^+ as a co-factor, PARPs and sirtuins cleave the glycosidic bond between ADP-ribose and nicotinamide thus consuming NAD^+ irreversibly (Kim *et al.*, 2005). Yet, PARP-1 is considered to be the major consumer of NAD^+ leading to dramatic changes in cellular NAD^+ pools upon its activation following DNA damage (Zhang, 2003). Indeed, in streptozocin-induced type-I diabetes mouse model, genetic ablation of the *PARP1* gene results in reduced β-cell death and protects mice against diabetes (Burkart *et al.*, 1999).

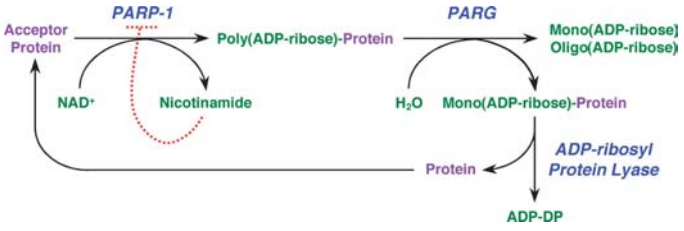


FIGURE 5-6. Dynamics and enzymology of protein ADP-ribosylation. Synthesis and degradation of PAR on an acceptor protein. PARP-1 catalyzes the polymerization of ADP-ribose units from donor NAD^+ molecules on target proteins, resulting in the attachment of PAR. PARG catalyzes the hydrolysis of PAR producing free mono and oligo(ADP-ribose). ADP-ribosyl protein lyase cleaves the final remaining ADP-ribose monomer from the target protein, releasing ADP-3''-deoxypentose-2''-ulose (ADP-DP)

Figure and legend adapted from Kim *et al.*, 2005.

Interestingly, increasing the levels of NPT1, an enzyme involved in the NAD^+ salvage pathway in yeast, leads to enhanced rDNA and telomeric silencing without changing steady-

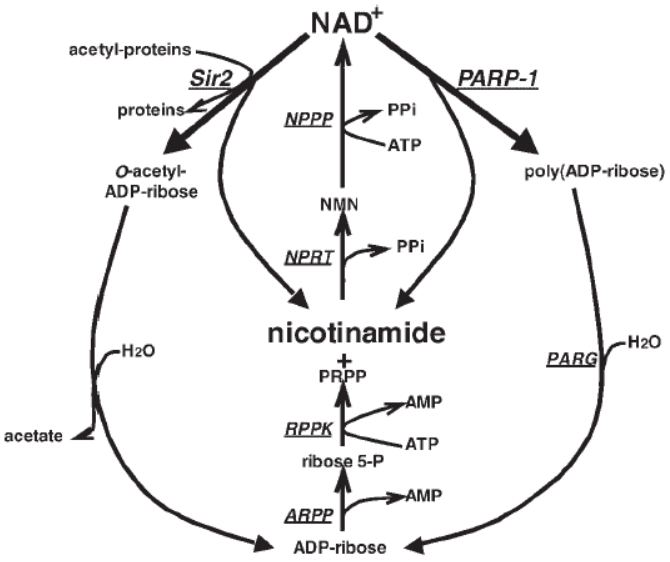


FIGURE 5-7. Interplay between sirtuin and PARP signaling pathways. The depletion of NAD^+ and the increase of nicotinamide by PARP-1 may suppress Sir2 deacetylation activity. PARP-1 and Sir2 share the NAD^+ salvage pathway. The rapid and cyclic turnover of poly(ADP-ribose) by PARG to ADP-ribose results in a drop of NAD^+ and the rise of nicotinamide, both of which are able to directly down-regulate Sir2 activity. The possible linkage of PARP-1 and Sir2 pathways may provide a network that couples energy production, oxidative stress, DNA damage, chromosome remodeling and gene silencing. ARPP: ADP-ribose pyrophosphatase; NAD^+: β-nicotinamide adenine dinucleotide; NPRT: nicotinate phosphoribosyl transferase; PARG: poly(ADP-ribose) glycohydrolase; PARP-1: poly(ADP-ribose) polymerase-1; Ppi: pyrophosphate; PRPP: 5-phosphoribosyl-1-pyrophosphate; RPPK: ribose phosphate pyrophosphokinase.

Figure and legend adapted from Zhang, 2003.

state levels of NAD^+ or the cellular NAD^+/NADH ratio (Anderson *et al.* 2002). This indicates that subtle changes in NAD^+ flux may be sufficient to affect sirtuin activity. Thus given the apparently disproportional relationship between the rates of NAD^+ consumption coupled to nicotinamide production by PARPs and sirtuins, it is likely that increased PARP activity may have an inhibitory effect on sirtuins by depleting local NAD^+ and producing the inhibitor nicotinamide (FIGURE 5-7). Some credence to this hypothesis is provided by the observation that ADP-ribosylation predominates in regions with hyperacetylated histones which could stem from increased local PARP activity coupled to SIRT1 inhibition (Vaquero *et al.*, 2003).

5.5 SIRT1 as a drug target

Nicotinamide and its acid form are collectively known as niacin or Vitamin B_3 (Denu, 2005). Diets poor in Vitamin B_3 and tryptophan which are used for the salvage and *de novo* NAD^+ production pathways respectively, lead to a condition called pellagra (DiPalma and Thayer 1991). As nicotinic acid has no effect on the activity of yeast Sir2 tested (Bitterman *et al.*, 2002), it is thought that any effects of Vitamin B_3 on sirtuins are the result of nicotinamide action.

Nicotinamide has been used as a treatment for some of the conditions mentioned above where cellular redox abnormalities have been known to play a role in pathogenesis (DiPalma and Thayer, 1991). Yet, there is little actual evidence to suggest that the beneficial effects of nicotinamide can be attributed to inhibition of either PARPs or sirtuins.

In vivo administration of nicotinamide or nicotinic acid leads to increased pyridine nucleotide content in kidney, liver, cardiac muscle, adipose tissue and spleen consistent with their role as precursors in cellular NAD^+ biosynthesis (Wahlberg *et al.*, 2002). Thus, whether nicotinamide inhibits directly PARPs/sirtuins or it actually enhances their activity by increasing the influx of NAD^+ anabolic pathways should be further elucidated. This is exemplified in addition by the fact that although a beneficial role for SIRT1 activity has been proposed in cardioprotection, niacin treatment has also a protective effect in the heart against ischaemia (Trueblood *et al.*, 2000).

The considerations presented above also highlight another dilemma burdening the field of sirtuin biology, namely whether pharmacological targeting for therapeutic purposes

should aim at enhancing or inhibiting sirtuin and in particular SIRT1 activity. This is a valid problem for several reasons which will be delineated below.

Model organism data suggest that caloric restriction enhanced Sir2 activity to extend life-span (Guarente and Picard, 2005). Based on this assumption, a chemical screen identified plant polyphenols as potent activators of sirtuins raising the exciting possibility that chemical activation of sirtuins may prolong life-span (Wood *et al.*, 2004; Howitz *et al.*, 2003). Interestingly, one such compound, resveratrol, is found in red wine!

Subsequent experiments into the mechanism of Sir2 activation revealed that the proposed effects of resveratrol are substrate-specific rendering the effects of the compound towards as-yet unidentified sirtuin substrates unpredictable (Kaeberlein *et al.*, 2005; Borra *et al.*, 2005). This was further elaborated by the finding that resveratrol inhibits the Sir-2.1-mediated transcriptional activation of certain genes in *C. elegans* (Viswanathan *et al.*, 2005). It is also of importance to note that other direct effects of resveratrol on distinct biomolecules such as DNA polymerases have been demonstrated (Locatelli *et al.*, 2005).

Clearly, these observations should not prevent a valuable therapeutic application of resveratrol, but they also demonstrate our limited understanding of the underlying mechanism of action. Indeed, resveratrol appers to have a beneficial effect in preventing inflammation, atherosclerosis and carcinogenesis (Locatelli *et al.*, 2005 and references therein). Whether this is due to sirtuin activation remains to be demonstrated.

Some evidence against the use of sirtuin activators is provided by current knowledge of sirtuin function. Sirtuin activation would prevent Bax transclocation to the nucleus by promoting its association with Ku70 conferring a survival advantage to cells (Cohen *et al.*, 2004). Similarly, activation of SIRT1 would lead to increased suppression of p53 activity, also leading to enhanced survival potential. Evasion of apoptosis is one of the hallmarks of cancer (Hanahan and Weinberg, 2000) thus SIRT1 activation in this context would promote tumourigenesis consistent with the relationship between enhanced longevity and cancer discussed in section 5.2.2.2.5.

Furthermore, SIRT1 was shown to partially mediate the transcriptional repression activity of the bHLH factors HES1/HEY2 (Takata and Ishikawa, 2003). HES1-mediated repression is required for progenitor cell proliferation during development because it binds to and represses transcription from the promoter of the CDK inhibitor p27 (Murata *et al.*, 2005). Thus, if

SIRT1 activity also contributes to p27 gene expression, in addition to the proposed regulation of p27 protein stability *via* Skp2-mediated ubiquitination (*see* section 5.2.2.2.5), based on this knowledge, deacetylase activation would also promote cell proliferation.

Conversely, lack of SIRT1 leads to reduced senescence due to suppression of the $p19^{ARF}$/p53 axis (Chua *et al.*, 2005). Evasion of senescence is itself a cancer attribute and in this case SIRT1 is important for accumulation of $p19^{ARF}$ to prevent a transforming phenotype to occur. Clearly, though, the complete physical absence of protein cannot by any means be deemed equivalent to pharmacological manipulation of its activity.

Among other implications, these data would suggest that the functions of SIRT1 may prove to be separable in which case promoting cell survival while preventing the associated tumour-promoting effects would render SIRT1 a suitable therapeutic candidate.

Finally, it is noteworthy to mention that sirtuins have also been implicated in the survival of pathogens such as *Leishmania* where nicotinamide is also effective in limiting their growth (Vergnes *et al.*, 2005; Sereno *et al.*, 2005). This demonstrates that the development of compounds targeting sirtuins would have a broader applicability in promoting human health.

5.6 Emerging nuclear roles of metabolic enzymes and metabolic intermediates

It has been widely believed that the major functional site of action of metabolic enzymes and intermediate metabolites may also reflect the compartmentalisation of metabolic functions such as energy production in the cytoplasm. However increasing evidence suggests that this may not need to be the case.

The first hint defying this notion comes from the fact that several metabolic enzymes have been identified associated with DNA (McKnight, 2003). Hall *et al.* reported that Arg5,6 which is cleaved to give rise to two mitochondrial proteins, Arg5 and Arg6, involved in arginine biosynthesis that bears a close link to nitrogen metabolism (Hall *et al.*, 2004). ChIP experiments identified both mitochondrial and nuclear loci bound by Arg5,6 where it serves to regulate their transcription.

Furthermore, several reports identified proteins that associate with DNA in a manner that is sensitive to the redox state of the cell and in particular NAD^+/NADH levels

demonstrating a role for the latter that extends beyond the obvious regulation of glycolytic and other redox-sensitive enzymes (Liu *et al.*, 2005; McKnight, 2003).

Dioum *et al.* found that the neuronal PAS domain protein 2 (NPAS2), a transcription factor required for the expression of genes involved in the mammalian circadian clock, contains a heme group. Furthermore, its *in vitro* association to DNA was promoted by NADH and under oxidative conditions was hintered (Dioum *et al.*, 2002). This suggested that alterations in neuronal cell metabolism may underlie transcriptional regulation of circadian rythms. Interestingly, the acetylation patterns of histone H3 were found to change in a periodic fashion that correlated with the expression of genes that control circadian rhythmicity (Etchegaray *et al.* 2003).

The C-terminal binding protein (CtBP) co-repressor harbours a NAD^+/NADH binding domain. NADH binding induces a conformational change on CtBP that stimulates its association with transcription factors thus promoting its transcriptional repression activity (Zhang *et al.* 2002). Importantly, an increase in NADH/NAD^+ levels induced under hypoxic conditions or the hypoxia-mimic $CoCl_2$ enhanced the CtBP-dependent transcriptional repression in cells, suggesting that CtBP may couple cellular redox status with transcription.

