# Analysis of the Structure and Function of Protein Phosphatase 2A

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This thesis is dedicated

to my family, friends

and

the memory of my father

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# I. Summary

One of the hallmarks of a living organism is the ability to respond to intra- or extracellular changes. These responses involve panoply of enzymes mediating signals through the cell and regulating distinct cellular functions. Protein kinases and protein phosphatases are important antagonists in this finely balanced process.

Protein phosphatase 2A (PP2A) is one of the major serine/threonine-specific phosphatases and has the most diverse substrate specificity of all protein serine/threonine phosphatases in the cell. PP2A consists of a core dimer made up of the 36-kDa catalytic subunit C tightly complexed with the scaffold regulatory subunit PR65/A. This complex associates with any one of the second or variable regulatory subunits PR55/B, PR61/B', PR72/B" or PR110/B''' to form an extensive array of trimeric holoenzymes. PP2A impacts on all major signaling pathways by reversing the functions of protein kinases and is, therefore, considered to be a central regulator of eucaryotic signal transduction. Dysfunction of this molecule may have severe consequences for the organism and it is, therefore, not surprising that PP2A has become an important target in the investigation of various diseases.

We investigated the function of invariant active-site residues of PP2A that are crucial for catalytic function of the enzyme. A baculovirus system using High Five insect cells was developed that allowed high level expression of active PP2A which was used for structural and functional analysis. Site-directed mutagenesis of PP2Ac and purification of mutant proteins from insect cells combined with functional analysis in yeast provided a powerful system for structure–function analysis of PP2Ac. Mutation of the active-site residues Asp$^{88}$ or His$^{118}$ within the human PP2A catalytic $\alpha$ subunit impaired catalytic activity *in vitro* and *in vivo* indicating an important role for these residues in catalysis.

As PP2A containing the PR55/B regulatory subunit is known to be involved in the pathogenesis of neurodegenerative disorders, we characterized the PR55/B family with particular emphasis on its distribution and developmental regulation in the brain. The

study revealed new aspects of genomic organization and variability, as well as hitherto unknown expression patterns of the PR55/B family in the brain. We also found distinct subcellular localizations of PR55/B isoforms in areas of the brain known to be affected by Alzheimer's disease. In addition, our results suggest a distinct role for PR55/Bα in astrocytosis, given that this isoform is highly expressed in activated astrocytes. Interestingly, astrocyte activation is an early step in the pathogenesis of Alzheimer's disease and related disorders.

In addition, we attempted to define the transcriptional effects of the PP2A-inhibitor okadaic acid (OA) on promoter complexes using Affymetrix GeneChips. Based on known target genes and further target genes that we identified, we suggest that OA mainly stimulates transcription activators and/or inhibits transcription repressors, probably by inhibition of PP2A. In order to investigate genes that are transcriptionally co-regulated by OA, we developed a software tool we named "StampCollector" that predicts potential transcription factor pairs (TF pairs) involved in the regulation of genes based on their promoter sequences.

Taken together, the results presented in this thesis underline the significance of PP2A in the regulation of cellular events. We combined various approaches in order to characterize the precise role of PP2A and its PR55/B regulatory subunits in gene regulation. Considering the putative role of PP2A in the pathogenesis of human disease, our results may lead eventually to the discovery of therapeutic agents for specifically counteracting PP2A dysfunction.

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… Nicky. I cannot imagine how I would have achieved anything without you. The last 5535 days with you have been just a blast.

# III. Abbreviations

| | |
|---|---|
| Aβ | β-amyloid peptide |
| AD | Alzheimer's disease |
| AGC | Containing PKA, PKG and PKC families |
| AP-1 | Activator protein-1 |
| APC | Anaphase-promoting complex |
| APCC | Adenomateus polyposis coli complex |
| APP | Amyloid precursor protein |
| BRD | Bromodomain kinase |
| CAMK | $Ca^{2+}$/calmodulin-dependent kinase |
| Cav-1 | Caveolin-1 |
| Cdc25 | Cell-division-cycle 25 |
| CMGC | Containing CDK, MAPK, GSK3 and CLK families |
| CDK | Cyclin dependent kinase |
| DARPP-32 | dopamine and adenosine 3', 5'-monophosphate-regulated phosphoprotein |
| DISC | Death inducing signaling complex |
| DNA-PK | DNA-dependent protein kinase |
| DSP | Dual-specific protein tyrosine phosphatase |
| EGFR | Epidermal growth factor receptor |
| ERK | Extracellular signal-regulated kinase |
| FHL2 | Four-and-a-half-LIM-only protein 2 |
| HEAT | Huntingtin/elongation/A subunit/TOR |
| HIV-1 | Human immunodeficiency virus 1 |
| Ig | immunoglobulin |
| IkB | Inhibitor of IkB |
| IKK | IkB kinase |
| IL | Interleukin |
| IFN | Interferon |
| JAK | Janus kinase |
| JNK | c-Jun N-terminal kinase |
| KAP | CDK-associated protein phosphatase |
| KSR | Kinase suppressor of RAS |
| MAP | Microtubule-associated protein |
| MAPK | Mitogen Activated Protein Kinase |
| Mdm2 | Murine double minute 2 |
| MEKK1 | MAPK/ERK Kinase Kinase 1 |
| MKK4 | Mitogen-activated protein kinase kinase 4 |
| MPF | M-phase-promoting factor |
| MSK1 | Mitogen and stress response kinase-1 |
| MT | Microtubules |
| NF-AT | Nuclear factor of activated T cells |
| NFkB | Nuklear factor kappa b |
| NIPP-1 | Nuclear inhibitor of protein phosphatase 1 |