Yet another example of redox regulation of mammalian transcription is exhibited by the coactivator complex OCA-S which regulates histone H2B gene expression (FIGURE 5-1). Surprisingly, OCA-S comprises at least two metabolic enzymes namely lactate dehydrogenase (LDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In this context, GAPDH is recruited to the H2B promoter during S-phase where histones are required to decorate the newly replicated DNA and this recruitment is essential role for OCA-S-mediated transactivation (Zheng *et al.*, 2003). In contrast to CtBP, association of GAPDH to DNA is favoured by NAD^+ and inhibited by NADH suggesting that either the activity or another subtle effect of pyridine nucleotide binding regulates this distinct nuclear function of a long-known metabolic enzyme.

In addition to the NAD^+-dependence of sirtuins, the distinct nature of the products derived from their enzymatic activity may also serve an as-yet elusive role in gene expression. The Sir2 protein deacetylation reaction yields, apart from the deacetylated protein, nicotinamide and 2',3'-*O*-acetyl-ADP-ribose. The latter product was shown to inhibit

starfish oocyte maturation implying that it may itself have a biological function (Borra *et al.*, 2002).

Interestingly, during its catalytic reaction the recently-identified JmjC-containing histone demethylase JHDM1 utilises iron and α-ketoglutarate to produce formaldehyde and succinate further underlying the involvement of intermediate metabolism products in nuclear activities (Tsukada *et al.*, 2006).

As only preliminary evidence exists to support a specific role or metabolic enzymes on DNA, it is tempting to think that they may constitute further evidence in support of the endosymbiotic theory of eukaryotic cell origin proposed by Lynn Margulis.

5.7 Functional interaction between sirtuins and HDACs

The existence of several deacetylase activities within mammalian cells raises questions about this apparent redudancy. Clearly, the molecular differences between the known deacetylases may account for the specificity of the processes they participate in by means of subcellular localisation and participation in distinct protein complexes. Nevertheless, an additional degree of complexity may be exerted by the combinatorial co-operation between deacetylases of the same or even different class.

At least two examples in the field of sirtuin research corroborate to this. Firstly, SIRT1 co-operates with HDAC4 to regulate the sumoylation and thus transcriptional activity of MEF2, (Zhao *et al.*, 2005). Secondly, the microtubule-associated SIRT2 interacts with HDAC6 and both have been shown to deacetylate microtubules (Hubbert *et al.*, 2002), yet the rationale behind this co-operation is less understood.

Moreover, there are numerous examples where TSA and nicotinamide have additive effects in rescuing protein acetylation (e.g. p53, FOXO, Bcl6) and in some cases as for p53 and FOXO, HDAC1 has also been shown to deacetylate them (Kouzarides, 2000).

Finally, it is conceivable that sirtuins themselves may collaborate to perform some cellular tasks. In yeast, overexpression of the cytoplasmic Hst2 affects the distribution of nuclear Sir2 thus enhancing rDNA silencing while alleviating telomeric silencing (Perrod *et al.*, 2001). The mechanistic details explaining this phenomenon are not clear but some hints of potential ways this can be achieved can be derived from studies of p53. p53 regulates

apoptosis through a nuclear pool that transcribes po-apoptotic genes and a cytoplasmic pool that, when activated, enhances mitochondrial permeability. In unstressed conditions cytolasmic p53 is bound to Bcl-X_L preventing it from activating apoptosis. Following genotoxic stress, nuclear p53 drives the expression of PUMA (for p53-upregulated modifier of apoptosis) which induces the release of cytoplasmic p53 from Bcl-X_L to induce BAX and thus mitochondrial permeability and apoptosis (Chipuk *et al.*, 2005), providing a striking example of functional co-operation between distinct pools of the same enzyme.

5.8 Conclusion

A surge of recent data have implicated mammalian sirtuins and in particular SIRT1 in the regulation of transcription factor activity. These effects are primarily exerted through the deacetylase enzymatic function of SIRT1 which has been widely suspected but only once experimentally demonstrated (Fulco *et al.*, 2003) to be potentially linked to cellular NAD^+/NADH changes. Thus further work would need to address how the NAD^+-dependance of SIRT1 is actually relevant to the transcriptional regulatory activity of the protein. For this, it is important to attain a deeper understanding of the metabolic pathways regulating NAD^+ biosynthesis as well as the role of other NAD^+ utilising enzymes such as PARPs (Zhang *et al.*, 2003). This is also relevant for elucidating the cellular mode of action of known small molecule regulators of SIRT1 such as nitotinamide which is at the same time an important intermediate in the NAD^+ salvage pathway (Lin *et al.*, 2003).

In addition, the existing preliminary evidence for a role of the sirtuin enzymatic product 2',3'-*O*-acetyl-ADP-ribose as a secondary messenger (Borra *et al.*, 2002) supported by the identification of 2',3'-*O*-acetyl-ADP-ribose-metabolising enzymes (Rafty *et al.*, 2002) paves the way for further penetrating into the roles of these metabolites in cellular physiology.

Clearly, despite the plethora of SIRT1 deacetylase targets already identified (FIGURE 5-2), the current approaches towards identifying novel SIRT1 substrates have been in part limited to predictions from sirtuin function in lower organisms. Yet, very little evidence exists to support a functional conservation between yeast Sir2 and mammalian SIRT1 excluding probably the documented actions on histone H1 deacetylation (Vaquero *et al.*, 2004) and its participation in a transcriptional repression complex (Kuzmichev *et al.*, 2005). For example, none

of the Sir2 protein complexes involved in silencing in *S. cerevisiae* have been identified in mammalian cells although the protein complex composition of many evolutionarily conserved proteins such as TOR and URI has been demonstrated to be also preserved throughout evolution (Wullschleger *et al.*, 2006; Gstaiger *et al.*, 2003).

What is more, should sirtuin activity be subjected to targeted pharmacological manipulation, it would be important to establish the interconnections between family members not excluding a detailed characterisation of structural similarities of these proteins as well as to other deacetylases. Clearly, current evidence from gene ablation studies in the mouse suggests that non-redundant functions of individual sirtuin members exist as distinct not compensated for phenotypes are evident (Mostoslavsky *et al.*, 2006; Cheng *et al.*, 2003).

Finally, the limitations in directly measuring intracellular NAD^+/NADH levels and demonstrating the direct link to sirtuin activity remains a great obstacle in studying sirtuin biology in their cellular *in vivo* context. Protein post-translational modifications (PTMs) and in particular phosphorylation constitute a universally applicable regulatory mechanism. Monitoring steady-state PTM levels e.g. by immunohistochemical techniques is indicative of specific signaling pathway activities in the context of tissues and organs, which in turn allows the correlation of signalling fluxes with particular disease states.

No known SIRT1 PTMs have been reported yet. In order to address this need, the work presented in this thesis has focused on the identification of molecular events and pathways with potentially regulatory roles for SIRT1 activity.

5.9 THESIS RESULTS: DISCUSSION AND FUTURE PERSPECTIVES

5.9.1 Regulation of SIRT1 by phosphorylation

5.9.1.1 SIRT1 is a nuclear phosphoprotein

To study the function and cellular localisation of SIRT1 one mouse monoclonal and two rabbit polyclonal antibodies raised against different antigenic regions of the protein were developed. All three antibodies were specific for SIRT1 and were succesfully used in different experimental applications including immunoprecipitation and immunolocalisation (FIGURES 4-1 TO 4-4). Importantly, these antibodies sustain SIRT1 enzymatic activity making them useful for purifying SIRT1 and assaying deacetylase activity *in vitro* (FIGURE 4-5).

A preliminary characterisation of the protein's subcellular localisation using either of the polyclonal antibodies in indirect immunofluorescence microscopy revealed a nuclear, occassionaly speckled, staining pattern (FIGURE 4-2). In contrast mAb12/1 gave a prominent cytoplasmic staining following different fixation/permeabilisation conditions (FIGURE 4.3). SIRT1 has been consistently reported as a nuclear protein (e.g. Vaquero *et al.*, 2004; Langley *et al.*, 2002) while mAb12/1 recognises a single band when used in immunoblotting attesting to its specificity (FIGURE 4-3). Thus, despite the absence of biochemical evidence in support of this, it could be postulated that a fraction of SIRT1 does localise to the cytoplasm but is only revealed with mAb12/1 due to a peculiarity of the antibody allowing it to recognise the cytoplasmic SIRT1 pool. This cytoplasmic SIRT1 pool would be predicted to be much smaller relative to its nuclear counterpart given the inability of the polyclonal antibodies to recognise the latter (FIGURE 4-2). Alternatively, despite its apparent specificity in immunoblotting applications mAb12/1 specificity may be skewed following cell fixation giving rise to an artifactual cytoplasmic signal.

An extensive attempt to compare the focal staining of SIRT1 with other known speckled structures in the nucleus such as lamin-containing replication foci (Kennedy *et al.*, 2000), BRCA1-containing nuclear foci (Scully *et al.*, 1996) and PML bodies (Seeler and Dejean, 1999) have indicated a partial co-localisation with PML bodies consistent with a previous study (Langley *et al.*, 2002), but not with lamin or BRCA1 (data not presented here).

Interestingly, the immunofluorescence signal corresponding to SIRT1 was markedly reduced in mitotic cells (FIGURE 4.6). To test and exclude the possibility that this did not simply result from signal diffusion due to nuclear envelope break-down the protein levels of endogenous SIRT1 were compared in unsynchronous and mitosis-arrested cells by immunoblotting. This approach revealed that SIRT1 migration in denaturing gels is sensitive to λ-PPase attesting to the fact that SIRT1 is a phosphoprotein (FIGURE 4-7). Furthermore, in mitotic cells, SIRT1 in nocodazole-arrested cells exhibited a more prominent electrophoretic mobility retardation relative to that in logarithmically growing cells suggesting the existence of mitosis-specific phosphorylation event(s) in addition to the interphase one(s) (FIGURE 4-7B).

To this end a multidisciplinary approach was followed to determine the interphase-specific as well as the mitotic phosphorylation events which SIRT1 is subjected to.

5.9.1.2 Identification of SIRT1 phosphorylation sites in interphase

Initial experiments revealed that the electrophoretic mobility of Sf9 (insect) cell-expressed recombinant GST-SIRT1 is sensitive to λ-PPase treatment indicating that it is also a phosphoprotein, similarly to endogenous human SIRT1 (FIGURE 4-8A). Interestingly, the *in vitro* deacetylase activity of insect cell-expressed GST-SIRT1 was significantly reduced following λ-PPase treatment (FIGURE 4-8B). As phosphorylation events are often conserved between insect and mammalian cells, this observation indicated that phosphorylation can regulate the enzymatic activity of SIRT1. Supporting this notion, other deacetylases have also been shown to be regulated at multiple levels by phosphorylation (e.g. Zhang *et al.* 2005; Pflum *et al.*, 2001). Thus an in-depth investigation into the identification and functional characterisation of the relevant phosphorylation sites was undertaken.

The phosphorylation sites of insect cell-expressed SIRT1 were determined by tryptic digestion of purified recombinant protein followed by mass spectrometric analysis (FIGURE 4-9). This approach revealed the existence of at least two serines modified by phosphorylation at positions 27 and 47 in the N-terminus of the protein. Individual mutations of either residues to non-phosphorylatable alanine indicated that neither of these were the phosphoshift sites and truncation of the N-terminus of the protein suggested that the phoshoshift site does not reside within the first 217 aminoacids (FIGURE 4-10). Nevertheless further credence to the fact

that these these phosphorylation events are conserved was provided by a study published subsequently and identified the same SIRT1 phosphorylation sites employing a large-scale study of HeLa cell nuclear phosphoproteins (Beausoleil *et al.*, 2004). Importantly, no other SIRT1 phosphorylation sites were reported in the same study.

The yeast sirtuin orthologue Hst2p has been reported to form homotrimers through N-terminal domain contacts. Furthermore, this homotrimerisation event is important for efficient enzymatic catalysis (Zhao *et al.*, 2003). Given the presence of the identified phosphorylation sites in the N-terminus of the protein, the possibility that S27 and/or S47 phoshorylation regulates human SIRT1 homopolymerisation was tested. Co-expression of differentially tagged SIRT1 followed by reciprocal immunoprecipitation confirmed the ability of SIRT for homotypic association (FIGURE 4-11A). Moreover, gel filtration analysis of endogenous SIRT1 in HeLa cells revealed a size distribution pattern consistent with the protein being in a monomeric to trimeric configuration (FIGURE 4-11B). Preliminary experiments to investigate the influence of S27 and S47 phosphorylation on this association gave ambiguous results but further work into this issue is underway.

An interesting observation stemming out of this line of investigation revealed that apart from the previously described putative nuclear localisation sequence (NLS) in residues 233-238, a second NLS must be present, possibly within residues 94-99, as a SIRT1 species lacking the N-terminal 217 aminoacids exhibits an exclusively cytoplasmic localisation (FIGURE 4-12).

In an alternative approach, the λ-PPase-sensitive electrophoretic mobility shift of endogenous SIRT1 was used as an indirect means of identifying the relevant interphase phosphorylation sites henceforth referred to as 'phosphoshift sites'. Sequential screens of truncated SIRT1 species revealed that the phosphoshift site(s) is (are) located within region 612-656 at the C-terminal extension of the protein (FIGURE 4-13A). A SIRT1 species with a deletion of aa's 612-656 showed an electrophoretic mobility which was not sensitive to λ-PPase confirming that the region in question carries the phosphoshift sites (FIGURE 4-13C). However, single mutations of all phosphorylatable aminoacids harboured within this region failed to abolish the λ-PPase-sensitivity of SIRT1 mobility (FIGURE 4-13B) suggesting that more than one sites are responsible for the observed shift.

Alternatively, none of these sites are phosphorylated to cause a mobility shift, rather the region in question is responsible for the recruitment of the relevant kinase which upon deletion of aa's 612-656 would be abolished resulting in the observed behaviour of the protein in FIGURE 4-13C.

To investigate this posibility, either endogenous or transfected SIRT1 was immunoprecipitated and incubated with ^{32}P-γ-ATP to allow its labeling by a putative stably-bound kinase. Although little activity was detected in immunoprecipitates of endogenous protein (FIGURE 4-14A), HA-tagged SIRT1 was strongly labelled with ^{32}P indicating the presence of a co-purifying kinase (FIGURE 4-14B). To test whether the putative co-purifying kinase associates through aa's 612-656 which cause the phosphoshift, the same assay as above was performed in full-length SIRT1 or a species lacking aa's 612-656. Deletion of aa's 612-656 did not abolish ^{32}P incorporation (FIGURE 4-14C) suggesting that the identity of the stably-bound kinase differs from that of the kinase causing the phosphoshift.

Thus, the above experimental approach identified a region in the C-temrinus of human SIRT1 that is required for the phosphorylation of the protein during interphase and a separate phosphorylation event mediated by a stably bound kinase. Further work into this issue will aim at identifying the site(s) of SIRT1 phosphorylation by the co-purifying kinase and probing the nature of the kinases involved. Towards this end, initial attempts to identify SIRT1 co-precipitating proteins by mass spectrometry has been hintered by insufficently pure immunoprecipitates which under the light of the gel filtration data is likely to stem from a very low abundance of such SIRT1-containing complexes (FIGURE 4-11B).

The emergence of data demonstrating that specificity of substrate recognition by kinases is determined by the primary sequence surrounding the phosphorylated residue, led to the development of searchable web-based databases which allow the user to probe for the presence of kinase target sites in a protein of interest. Following this approach one putative site for CK2 kinase was identified at residue S693. Further observation of the C-terminal sequence of the protein revealed at least two other sites (S659 and S661) that complied with the criteria of a CK2 site (FIGURE 4-15A).

In vitro kinase assay using recombinant CK2 cofirmed that all three SIRT1 sites are phosphorylated by CK2. Mutation of all three sites did not affect phosphorylation by the co-precipitating kinase (FIGURE 4-14B) nor did it abolish the λ-PPase-sensitive shift of interphase

cells (FIGURE 4-16). This indicated that, if CK2 phosphorylates SIRT1 *in vivo*, this constitutes yet a third phosphorylation event.

Given the strength of SIRT1 phosphorylation by CK2 *in vitro* it is important to determine whether this also occurs *in vivo*. Several lines of evidence support the value of pursuing this observations further. To begin with, a large-scale two-hybrid analysis of the *Drosophila* proteome revealed a direct association of dSir2 with the the regulatory β subunit of CK2 (Giot *et al.* 2003). Similarly, the entire CK2 complex is a third order interactor of the yeast Sir2 in another large-scale screen (Ho *et al.*, 2002). In human cells CK2 phosphorylates and activates another nuclear deacetylase, HDAC1. Furthermore, this phosphorylation enhances the formation of HDAC1-containing transcriptional complexes (Pflum *et al.* 2001).

For a long time CK2 was thought to be a constitutively active enzyme and its substrate repertoire numbers hundreds of proteins (Meggio and Pinna, 2003; Ahmed *et al.*, 2002). Yet, recent evidence has demonstrated additional roles for this kinase where it acts in an inducible manner (Loizou *et al.*, 2004). In this respect it is also important to note that the specificity of CK2 towards different substrates depends on the context of the protein complex which CK2 participates in. Thus purified CK2 phosphorylates different substrates than when bound to the DNA damage-inducible FACT complex (Keller *et al.* 2001). Also, CK2 phosphorylates components of the RNAPolIII holoenzyme with either inhibitory or activating effects on its transcriptional activity depending on the cell cycle phase (Hu *et al.*, 2004). This may partially explain the apparent *in vitro* promisquity of CK2 towards other proteins but also underlines the fact that despite its apparently mudane cellular tasks, it may perform as-yet unidentified exciting novel functions, in particular through SIRT1 phosphorylation under specific condition and molecular context.

The crystal structure of the Hst2p yeast sirtuin revealed that the C-terminal extension of the protein forms contacts with the NAD^+ binding pocket in the enzymatic active site (Zhao *et al.*, 2003). Intramolecular contact formation is a mechanism commonly employed by enzymes to regulate their activity. The example of Src kinase readily demonstrates this (Harrison, 2003). Phosphorylation of the C-terminal tail of Src leads to recognition and binding of the phosphorylated tyrosine residue by the SH2 domain of th e kinase, inducing a compact inactive conformation of the enzyme (FIGURE 5-8).

If the intramolecular interaction between the C-terminus of Hst2p is also conserved in SIRT1, it could be envisioned that phosphorylation of the C-terminus of the protein leads to dissociation of the C-terminus from the active site rendering it more accessible to substrates. Conversely, the phosphate groups may mimic the phosphates found in NAD^+ and thus promote the interaction of the SIRT1 C-terminal tail with the NAD^+ binding pocket thus competing for NAD^+ binding. This would increase the K_m of NAD^+ for enzyme binding and would consequently modulate the sensitivity of SIRT1 towards cellular redox changes.

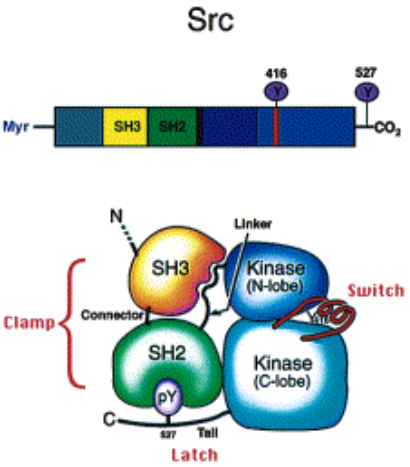


FIGURE 5-8. Intramolecular interactions in Src kinase regulation. Intramolecular interactions mediated by the SH2 and SH3 domains ("clamp") of Src fix the bilobed kinase domain in an inactive conformation. Phosphorylation of SIRT1 in its C-terminal extension may serve a similar role. *See* text for further details.

Adapted from Harrison, 2003

Interestingly, CK2 was also shown to phosphorylate the protein IκB which is a negative regulator of NFκB transcription factor (Kato *et al.*, 2003) similarly to SIRT1 (Yeung *et al.*, 2004). The IκB sites are located within the C-terminal part of the protein (FIGURE 4-17) in a region known as PEST, named after the abundance of P, E, S and T residues it exhibits (Rechsteiner and Rogers, 1996). Upon UV treatment, CK2 phosphorylates several residues in the IκB PEST region in a manner depending on the kinase p38 to target IκB for degradation (Kato *et al.*, 2003). Importantly, this is a distinct mechanism of NFκB activation compared to the one employed upon other stresses such as inflammatory cytokines and ionising radiation in which case an N-terminal phosphorylation event is required (Hayden and Ghosh, 2004). It would thus be conceivable that under particular circumstances, the co-ordinate regulation of both IκB and SIRT1 is orchestrated by CK2 as a determinant of NFκB activity.

This theory was supported by the *in silico* identification of a PEST region in the C-terminus of SIRT1 itself, which encompasses aa's 653-711. Importantly, all three identified *in vitro* CK2 phosphorylation sites are located within this region (FIGURE 4-18). This discovery further validated the CK2-SIRT1 link and prompted the invastigation of a putative role of SIRT1 in the UV reponse.

5.9.1.3 SIRT1 in cellular responses to UV irradiation

Epidemiological as well as experimental studies corroborate to the fact that UV light contributes profoundly in the aetiology of skin cancer (Bode and Dong, 2003). Several kinase signaling cascades have been shown to be activated in response to UV irradiation contributing to a proliferation-like response, possibly reflecting the perceived need to compensate for the damaged tissue (Bode and Dong, 2003).

Jun N-terminal kinase (Jnk) was identified as a UV-inducible kinase by its ability to associate with c-jun (Derijard *et al.*, 1994) similarly to the identification of CK2 as the UV-inducible IκB kinase (Kato *et al.*, 2003). Based on this paradigm and equipped with the knowledge that SIRT1 associated stably with a kinase, the possibility that this kinase is also UV-inducible was investigated. However, although recombinant GST-SIRT1 could co-precipitate kinase activity, this did not appear to be UV-inducible (FIGURE 4-19).

Altered SIRT1 protein turnover in response to UV irradiation

Cycloheximide chase studies indicated that SIRT1 is more unstable following UV irradiation compared to mock treatment in agreement with the model presented above which draws parallels with the regulation of IκB by CK2 (FIGURES 4-20, 4-21). In contrast to this result, further analyses revealed that SIRT1 protein abundance increases in a time- and dose-dependent manner (FIGURE 4-22). Importantly, this increase is independent of the presence of p53 protein which mediates the expression of several genes following UV irradiation (Latonen and Laiho, 2005).

These results, although at first sight conflicting need not be considered as such. Cellular responses to DNA damage evoke cell cycle arrest allowing time for DNA repair to take place and prevent genomic instability. In the case of irrepairable damage apoptosis is elicited. Cellular responses following DNA damage also involve *de novo* gene transcription which for intrinsic reasons requires significantly longer time than the immediate arrest required to prevent engamement into an abberant and possibly detrimental process.

This has been well-documented in the response to ionising radiation. Long-term cell cycle arrest is achieved by transcriptional upregulation of the CDK inhibitor p21 which

depends on the tumour suppressor p53. Immediately after damage though, cell cycle arrest is achieved by proteolytic destruction of cyclin-D which allows the release of p21 from CDK4 and its association to CDK2 leading to cell cycle arrest (Agami and Bernards, 2000). This work clearly demonstrated that the mechanisms employed to implement initiation and maintenance of cellular responses to DNA damage are distinct.

In response to UV irradiation, several transcription factors are activated including p53, NFκB and AP-1 (Bode and Dong, 2003). Yet, SIRT1 appears to inhibit the transcriptional activity of at least p53 and NFκB (Yeung *et al.*, 2004; Vaziri *et al.*, 2001; Luo *et al.*, 2001). It is possible that an initially high proteolytic turnover of SIRT1 alleviates such repressive effects on transcription to allow for an early boost of gene expression required for the damage response. At later times, SIRT1 net protein levels increase, probably despite continuously high proteolytic turnover reflecting a dynamic system adaptable to intracellular cues monitoring the extend of repair. This is consistent with the results in FIGURE 4.22 that indicate no accumulation of SIRT1 protein at high doses of UV irradiation where the extend of damage is presumably insurmountable for the cell.

Importantly although the kinetics of SIRT1 accumulation closely resemble those of p53 there is no indication that SIRT1 expression is a target of p53 in this context based on FIGURE 4.23. However this possiblity should be tested repeatedly to confirm the validity of the latter experiment especially under the light of recent evidence that the *SIRT1* gene promoter harbours p53 binding sites which drive SIRT1 expression in response to starvation (Nemoto *et al.*, 2004).