| | |
|---|---|
| NMDA | N-methyl-d-aspartate |
| OA | Okadaic acid |
| PAK | p21-activated kinase |
| PDHK | Pyruvate dehydrogenase kinase |
| PHF | Paired helical filaments |
| PI3K | Phosphatidylinositol 3- Kinase |
| PIAS | Protein Inhibitor of Activated STAT |
| PIKK | PI3K-related kinase |
| PKR | Double-stranded-RNA-dependent protein kinase |
| PKB | Protein kinase B |
| PKC | Protein kinase C |
| PP | Protein phosphatase |
| PPME | Protein phosphatase methylesterase |
| PPMT | Protein phosphatase methyltransferase |
| pRb | Retinoblastoma protein |
| PRMT-1 | Protein Arginine Methyltransferase-1 |
| PSTP | Protein serine-threonine phosphatase |
| PTEN | Phosphatase and tensin homologue deleted from chromosome ten |
| PTP | Protein tyrosine phosphatase |
| SCA | Spinocerebellar Ataxia |
| SCID | Severe combined immunodeficiency |
| SH2 | Src homology domain 2 |
| SHP2 | Src homology domain 2 protein tyrosine phosphatase |
| SMN | Survival of motor neurons |
| STE | Homolog of yeast sterile 7, 11, 20 kinase |
| STAT | Signal transducer and activator of transcription |
| SUMO | Small ubiquitin-related modifier |
| TAK1 | TGFβ-activated kinase 1 |
| TC-PTP | T cell protein tyrosine phosphatase |
| TCR | T cell antigen receptor |
| TGFβ | Transforming growth factor β |
| TOR | Target of rapamycin |
| TPPII | Tripeptidyl peptidase II |
| TPR | Tetratrico-peptide repeat |
| TRIP-1 | TGF-β receptor II interacting protein-1 |
| Ub | Ubiquitin |
| UBC9 | Ubiquitin conjugating enzyme 9 |

# IV. General Introduction

**Reversible Phosphorylation**

The reversible phosphorylation of proteins controlled by protein kinases and protein phosphatases is a major regulation mechanism in all eucaryotic cells. All known protein phosphatases reverse the function of protein kinases in intracellular processes of eucaryotic cells by catalyzing the dephosphorylation of posttranslational modified serine, threonine and tyrosine residues in phosphoproteins (Fig. 1).