Furthermore, although the osteosarcoma cell line used here has been extensively employed as a system to study cellular UV responses, it is conceivable that it does not reflect a physiologically relevant system to study such pathways as bone cells are not subjected to direct UV exposure. Thus a more suitable cell line to investigate these processes would be one derived from skin such as HaCaT keratinocytes. This is prarticularly important as the presence of additional transcription factors may determine the exact outcome of SIRT1 action.

Finally, following a different line of investigation, it was discovered that mAb12/1 used for all the above experiments shows phosphorylation-sensitive recognition of the corresponding SIRT1 epitope (*see* later this section). Thus a re-evalutation of these results is

necessary to confirm the conclusions presented above as it is possible that what were interpreted to be changes in protein abundance may actually reflect phosphorylation changes. At this point, this could be deemed unlikely as the only circumstances under which mAb12/1 was shown to be altered were in mitosis. Although UV treatment causes a G2/M arrest this requires the *de novo* protein synthesis (Shaulian *et al.*, 2000) and would thus be incompatible with the cycloheximide and actinomycin-D treatment results (FIGURE 4-22).

SIRT1 interacts with and deacetylates the co-activator p300

The co-activator p300 acetylates and thus increases the transcriptional activity of several transcription factors, including p53 and NFκB (Goodman and Smolik, 2000). Furthermore, p300 acetyltransferase (AT) activity depends on autoacetylation of the AT domain (Thompson *et al.*, 2004). Given the need to co-ordinately regulate several transcription factors in a coherent manner, it was considered plausible that SIRT1 deacetylates and thus inhibits p300 AT activity to induce hypoacetylation of downstream transcription factors.

In support of this scenario, SIRT1 and p300 were shown to interact both *in vivo* and *in vitro* (FIGURE 4.24) through the AT domain of p300 (residues 1197-1673). Furthermore, SIRT1 deacetylates p300-AT both *in vivo* and *in vitro* (FIGURE 4.25). However, following UV treatment in U2-OS cells, no change in the ability of the two proteins to interact was observed (FIGURE 4.26) suggesting that any transcriptional effects of SIRT1 are not likely to stem from differential p300 association to SIRT1.

Nevertheless, the functional consequences of SIRT1-regulated p300 deacetylation should be further

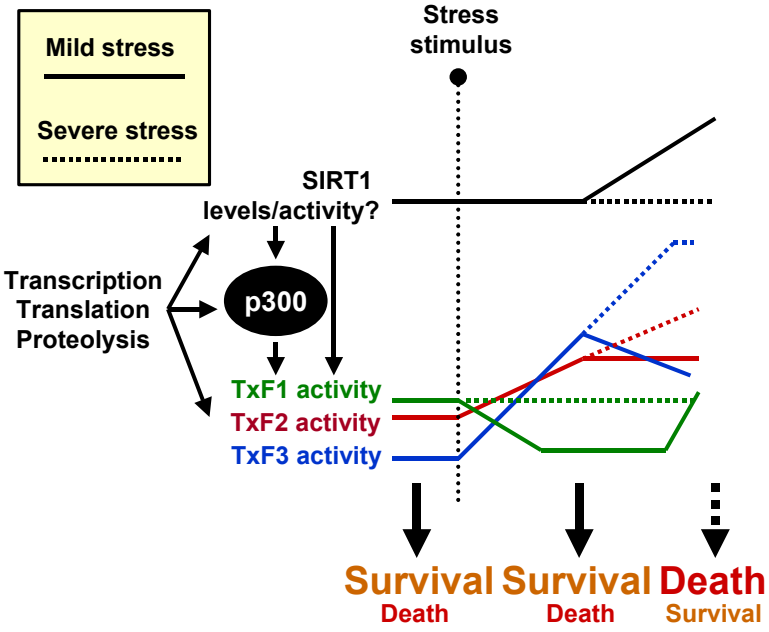


FIGURE 5-9. Co-ordinate regulation of transcription factors and co-activators by SIRT1. *See* text for details.

investigated. Indeed, systematic analyses of transcriptional responses to stress stimuli have identified extensive cross-talking between transcription factors that underlie the corresponding gene expression changes (Janes *et al.*, 2005; Janes *et al.*, 2006). This is conceptually reasonable as given the plethora of environmental stimuli a cell faces at any given time, the pleiotropic effects elicited by different transcription factors need to be co-ordinated so that a coherent and appropriate response is elicited. Alternatively, it is the very fine balance between the activities of these transcription factors that determines the final outcome of the response. In any case, despite our understanding of individual regulatory evetns, the underlying molecular mechanisms responsible for the co-ordination thereof remain largely unknown. Thus, it is conceivable that SIRT1 may play such a co-ordinating role through its dual function in deacetylating both transcription factors directly and an upstream acetyltransferase, in parallel to additional regulatory mechanisms (FIGURE 5-9).

UV-inducible changes in SIRT1 subnuclear localisation

In addition to changes in gene expression, UV irradiation results in significant structural rearrangements within the cell, possibly reflecting the need to accommodate the ensuing changes in gene transcription and DNA replication or other regulatory mechanisms (discussed below). Thus the localisation of SIRT1 in response to UV irradiation in U2OS cells was investigated by indirect immunofluorescence microscopy.

Strikingly, following UV irradiation SIRT1 redistributed from showing a diffuse nuclear localisation to form characteristic ring-like structures within the nucleus surrounding regions staining poor for DAPI and thus being predicted to be nucleoli (FIGURE 4.28). Clearly further evaluation of this conclusion should be pursued in the form of co-localisation studies with nucleolar markers as well as biochemical confirmation of this (Andersen *et al.*, 2002).

The nucleoli are structures within the nucleus where rDNA is transcribed by RNAPolI and ribosomes are assembled, i.e. they are the sites of ribosomal biogenesis (Grummt, 2003). Further to this well-established role, though, other nucleolar functions have been postulated as several proteins with no apparent role in ribosomal biogenesis have been found therein (Olson, 2004).

In particular, nucleolar integrity is important for regulation of p53 tumour suppressor protein stability by sequestering the p53 E3 ubiquitin ligase mdm2/hdm2. Consistent with this, the majority of stress stimuli that lead to p53 stabilisation tested to-date are associated with disruption of nucleolar structure (Olson, 2004; Rubbi and Milner, 2003). Direct disruption of nucleolar structures by microinjection of antibodies against nucleolar structural proteins or localised UV irradiation of nucleoli established that nucleolar integrity acts as a cellular sensor for stress to induce p53 stability (Rubbi and Milner, 2003). Three apparently distinct mechanisms that link nucleolar structure and p53 stabilisation have been proposed.

The first one involves the $p19^{ARF}$ tumour suppressor which binds to mdm2 and sequesters it to the nucleolus thus preventing p53 degradation (Weber *et al.*, 1999). Interestingly, $p19^{ARF}$ progressively accumulates to the nucleoli of serially passaged primary mouse fibroblasts correlating with the onset of senescence which is known to require p53 (Weber *et al.*, 1999). Furthermore, absence of SIRT1 prevents $p19^{ARF}$ accumulation and subsequent p53 stabilisation allowing MEFs to overcome senescence (Chua *et al.*, 2005).

The second mechanism proposes exactly the same role for the PML protein which can recruit mdm2 to the nucleolus in a manner independently of $p19^{ARF}$ (Bernardi *et al.*, 2004). Interestingly, PML was also shown to associate with SIRT1 and regulate the stability of another SIRT1 target, FOXO1 in pancreatic β-cells in response to hyperglycaemia-induced ROS (Kitamura *et al.*, 2005; Langley *et al.*, 2002).

The third mechanism involves the ribosomal assembly and transport protein nucleophosmin (NPM, a.k.a. B23, numatrin or NO38). NPM is detected in the nucleoli of unstressed cells although it undergoes constant nucleocytoplasmic transport. In response to UV, though, NPM shows a diffuse nucleoplasmic distribution. This correlates with disruption of hmd2/p53 interaction and consequently increased p53 stability (Kurki *et al.*, 2004). In this context, NPM has also been shown to direclty interact with p53 and act as a repressor of its transcriptional activity thus setting a threshold for UV-induced p53 activation (Maiguel *et al.*, 2004).

Based on these data, a general model of how nucleolar structure regulates p53 stability has been proposed. According to this, following stress, disruption of the nucleolus results in the release of nucleolar proteins such as $p19^{ARF}$ and NPM which bind to mdm2 and prevent p53 degradation (Olson, 2004).

Additional evidence corroborating to a role of SIRT1 in nucleolar function is provided by the well-studied biology of Sir2 in yeast. In *S. cerevisiae* Sir2 is part of a multisubunit complex known as RENT. The RENT complex is required for silencing at the rDNA loci and mitotic exit (Straight *et al.*, 1999; Shou et el., 1999). Net1, an integral part of the RENT complex, is required for nucleolar integrity. Interestingly, Sir2 is associated throughout the cell cycle with nucleoli until the anaphase/telophase transition when it disperses away from the nucleoli, which undergo structural changes (Straight *et al.*, 1999; Shou et el., 1999; Gotta *et al.*, 1997). Consistent with these, nucleolar fragmentation correlates with ageing in yeast cells (Sinclair *et al.*, 1997) while ageing-associated changes in nuclear structure in general are also observed in mammalian cells (Lans and Hoeijmakers, 2006).

Finally, in response to various cellular stresses, the transcription of rDNA genes and therefore ribosomal biogenesis is downregulated resulting in a general reduction in protein biosynthetic capacity (Grummt, 2003). This is mediated, at least in part, by the Jnk2 kinase which phosphorylates the RNAPolI basal transcription factor TIF-IA to inhibit RNAPolI holoenzyme formation (Mayer *et al.*, 2005). Thus post-translational modifications regulate pre-initiation complex formation to control rRNA synthesis in response to stress. Similarly, deacetylation of TAF_I68, a subunit of TIF-IB, another RNAPolI basal transcription factor, decreases its ability to bind to rDNA promoters and rRNA synthesis (Muth *et al.*, 2001).

Under the light of this, it can be proposed that the recruitment of SIRT1 to nucleoli following UV irradiation may contribute to the suppression of RNAPolI-mediated transcription by preventing the assembly of the RNAPolI transcriptional machinery in the rDNA promoter. It would thus be interesting to investigate the effects of SIRT1 on the efficiency of rDNA transcription in response to UV or other stresses.

5.9.1.4 Phosphorylation of SIRT1 in mitosis

The eucaryotic cell cycle (FIGURE 4-29A) comprises a sequence of spatiotemporaly ordered events that aim at the duplication of the genome and its subsequent segragation to daugther cells in a faithful manner that maintains genomic integrity. During mitosis major structural changes occur in the cell such as nuclear envelope break-down and chromosomal

condensation. The reversibility of these changes is crucial to the outcome of the commitment of cells to division.

Several aspects of chromosome condensation have been described in relation to the gross structural changes that chromatin undergoes during mitosis (Swedlow and Hirano, 2003). The condensed nature of mitotic chromatin is reminiscent of heterochromatin, the transcriptionally inactive part of the genome which correlates with at least one distinct state of histone modification, that of hypoacetylation (Grewal and Moazed, 2003). The role of histone modifications in mitotic chromatin condensation is less clear. Phosphorylation of histone H3 at S10 is considered to be a hallmark or mitotic chromatin, yet its exact function is not well understood (Swedlow and Hirano, 2003).

Global acetylation in mitosis is thought to serve as an epigenetic memory mark for transcriptionally active genes (Jeppesen, 1997) yet both HATs and HDACs dissociate from mitotic chromatin being unable to modify histones in the context of mitotic chromosomes (Kruhlak *et al.*, 2001). Whether, though global deacetylation is a prerequisite for mitotic chromosome condensation remains unclear.

SIRT1 was found to be hyperphosphorylated in nocodazole-arrested mitotic cells (FIGURE 4-7) suggestive of a cell cycle-specific modification and potentially also function of the protein. Further corroborating to this, a single CDK consensus site was identified in the C-terminus of SIRT1 (S540) and recombinant cyclinB/Cdk1 complexes can phosphorylate insect cell-expressed GST-SIRT1 *in vitro* (FIGURE 4-30). This phosphorylation does not cause the characteristic electrophoretic mobility shift of SIRT1 seen in mitosis (FIGURE 4-30C) implying that Cdk1 is not the relevant kinase, that this site is not at all phosphorylated *in vivo*, or if it is, it does not change SIRT1 mobility or, indeed, that there are other unidentified phosphorylation sites and kinases modifying SIRT1 in mitosis.

Nocodazole is a microtubule destabilising drug by virtue of its ability to bind to microtubules and inhibit their polymerisation. This disrupts the mitotic spindle, prevents kinetochore attachment to it and thus elicits the mitotic spindle checkpoint (Rieder and Maiato, 2004). The ultimate goal of this process is to prevent the degradation of specific substrates such as cyclins and securin by the APC ubiquitin ligase a process which is normally necessary for the metaphase to anaphase transition causing the observed cell cycle arrest in what bears the characterisitics of a prometaphase (Peters, 2002).

Unexpectedly, using mAb12/1, SIRT1 protein levels appeared to be reduced in nocodazole-arrested mitotic cells but not in cells synchronised in mitosis following release from a thymidine/aphidicolin block (FIGURES 4-31 & 4-37). This implicated that the epitope recognised by mAb12/1 is potentially modified in response to mitotic spindle checkpoint activation rendering it unrecognisable by the antibody.

Detailed mapping of the mAb12/1 epitope revealed that aa's 16-18 are minimally required for recognition by the antibody (FIGURE 4-38). Interestingly, residue 16 is a serine which can be modified by phosphorylation. Subsequent mutagenesis analyses, revealed that modification of SIRT1 S16 by phosphorylation prevents its recognition by mAb12/1 explaining the observed loss of SIRT1 signal in nocodazole-arrested cells (FIGURE S 4-38 & 4-39A). Furthermore, given the almost complete loss of signal in nocodazole-treated cells, it can be concluded that this phosphorylation is stoichiometric.

In addition, it was considered possible that, due to its proximity, S14 could also have an impact on S16 phosphorylation. Indeed, even in logarithmically growing cells, substitution of S14 with alanine resulted in increased mAb12/1 signal, consistent with reduced S16 phosphorylation, suggesting that S14 phosphorylation is required for efficient S16 phosphorylation (FIGURE 4-39A). Interestingly, this implied that a basal level of S16 phosphorylation also occurs in intephase, consistent with the rescue of mAb12/1 immunoreactivity by λ-PPase treatment of log-phase cells (FIGURE 4-38A).

Attempts to create phospho-specific polyclonal antibodies recognising either phosphorylated S16 or phosphorylated S14/S16 have been until now unfruitful. This may extend beyond the technical difficulties of creating successful phospho-specific antibodies as treatment of nocodazole-arrested cells with the prolyl isomerase Pin1 inhibitor juglone rescued mAb12/1 immunoreactivity (FIGURE 4-39B). Pin1 recognises proline to induce its isomerisation from the *trans* to the *cis* configuration when it is preceded by a phosphorylated serine or threonine (FIGURE 4-27A) (Yaffe *et al.*, 1997).

In a synthetic view of the above results, a model can be proposed where SIRT1 S14 phosphorylation allows recognition of P15 to induce its isomerisation. The resulting regional conformational change imposed drives the recognition of the site by possibly another kinase which then phosphorylates S16 (FIGURE 5-10). Alternatively, this sequence of events renders S16 inaccessible to a phosphatase thus attenuating S16 dephosphorylation kinetics.

Provided that this is a valid interpretation, the use of a doubly phosphorylated synthetic peptide for the creation of phospho-specific antibodies is doomed to failure as the predicted Pin1-imposed conformational change in the native protein, not present in the antigenic peptide used for immunisation, would hinter the recognition of the endogenous protein due to the associated steric hindrance effects stemming from P15 isomerisation.

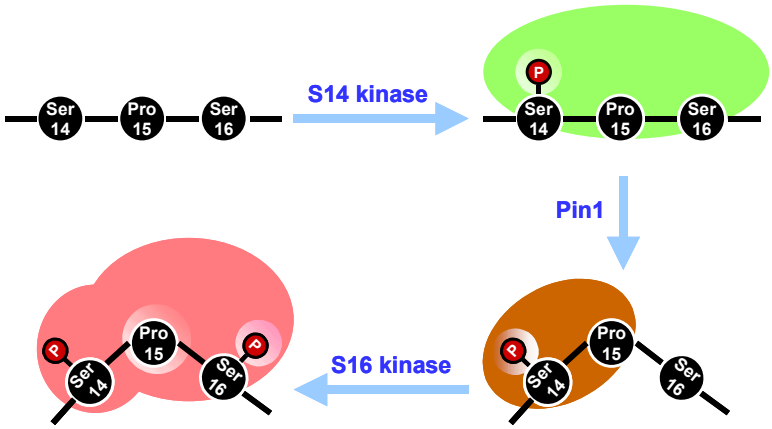


FIGURE 5-10. Proposed mechanism of conformation-dependent SIRT1 S16 phosphorylation. *See* text for details.

Structural analyses of substrate recognition by kinase domains as well as oriented peptide library screens have revealed that several residues flanking the phosphorylatable aminoacids contribute to the specificity of the kinase further supporting the proposed concept that S14 influences S16 phosphorylation (O'Rourke and Ladbury, 2003). Surveillance of known phosphoproteins in the litterature as well as protein phosphorylation databases revealed the presense of several S-P-S sites (e.g. Beausoleil *et al.*, 2004) nevertheless the knoweldge concerning the interplay between the neighbouring serine modifications is remarkably limited.

A representative example of such an interconnection is provided by the regulation of Cdc25C. The Cdc25 family of proterins are dual-specificity phosphatases whose best-characterised role is in the dephosphorylation of CKD's at the inhibitory T14 and Y15 sites contributing to their activation at various stages of the cell cycle (Busino *et al.*, 2004). Following DNA damage, Cdc25C is phosphorylated at S216 and is sequestered by 14-3-3 proteins leading to its inactivation thus preventing cell cycle progression (Donzelli

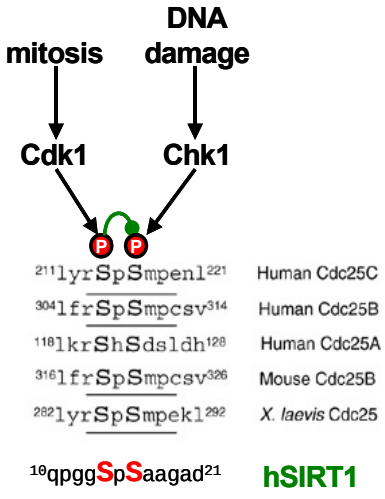


FIGURE 5-11. Mechanism of Cdc25 regulation by dual phosphorylation at an S-P-S site.
Adapted from Bulavin *et al.*, 2003

and Draetta, 2003). However, during mitosis, the preceding S214 is phosphorylated preventing S216 phosphorylation and thus maintaining Cdc25C capable to activate CDKs and allow cell cycle progression (FIGURE 5-11) (Bulavin *et al.*, 2003). Interestingly, S214 and S216 flank a proline residue. Although in this particular example, neighbouring phosphorylation has an inhibitory rather than a promoting effect contrasting what is proposed for the SIRT1 S-P-S site, it strongly supports the merit of further investigating the interplay between S14 and S16.

Investigation of SIRT1 S16 phosphorylation by mitotic checkpoint kinases

SIRT1 was found to be phosphorylated in response to activation of the mitotic spindle checkpoint providing a hint for the possible kinases involved. To this end, three previously characterised checkpoint kinases, Bub1, BubR1 and Mps1 (Kops *et al.*, 2005) were depleted by siRNA, cells treated with nocodazole and the resulting effects on S16 phosphorylation were monitored with mAb12/1. This approach initially indicated that depletion of BubR1 but not Bub1 or Mps1 rescues mAb12/1 immunoreactivity in nocodazole-arrested cells suggesting that BubR1 is the relevant S16 kinase. However, various lines of evidence indicate that this was an erroneous conclusion.

Firstly, BubR1 fails to phosphorylate recombinant SIRT1 *in vitro* (FIGURE 4-45) despite exhibiting a robust autophosphorylation activity which suggests that it is an active kinase. Secondly, BubR1 depletion itself suffices to prevent mitotic checkpoint signaling resulting in failure of cells to arrest in prometaphase in response to nocodazole treatment (Meraldi *et al.*, 2004). Thus, the apparent rescue of mAb12/1 immunoreactivity is likely to stem from the fact that the cells are actually not fully arrested in mitosis and thus not phosphorylated on S16 rather than from the inactivation of BubR1 *per se*.

Although the ability of cells to arrest in mitosis in response to nocodazole treatment should also be compromised by Bub1 and Mps1 depletion leading to a mAb12/1 immunoractivity rescue similar to BubR1 depletion, this is not the case here. This is likely to be due to the inefficient downregulation of the respective protein levels by the siRNAs employed in this study as this is known to allow sufficient residual protein at the kinetochores to maintain a functional checkpoint (P. Meraldi, ETH, personal communication). Thus, when the

above observations are considered in their totality, it is likely that the approach followed for the identification of the relevant S16 kinase is problematic.

Further investigation led to the observation that when the mitotic cells were separated from the G2 cells in a synchronous population following release from a thymidine/aphidicolin block, SIRT1 also showed stoichiometric phosphorylation of S16 (FIGURE 4-46). This firmly established that S16 phosphorylation is not specifically elicited in response to spindle checkpoint activation, but rather it occurs during normal mitosis. Further support for this was provided by the fact that cells arrested in metaphase by MG132 treatment in the absence of spindle checkpoint stimulation also exhibited S16 phosphorylation (FIGURE 4-46B).

A potentially more appropriate approach to this problem would involve the use of cell-free mitotic extracts accompanied by immunodepletion of candidate kinases and subsequent use of these preparations for *in vitro* kinase assays using SIRT1 as a substrate (Nakagawa *et al.*, 1989). This would overcome the defects in mitotic arrest/entry which are tightly associated with siRNA-mediated depletion of mitotic and checkpoint kinases as demonstrated above.

The modification of SIRT1 in mitosis implies a role of the protein in cell cycle. To probe this possibility, SIRT1 was depleted by siRNA and the effects on mitotic markers following arrest with nocodazole were analysed. Cyclin B1 accumulation and phosphorylation of S10 on histone H3 showed an indistinguishable pattern in either the presence or absence of SIRT1 (FIGURE 4.47A) suggesting that SIRT1 is not likely to be an integral component of spindle assembly checkpoint. It is also possible, however, that similar to incomplete depletion of Bub1 and Mps1, residual SIRT1 may mask such an effect.

This is supported by the finding that *SIRT1*$^{-/-}$ or *SIRT1*$^{+/-}$ MEFs are partially impaired in their ability to arrest in G2/M following nocodazole treatment (FIGURE 4-48). Similarly, though this experiment may also hide some pitfalls as the observed effect can reflect reduced proliferation rates of the *SIRT1*$^{-/-}$ or *SIRT1*$^{+/-}$ MEFs.

Furthermore, mouse cells are particularly resistant to nocodazole treatment as the drug does not seem to build up in such high intracellular concentrations as in human cells. This has been partially attributed to increased drug efflux *via* the P-glycoprotein mutlidrug resistance protein (Rieder and Maiato, 2004). Recently, SIRT1 was shown to possitively regulate the expression of P-glycoprotein multidrug resistance gene *mdr1* in cancer cells (Chu *et al.*, 2005). If this also applies to mouse cells, one would predict that reducing or completely

eliminating expression of SIRT1 in MEFs would result in impaired nocodazole efflux, leading to higher intracellular amounts of the drug and thus increased sensitivity of these cells to nocodazole-induced mitotic arrest. This would contradict the observed effects and would consequently dismiss this interpretation.

Despite the evidence presented above, it is noteworthy that the initial line of investigation to explain the loss of SIRT1 immunoreactivity in mitosis was based on the assumption that it was a consequence of proteolytic degradation of SIRT1. This was strongly supported by the identification of specific sequences that have been previously shown to target proteins for proteolysis during the M-phase, namely the D-box which serves as a recognition motif for APC/Cdc20 (Glotzer *et al.*, 1991) and the β-TrCP phosphodegron which is recognised by the $SCF^{\beta\text{-}TrCP}$ E3 ligase (FIGURE 4-32) (Watanabe *et al.*, 2004; Moshe *et al.*, 2004; Margottin-Goguet *et al.*, 2003; Busino *et al.*, 2003; Jin *et al.*, 2003).

This prediction was further supported by clear experimental evidence that demonstrated a direct *in vitro* interaction of SIRT1 with both substrate recognition subunits of the APC, Cdh1 and Cdc20 *in vitro* as well as a phosphorylation-dependent interaction of SIRT1 with β-TrCP (FIGURES 4-33 & 4-35). However, subsequent experiments were unable to demonstrate a functional consequence of the presence of these E3 ligase recognition motifs (FIGURES 4-34 & 4-36).

In retrospect, the inability of β-TrCP to target SIRT1 for degradation is not surprising. The crystal structure of β-TrCP in complex with its substrate β-catenin coupled to biochemical evidence demonstrated that the spacing between the β-TrCP phosphodegron and the lysine residue targeted for ubiquitination by the E3 ligase complex is crucial and varies between 9-11 aminoacids in known β-TrCP substrates (Wu *et al.*, 2003). No lysine residues lie in the proximity of the SIRT1 phosphodegron providing an explanation for the inability of β-TrCP to target the protein for degradation. The question, though, what, if any, is the function of this sequence motif remains unanswered.

With respect to the D-box it could be argued that if SIRT1 were an APC substrate, mitotic arrest with nocodazole would result in SIRT1 accumulation rather than degradation due to APC inactivation. Although this is the case for most APC substrates, it is of interest to note that the degradation of cyclin A by the APC is not inhibited by the spindle assembly checkpoint as with other APC substrates (Geley *et al.*, 2001).

Normally, UbcH10, the E2 protein required for the degradation of cyclin A is subjected to auto-ubiquitination and degradation by the APC and this is inhibited by other APC substrates (Rape and Kirschner, 2004). Thus, as APC is inactivated by the spindle-assembly checkpoint, APC substrates accumulate, UbcH10 is stable and cyclin A is degraded probably because the APC/UbcH10 complex shows different sensitivity to checkpoint inhibition compared to other APC/E2 complexes.

Based on these, SIRT1 degradation in the presence of an active checkpoint would be feasible, encouraging further investigation of the D-box function. Although the ineffectiveness of Cdh1 and Cdc20 depletion to elicit any changes in SIRT1 abundance puts this theory at doubt it is noteworthy that they both significantly interact with SIRT1 *in vitro* (FIGURE 4-35).

Furthermore, bearing in mind the considerations presented in section 5.9.1.1, it is also possible that the degradation mechanisms proposed above only apply to a specific SIRT1 pool of low abundance. In this case, changes in protein turnover would occur in a significantly under-represented protein population and thus would be not be readily detectable using the polyclonal antibodies.

position	-2	0	2	4	6	8	10
eIF4GI	K K R	**Y**	D R E F	**L L**	G F Q F		
eIF4GII	K K Q	**Y**	D R E F	**L L**	D F Q F		
4E-BP1	R I I	**Y**	D R K F	**L M**	E C R N		
4E-BP2	R I I	**Y**	D R K F	**L L**	D R R N		
4E-BP3	R I I	**Y**	D R F F	**L L**	E C K N		
SIRT1	A I G	**Y**	**R** D N	**L** **L**	**F** G D	**E**	I
consensus		**Y**		**L**	**Φ**		

FIGURE 5-12. Overlap of the SIRT1 D-box and eIF4E consensus binding sites. An alignment of the eIF4G and 4E-BP In **bold red**: conserved residues of the eIF4E consensus site. In **bold green**: conserved residues of the consensus D-box (in light green rectangle).

An additional consideration is also of note: while attempting to identify functional motifs in the N- and C-terminal SIRT1 extensions, it was observed that the identified D-box overlaps with a consensus eIF4E binding site found in 4E-BPs and eIF4G, an integral component of the translation initiation machinery (FIGURE 5-12) (Gingras *et al.*, 1999). The association of 4E-BP to eIF4E inhibits translation as it prevents the latter from binding to the 5' cap of mRNAs (Gingras *et al.*, 1999). The significance of this overlap is unknown, however it is particularly intriguing in view of the presence of a TOS motif in SIRT1 because 4E-BPs also have one which is required for their phosphorylation by TOR kinase leading to dissociation from eIF4E and consequently to translation initiation (Schalm and Blenis, 2002).

Finally, the presence of two distinct degradation-targeting motifs is not unprecedented. Apart from the aforementioned IκB which has both a β-TrCP phosphodegron and a PEST

region (Kato *et al.*, 2003), Cdc25A also harbours a KEN box targeting the protein to APC^{Cdh1} as well as a β-TrCP motif (Busino *et al.*, 2004). Moreover, treatment of logarithmically growing cells with the proteasome inhibitor MG132 results in SIRT1 protein accumulation demonstrating that the protein is subjected to constant proteolytic turnover (FIGURE 4-31B). Given the fact that SIRT1 protein accumulates in response to at least one stimulus, namely nutrient starvation (Rodgers *et al.*, 2005) the mechanism of reversing this effect is of importance and based on the observations presented here is likely to be *via* proteasome-mediated degradation.

5.9.1.4.1 Potential functions of SIRT1 in mitosis

Evidence from the literature indicates various aspects of mitosis where functions perfomed by SIRT1 could be envisioned.

The potential role of SIRT1 as a histone deacetylase in mitotic chromosome condensation has been discussed in Chapter 4. Its recent demonstration as a histone H1 deacetylase specifically, adds further to this notion (Vaquero *et al.*, 2004). H1 is a linker histone which has been shown to be required for proper chromosome structure in Xenopus egg extracts. Depletion of histone H1 leads to incompletely condensed chromosomes that extend out of the metaphase plate (Maresca *et al.*, 2005). It is not clear whether the acetylation status of histone H1 is important for this function, yet this is a line of investigation that should be further explored. Interestingly, histone H1 interacts with the N-terminus of SIRT1 which also harbours the mitotic phosphorylation site S16 described in this work. Thus, it is possible that S16 phosphorylation controls this interaction to regulate histone H1 function in mitotic chromosome structure apart from its proposed role in transcriptional regulation (Vaquero *et al.*, 2004).

Additional potential roles of SIRT1 in mitosis are discussed below.

Acetyl-CoA metabolism and mitosis

To begin with, at least two distinct acetyltransferases have been implicated in mitotic progression, namely Eco1 and p300. The Eco1 acetyltransferase was firstly described in

yeast as a protein required for the establishment of chromatid cohesion during S phase. Bioinformatic analysis identified an acetyl-CoA binding motif within the Eco1 sequence and subsequent experiments demonstrated an *in vitro* acetyltransferase activity which targets several components of the cohesin complex (Ivanov *et al.*, 2002). The importance of this activity has been subsequently questioned as yeast expressing acetyltransferase-defective Eco1 is viable (Brands and Skibbens, 2005). Although the exact mode of action of Eco1 remains unclear, orthologues have been identified in higher organisms and were also shown to be required for cohesion (Hou and Zou, 2005; Williams *et al.*, 2003).

Recently, a further link between silencing and chromatin cohesion involving the Sir protein was established. Using a system that allowed the visualisation of cohesion outside the context of the chromosome, Chang *et al.* could show that in *S. cerevisiae* the Sir proteins are required for cohesion of the *HMR* loci, but not for other chromosomal regions, by directly recruiting the cohesin complex (Chang *et al.*, 2005). In addition, in *S. pombe*, Swi6 is required for the recruitment of the cohesin complex to centromeric heterochromatin but does not appear to participate in chromosome arm cohesion (Huang and Moazed, 2006). A Swi6 orthologue has been identified in both *Drosophila* and humans and is known as heterochromatin protein 1 (HP1). As the topology of chromatid cohesion remains debatable (Huang and Moazed, 2006), it is tempting to speculate that in a conserved similar function of SIRT1 is also present in mammalian cells.

Furthermore, the dynamics of acetyl-CoA and related metabolic activities appear to have a broader impact on mitosis. In *S. pombe*, mutations in acetyl-CoA carboxylase (ACC) and fatty acid synthase result in defective mitosis (Saitoh *et al.*, 1996). The situin orthologue CobB has been implicated in acetyl-CoA biosynthesis in bacteria by activating the acetyl-CoA synthase (Starai *et al.*, 2002). Also SIRT1 regulates lipid metabolism in rodents where the proposed mechanism of action though is thought to be through transcriptional regulation (Picard *et al.*, 2004). Given the apparent involvement of SIRT1 in mitosis presented here, it would be really exciting to investigate how acetyl-CoA metabolism and lipid biosynthetic processes impact cell division and what the potential role of sirtuins in these processes might be.

The acetyltransferases CBP/p300 have also been shown to be required for proper mitotic progression by associating with the APC components Apc5 and Apc7 via an E1A-like

region present in the latter two (Turnell *et al.*, 2005). Both Apc5 and Apc7 potentiate CBP/p300 transcriptional activation while CBP is required for the ubiquitin ligase function of APC and also for proper mitotic exit. It is important to note, though, that this latter function is attributed to the E4 ligase activity of CBP. Thus, in addition to acting as a deacetylase for the putative substrates of the proteins described above, SIRT1 may also modulate the mitotic role of CBP/p300 by deacetylation as shown in this thesis.

SIRT1 and mitotic exit

Further evidence for potential roles of SIRT1 in mitosis lies within our current knowledge of sirtuin biology in lower organisms. In *S. cerevisiae* Sir2p is part of the RENT complex as described previously, which also includes the Cdc14 phosphatase that inactivates mitotic CDKs to promote mitotic exit (Shou *et al.*, 1999; Straight *et al.*, 1999). Two mammalian orthologues of Cdc14 have been identified named Cdc14A and Cdc14B and abberant expression of either protein leads to mitotic defects althought their exact mechanism of action reamisn unclear (Mailand *et al.*, 2002).

Interestingly, SIRT1 S16 dephosphorylation appears to require the anaphase-to-metaphase transition implying that the dynamics of SIRT1 phosphorylation during mitosis may be linked to mitotic exit (FIGURE 4.46B). It would thus be of interest to probe the evolutionary conservation and potential functional significance of the SIRT1/Cdc14 association in particular in the context of mitotic exit. This is also of broader interest as despite the accumulating knowledge about mitosis-associated events, relatively little is known about mitotic exit in mammalian cells.

Survival during mitosis

Progress through mitosis is associated with dramatic cytoarchitectural changes which have a broad impact on the organisation of the cell and thus require sustaining survival signaling in order to prevent cell death. Recent evidence suggests that upon treatment of cells with microtubule poisons, the NFκB pathway is activated and this is required for survival (Mistry *et al.*, 2004). As SIRT1 has been shown to deacetylate and thus inactivate the p65

subunit of NFκB, it is likely that is also has a similar role in this context. Furthermore, this might also have therapeutic implications as microtubule poisons such as taxol are used for cancer treatment. If the above prediction is correct, concomitant inactivation of survival pathways by SIRT1 activation in addition to microtubule poisons would not only attenuate the high proliferation rate of cancer cells but would also enhance their propensity to undergo apoptosis.

It is noteworthy that activation of mitotic proteins such as CDKs is also linked to cell death. The CDK inactivating proteins Wee1 kinase and the Cdc27 subunit of the APC are cleaved in a caspase-dependent manner following apoptotic stimulation leading to CDK activation (Zhou *et al.*, 1998). Conversely, induced activation of CDK1 in post-mitotic neurons promotes neuronal apoptosis (Stegmuller and Bonni, 2005). In either cases, the exact purpose and ensuing consequences of CDK activation remains unclear but these examples underline yet another aspect of the close interplay between cell division and apoptosis. Again, the preceding knowledge of SIRT1 involvement in cell survival regulation coupled to the proposed involvement in mitosis strongly indicate another path of investigation along these lines.

Priming S phase events in mitosis

One of the success strategies of the cell cycle programme is based on mechanisms that couple each phase to the other thus allowing unidirectional progression. Thus events that are associated with a particular cell cycle phase have their foundations in a preceding phase.

One such example is the formation of pre-replication complexes (pre-RCs) during late mitosis and early G1. Starting already at anaphase, pre-RCs assemble at replication origins to establish replication competence, although by themselves are not sufficient for replication (Prasanth *et al.*, 2004). In *S. cerevisiae* Sir2p deletion suppresses the phenotype of a pre-RC assembly mutant (Cdc6p - required for loading of the pre-RC component MCM helicase to origins of replication) indicating that Sir2p is a negative regulator of replication (Pappas *et al.*, 2004).

It would be of interest to investigate whether SIRT1 has a similar role in mammalian cells. In this case, SIRT1 would need to be inactivated to allow efficient pre-RC formation. Following mitotic exit, re-activation of SIRT1 would function in preventing pre-RC formation and thus aberrant genome re-replication until the next cell cycle. An experimental issue to be taken into consideration is that based on such a model, depletion of SIRT1, e.g. by siRNA, would have minimal impact on mitosis itself. If the identified SIRT1 S16 phosphorylation plays a role in this process a more appropriate approach would require the investigation of how SIRT1 S16 mutants affect S phase entry/progression kinetics.

Furthermore, S phase onset is regulated by the levels of the CDK inhibitor p27. Degradation of p27 by the SCF^{Skp2} ubiquitin ligase underlies S-phase entry (Sutterluty *et al.*, 1999) while Skp2 levels are also under the negative control of APC^{Cdh1} to regulate G1 duration by allowing p27 accumulation and thus prevent premature S phase entry (Bashir *et al.*, 2004; Wei *et al.*, 2004). As previously discussed (section 5.2.2.5), SIRT1 may contribute to transcriptional repression of the *p27* gene in collaboration with the Hes1/Hey2 transcriptional repressors or alternatively, promote Skp2-mediated p27 degradation by deacetylating and thus exposing lysine residues in p27 for ubiquitination.

Mitosis and ageing

Recent data suggest a close link between molecular components regulating mitosis as well as the spindle assembly checkpoint and the ageing process (Baker *et al.*, 2005).

Comparison of mRNA transcript levels between fibroblasts derived from young and old invividuals as well as people who suffer from a progeroid syndrome revealed that a striking proporion of genes that displayed differential expression are involved in mitotic progression. These genes included cyclins A, B and F, Polo-like kinase and the APC component Cdc20 which were all downregulated in the fibroblasts derived from old/prematurely aged individuals (Ly *et al.*, 2000).

Mouse models further support these findings. Mice genetically engineered to express a *BubR1* hypomorphic allele (leading to reduced but not completely abolished BubR1

expression) suffer from premature ageing which correlates with increased expression of senescence markers (reviewed in Fernandez-Capetillo and Nussenzweig, 2004).

In conclusion, the role of SIRT1 in mitosis proposed by this work is unclear and only inferred by the observation that SIRT1 is stoichiometrically phosphorylated at least at S16 in this phase of the cell cycle. The ability of SIRT1 to modulate mammalian ageing has been poorly investigated and largely unproven. Understanding the significance of SIRT1 modification in mitosis would provide further insights into a novel function for SIRT1 and a potential link to the regulation of organismal life-span.

5.9.2 Regulation of SIRT1 by caspase-mediated cleavage

During the course of these studies and on several occasions a fast-migrating protein band strongly immunoreactive to mAb12/1 and to a lesser extend to GST-S1 was observed (e.g. FIGURES 4-7, 4-22, 4-31). The appearence of this band, initially termed $p110^{SIRT1}$ by virtue of its apparent molecular weight, invariably correlated with the presence of apoptotic figures observed by light microscopy.

Systematic investigation of various apoptosis-inducing stimuli further supported this observation (FIGURE 4-50). Furthermore, the use of caspase inhibitors as well as *in vitro* caspase cleavage assays established that $p110^{SIRT1}$ derives from full-length SIRT1 by caspase-mediated proteolytic processing (FIGURES 4-51 & 4-56).

Comparison of SIRT1 antibody recognition patterns in extracts of cells stimulated to undergo apoptosis indicated that caspase cleavage occured in the C-terminal site of SIRT1 (FIGURE 4-52). Systematic mapping and mutagenesis of consensus caspase cleavage sites on SIRT1 identified D707 as the relevant processing site giving rise to $p110^{SIRT1}$ which was henceforth referred to as SIRT1(1-707) (FIGURES 4-53, 4-54, 4-56).

Based on these results, the GST-S1 epitope is preserved in SIRT1(1-707) which would thus be predicted to be fully recognised by this antibody. However, while GST-S1 only weakly recognises SIRT1(1-707) in immunoblotting, mAb12/1 exhibits significantly higher relative immunoreactivity towards this species (FIGURE 4-52). Under the light of the phosphorylation-sensitive recognition of SIRT1 by mAb12/1, these results also bear

additional significance: they imply that SIRT1(1-707) is dephosphorylated, enhancing its recognition by the monoclonal antibody. As our understanding of SIRT1 S16 phosphorylation remains limited, it is difficult to appreciate the significance of this observation, nevertheless, it should be taken into consideration in subsequent studies.

It is also worth mentioning that there exist several examples in the literature where phosphorylation of residues in the proximity of caspase cleavage sites was shown to impede processing by caspases. This was shown to be the case for the class II deacetylase HDAC4 (Paroni *et al.*, 2004, Liu *et al.*, 2004), the transcription factor Max (Krippner-Heidenreich *et al.*, 2001), the tumour suppressor lipid phosphatase PTEN (Torres *et al.*, 2003) and the apoptosis factor Bid (Desagher *et al.*, 2001). Intriguingly, in all these cases, the relevant kinase is CK2. However, none of the mutant CK2 sites confered differential cleavage sensitivity to SIRT1 (not shown) suggesting that this mechanism may not apply for this protein under the experimental conditions employed for these experiments.

5.9.2.1 Functional significance of caspase-mediated SIRT1 cleavage

Importantly, SIRT1(1-707) does not exhibit altered *in vitro* enzymatic activity nor does it localise differently than its full-length counterpart (FIGURES 4-59 & 4-60). These results raise the obvious question what is the functional significance of this processing event.

Initial approaches towards addressing this issue focused on the potential impact of SIRT1 C-terminal cleavage on its transcriptional activities. For this purpose, a heterologous luciferase reporter assay was employed where 3 consensus NFκB binding sites were located upstream of a consistutive promoter to render luciferase expression sensitive to NFκB activity. This is a well-suited system for such studies as it provides a straightforward means of not only monitoring transcriptional effects of SIRT1 but also allows to assay various SIRT1 mutants and assess their functional significance.

The choice of NFkB reporter was based on previous work where SIRT1 was shown to negatively regulate NFκB transcriptional activity in response to TNFα stimulation (Yeung *et al.*, 2004), but also on the fact that most of the stimuli that were shown to induce SIRT1 cleavage are also known to be potent NFκB activators (FIGURE 4-50). Of note is also the example of hematopoietic progenitor kinase 1 (HPK1) which is an activator of NFκB , yet under apoptotic

conditions it undergoes caspase-mediated cleavage which converts it into an inhibitor of NFκB (Arnold *et al.*, 2001). Thus it was postulated that under such conditions, modulation of SIRT1 activity may in turn impact the activation status and/or magnitude of NFκB.

Following extensive optimisation of assay conditions, SIRT1 was found to consistently suppress NFκB reporter activity in response to TNFα stimulation even at very low amounts of transfected SIRT1 which ranged in the few ng of DNA level or up to 1/500 SIRT1-to-reporter plasmid ratio. However, no significant difference in the ability of SIRT1(1-707) to suppress TNFα-induced NFκB activity was observed (FIGURE 4-61). These data implied that caspase-mediated processing of SIRT1 is unlikely to impact its transcriptional repressor activity under the assay conditions used.

It is worthwhile, however, to critically evaluate the validity of this conclusion with respect to the limitations that such an experimental system comprises. To begin with it is possible that the reporter construct employed here does not bear the necessary features required for a functional consequence of SIRT1 cleavage to be revealed. This would be particularly the case if an additional protein interaction mediated by the cleaved C-terminal region is required for such an effect. It is of interest to note that in the original work reporting the SIRT1-NFκB interaction, treatment of cells with the SIRT1 activator resveratrol resulted in suppression of NFκB gene espression attributed to the prolonged occupancy of NFκB target gene promoters by SIRT1 (Yeung *et al.*, 2004). The molecular mechanism of this is intriguing but remains elusive. It is possible that this can be accounted by a conformational change elicited by resveratrol. Similarly, any of the modifications of SIRT1 discovered in this work, including the caspase-mediated cleavage may have a similar *modus operanti*.

Furthermore, Starai *et al.* reported that CobB, a bacterial sirtuin deacetylates acetyl-CoA synthase leading to its activation (Starai *et al.*, 2002). As the authors point out, the catalytic lysine targeted for deacetylation constitutes a functionally conserved residue in AMP-forming enzymes which include luciferase. Thus it is conceivable that the expression of SIRT1 in the NFκB reporter assays results in a steady-state modification of luciferase that alters its activity leading to erroneous conclusions.

It is also clear that additional levels of NFκB activity modulation exist such as the deacetylation-dependent nucleocytoplasmic transport of the p65 subunit (Chen *et al.*, 2001). Thus, it is possible that SIRT1(1-707) does have a different function than that of full-length

SIRT1 however the steady-state conditions under which the reporter assays are performed are unsuitable for detecting the potentially subtle differences incurred.

Examining the nuclear translocation kinetics of NFκB as well as NFκB target gene expression following TNFα stimulation is likely to be more informative in this context. The evaluation of MEFs lacking SIRT1 for such an experiment is underway. Provided that the presence of SIRT1 proves to be a determinant of NFκB activation amplitude, re-introduction of SIRT1 and mutants thereof by retroviral transduction will be employed to assess the differential effects of SIRT1 species.

A significant hint as to the potential significance of SIRT1 cleavage was also provided by two other pieces of data. Firstly, inhibition of proteasome activity by MG132 under conditions that induced apoptosis, resulted in rescue of SIRT1(1-707) immunoreactivity (FIGURE 4-57). What is more, *in vitro* cleavage of SIRT1 by activated cell extracts indicated that under these conditions, SIRT1(1-707) was sensitive to further proteolytic processing, while mutation of the caspase cleavage site not only prevented SIRT1(1-707) formation, but it also blocked SIRT1 degradation overall (FIGURE 4.56).

Taken together, these data suggest that a potential role of SIRT1 cleavage by caspases following activation of the apoptotic programme is to render the protein more susceptible to further proteolysis. The impact of a SIRT1 non-cleavable mutant on the sensitivity of cells to apoptotic stimuli is currently under examination.

Interestingly, the C-terminal region removed following caspase cleavage harbours a consensus sumoylation site (^{733}V<u>K</u>QE^{736})(Seelre and Dejean, 2003). Modification of proteins by SUMO has been linked, among others, to transcriptional regulation and interestingly, SUMO is found concentrated at PML bodies, a site where also SIRT1 was shown to localise under specific conditions (Seelre and Dejean, 2003; Langley *et al.*, 2002). It is also of particular interest that p300, has a transcriptional repression domain called CRD1 (for <u>c</u>ell cycle <u>r</u>egulatory <u>d</u>omain <u>1</u>) which harbours two sumoylation sites K1020 and K1024. SUMO modification at these lysines is essential for its transcriptional repressor function (Bouras *et al.*, 2005). Interestingly, SIRT1 represses p300-mediated transactivation in a manner that depends on the p300 CRD1 domain because SIRT1 deacetylates K1020 and K1024 allowing them to be modified by SUMO ligases and promoting the transcriptional repressor function of p300 (Bouras *et al.*, 2005).

Increased caspase activity during ageing

Several of the ageing phenotypes can be attributed to loss of tissue mass due to increased apoptosis. This has been well documented for different post-mitotic tissues including the heart, muscle and the central nervous system where apoptosis is one of the underlyng disease mechanisms in Alzheimer's and Parkinson's disease, two age-related neurological disorders (Zhang *et al.*, 2003).

Overall, there is a striking correlation between caspase activity and ageing-associated apoptosis. Studies in humans as well as mice have shown increased activity as well as mRNA levels for various caspases in cells of the liver, spleen, lung and the immune system (Zhang *et al.*, 2003). Strikingly, mouse models that exhibit premature ageing also have increased caspase activity.

A recent example of this are mice with a mutation in the nucleus-encoded catalytic subunit of mitochondrial DNA (mtDNA) polymerase PolgA that abolishes its proofreading activity and thus renders it more prone to errors during mtDNA replication. These mice have high mtDNA mutation rates and, at the organismal level, they suffer from classic symptoms of premature ageing such as weight loss, hair loss, skeletal abnormalities, osteoporosis, anemia and reduced fertility (Kujoth *et al.*, 2005; Trifunovic *et al.*, 2004). Such mice also exhibit increased occurrence of apoptotic markers, including cleaved and thus activated caspase-3 especially in tissues with high cell turnover such as the thymus, intestine and testis (Kujoth *et al.*, 2005).

Intriguingly, increased apoptosis in kidneys of aged rats can be suppressed by caloric restriction (Lee *et al.*, 2004). This was attributed to the decrease of oxidative damage elicited by diet and, in support of this, markers of lipid peroxidation resulting from oxidative damage, were higher in animals fed *ad libitum* compared to those subjected to caloric restriction (Lee *et al.*, 2004).

Finally, progressive apoptosis of oocyte population acquired during embryonic development in the ovaries leads to reduced reproductive capacity and ultimately what is known as the menopause in females (Tilly, 2000). A recent report has drawn a link between cellular metabolic status and oocyte death. NADPH production by the pentose phosphate

pathway promotes the activity of calcium/calmodulin-dependent protein kinase II (CaMKII) which in turn phosphorylates and inhibits caspase-2, a key executor of the apoptotic programme in oocytes (Nutt *et al.*, 2005). This provides evidence that the nutrient status of the environment experienced by the reproductive system determines to a significant extend the reproductive capacity of an animal.

In conclusion, the work described above as well as the demonstration that SIRT1 is a substrate for caspases, constitute compelling evidence in favour of investigating the link between ageing-associated apoptosis and SIRT1 processing especially in the light of the proposed role of SIRT1 in ageing. Moreover, undertanding the functional significance of SIRT1 processing is likely to provide an entirely novel mechanism of SIRT1 regulation and thus shed light into unknown aspects of the protein's role in physiological conditions where a role for apoptosis has been documented.

5.9.3 Prediction of signalling pathways which SIRT1 may participate in

In addition to the experimental evidence provided in this work with regards to novel potential SIRT1 regulatory mechanisms, current knowledge derived from the literature as well as the *in silico* identification of putative regulatory motifs in the primary sequence of SIRT1 cast some light into which signaling pathways are likely to be involved in modulating SIRT1 function (FIGURE 5-13).

One of the phenotypes of *SIRT1*$^{-/-}$ mice is persistent eyelid closure accompanied by developmental abnormalities of the eye (Cheng *et al.*, 2003; McBurney *et al.*, 2003). The EGF signaling pathway is a well-established mediator of eyelid development in the mouse and genetic inactivation of its components such as Jnk1 and Jnk2 or c-jun invariably leads to eyelid closure defects (Weston *et al.*, 2004; Zenz *et al.*, 2003; Li, 2003). This is due to the inability of two fronts of epithelial cells to fuse during embryonic development attributed to reduced EGF receptor signaling. Furthermore, EGF signaling is also important for the major structural changes leading to opening of the eyelid post-natally (Zieske, 2004).

The persistence of eyelid closure in *SIRT1*$^{-/-}$ mice post-natally indicates that SIRT1 plays an important role in the eyelid opening process and this is likely to be linked to its ability to participate in signaling downstream the EGF receptor, possibly by modulating the

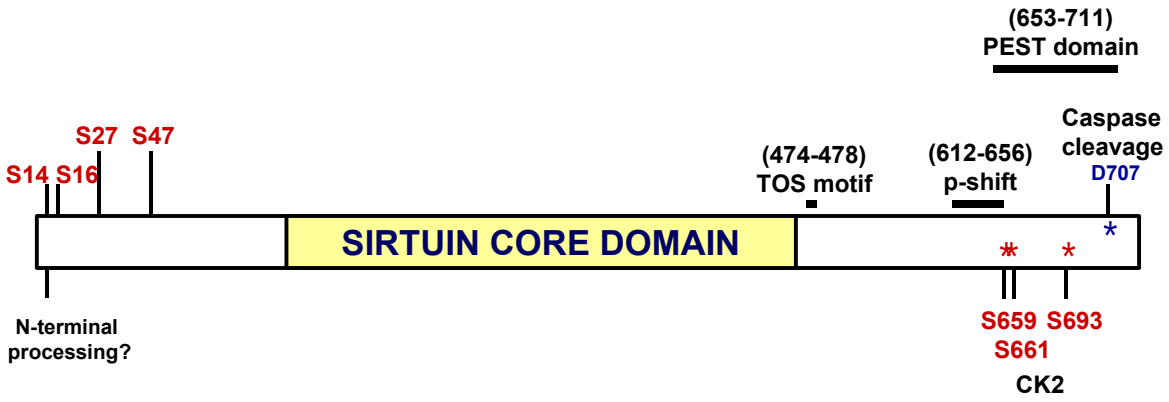


FIGURE 5-13. Overview of the newly-identified and predicted SIRT1 modifications with potential regulatory roles.

expression of genes required for this developmental process (Zieske, 2004). Interestingly, among the factors involved in eyelid development (such as Pax6 and E2F transcription factors) are members of the forkhead transcription factor family as well as IKKα (Zieske, 2004).

Furthermore, genetic evidence supports a role for Sir-2.1, the *C. elegans* SIRT1 orthologue, in negative regulation of the insulin/IGF signaling pathway (Tissenbaum and Guarente, 2001). Similarly, SIRT1 was shown to regulate the transcriptional activity of forkhead transcription factors which are under the negative control of insulin/IGF signaling. (Giannakou and Partridge, 2004). It is thus conceivable that SIRT1 activity itself is under the control of this pathway in order to ensure a functionally coordinated modulation of the forkhead mediated transcriptional response *via* mechanisms discussed in other parts of Chapter 5.

Similarly, in addition to SIRT1 regulating the transcriptional activity of NFκB it is conceivalbe that SIRT1 activity also receives inputs from upstream components of the NFκB pathway such as IKKβ. This again would ensure the co-ordinate regulation of transcriptional activity by modulating the amplitude, duration and reversibility of NFκB activity.

The TOR signaling pathway is proposed to function as an integrator of energy, nutrient and growth factor cues. Energy depletion leads to TOR inactivation *via* the AMPK kinase cascade, insulin receptor signaling activates TOR kinase *via* the PKB/Rheb signaling axis while oxygen levels also modulate TOR activity through different mechanisms (Wullshleger *et al.*, 2006). Interestingly, SIRT1 harbours a TOS motif in the proximity of its sirtuin core domain. TOS motifs are present in all major TOR substrates tested to-date and have been shown to be required for their interaction with the TORC1 component raptor (Schalm *et al.*, 2003; Schalm

and Blenis, 2002). It would thus be intriguing to consider a scenario where SIRT1 and TORC1 components interact functionally, biochemically or even physically in a manner that potentially requires their corresponding enzymatic activities possibly through their enzymatic activities.

Finally, the dependence of SIRT1 deacetylase function on NAD^+ in addition to the proposed inhibitory role of NADH on its activity make it suitable for responding to cellular metabolic changes incurred by changing oxygen tension. Hypoxic conditions lead to a decrease in NAD^+/NADH ratio (Zhang *et al.*, 2002) suggesting that under limiting oxygen conditions, SIRT1 activity would be impaired. The cellular redox status has been shown to modulate transcriptional activities of other factors as discussed in section 5.4. Thus it is a valid to predict that SIRT1 may one way or another participate in the oxygen homeostasis signaling pathway.

5.9.4 Conclusion

There is an increasing appreciation of the significance behind the extensive interconnections between basic cellular homeostatic pathways. In an ever-changing environment, a dynamic system must be in place to ensure the functional co-ordination between diverse signaling cascades and implement a physiologicaly coherent outcome.

Proteins that integrate diverse signals and can in turn pleiotropically affect downstream effectors would be key for this strategy. The emergence of SIRT1 as a regulator of several aspects of homeostatic signaling pathways (FIGURE 5-14) makes it a likely candidate for this function. Thus understanding the mechanistic details of its regulation will provide a means to test its proposed role as a master co-ordinator of responses to environmental cues. The work presented here provides the first examples of SIRT1 regulatory mechanisms and is likely to yield new insights into the signaling pathways that modulate SIRT1 activity.

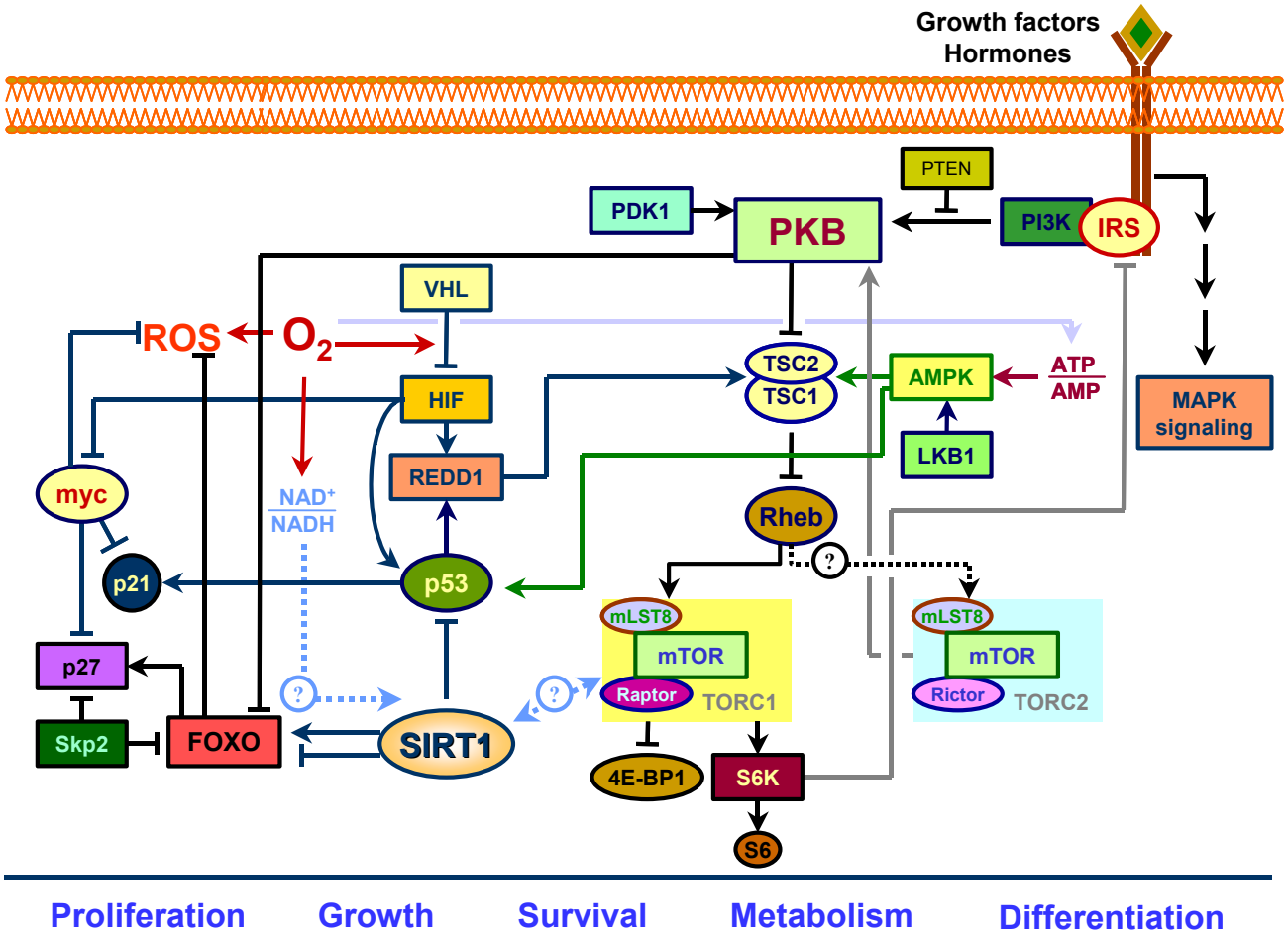


FIGURE 5-14. Functional interaction of SIRT1 with major homeostatic cellular pathways. A putative role in the co-ordination of such signaling network may underlie the pleiotropic character of the SIRT1 substrates set. In addition, interconnections between the FOXO, p53 and NFκB signaling cascades have been reported but are not depicted here. *See* text for further details.

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