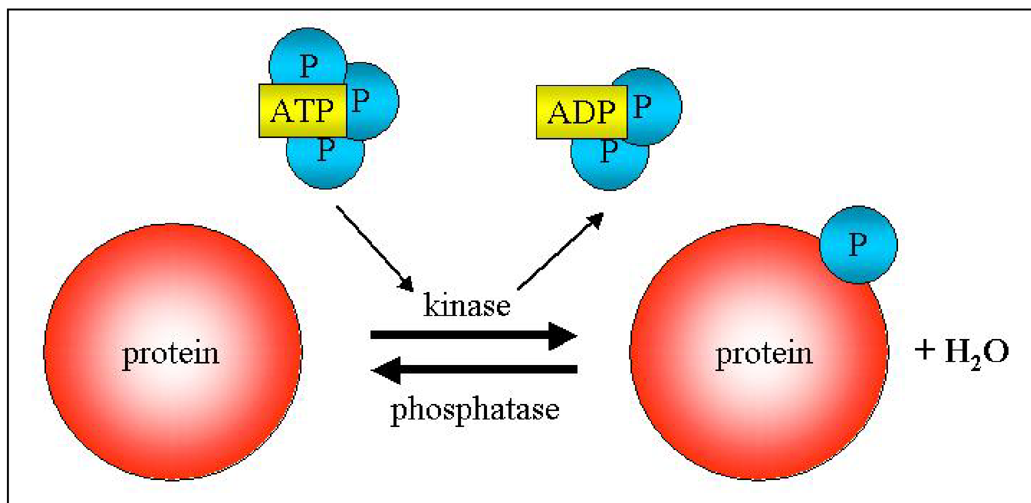


**Fig. 1**: Reversible protein phosphorylation involves the function of protein kinases and protein phosphatases.

**Protein Kinases**

The human genome sequence has revealed that estimations from other species (*Saccharomyces cerevisiae* and *Caenorhabditis elegans*) of the total number of genes, and consequently the number of genes encoding protein kinases must be corrected downward. Of the 30,000 genes present in the human genome, about 518 are encoded as protein kinases. In the human kinome map they are clustered into 7 functional groups primarily by sequence comparison of their catalytic domains, aided by sequence similarity and domain structure outside of the catalytic domains, known biological functions, and a similar classification of the yeast, worm, and fly kinomes (Table 1) (Manning *et al.*,

2002a; Manning *et al.*, 2002b). Protein kinases control protein activity by catalyzing the addition of a negatively charged phosphate group to other proteins. They modulate a wide variety of biological processes, especially those carrying signals from the cell membrane to intracellular targets, and coordinate complex biological function. Based on their substrate specificity, protein kinases are divided into three major groups: (i) the protein serine/threonine kinases, (ii) the protein tyrosine kinases, and (iii) the dual-specific protein kinases that phosphorylate serine, threonine and tyrosine residues. As many as 90% of phosphorylation events occur on serine, about 10% on threonine and less then 1% on tyrosine residues. The level of tyrosine phosphorylation is often higher in virus-infected cells, linking tyrosine phosphorylation to cell proliferation and transformation (Sefton *et al.*, 1981). In agreement with this, many receptor tyrosine kinases, which comprise most of the growth factor receptors, have been identified as oncogenes (Hunter and Cooper, 1985).

| Group | Families | Subfamilies | Yeast kinases | Worm kinases | Fly kinases | Human kinases | Human pseudogenes | Novel human kinases |
|---|---|---|---|---|---|---|---|---|
| AGC | 14 | 21 | 17 | 30 | 30 | **63** | 6 | 7 |
| CAMK | 17 | 33 | 21 | 46 | 32 | **74** | 39 | 10 |
| CK1 | 3 | 5 | 4 | 85 | 10 | **12** | 5 | 2 |
| CMGC | 8 | 24 | 21 | 49 | 33 | **61** | 12 | 3 |
| Other | 37 | 39 | 38 | 67 | 45 | **83** | 21 | 23 |
| STE | 3 | 13 | 14 | 25 | 18 | **47** | 6 | 4 |
| Tyr kinase | 30 | 30 | 0 | 90 | 32 | **90** | 5 | 5 |
| Tyr kinase-like | 7 | 13 | 0 | 15 | 17 | **43** | 6 | 5 |
| RGC | 1 | 1 | 0 | 27 | 6 | **5** | 3 | 0 |
| Atypical-PDHK | 1 | 1 | 2 | 1 | 1 | **5** | 0 | 0 |
| Atypical-Alpha | 1 | 2 | 0 | 4 | 1 | **6** | 0 | 0 |
| Atypical-RIO | 1 | 3 | 2 | 3 | 3 | **3** | 1 | 2 |
| Atypical-A6 | 1 | 1 | 1 | 2 | 1 | **2** | 2 | 0 |
| Atypical-Other | 7 | 7 | 2 | 1 | 2 | **9** | 0 | 4 |
| Atypical-ABC1 | 1 | 1 | 3 | 3 | 3 | **5** | 0 | 5 |
| Atypical-BRD | 1 | 1 | 0 | 1 | 1 | **4** | 0 | 1 |
| Atypical-PIKK | 1 | 6 | 5 | 5 | 5 | **6** | 0 | 0 |
| Total | 134 | 201 | 130 | 454 | 240 | **518** | 106 | 71 |

**Table 1:** Kinase distribution by major groups in human and model systems (taken from (Manning et al., 2002b)).

**Protein Phosphatases**

About 150 genes in the human genome encode protein phosphatases, including up to 40 protein serine/threonine phosphatases (Cohen, 2002). Protein phosphatases are classified into the broad classes of (i) the protein serine/threonine phosphatases and (ii) the protein tyrosine phosphatases, which include the dual-specific phosphatases dephosphorylating serine, threonine and tyrosine. It is now possible to discern several major subgroups in both classes (Fig.2).

The abundant functions of protein phosphatases and their involvement in major signal transduction pathways make them important targets in the investigation of many different diseases. Defective or inappropriate signal transduction can result, for example, in diabetes, cancer and immune dysfunction. Also very important is phosphatase interaction with brain-specific phosphoproteins, the dysfunction of which may lead to severe disorders such as Alzheimer's. Since deregulated protein kinases may function as dominant oncogenes, some protein phosphatases are expected to function as tumor suppressors. Some properties of major representatives of each subgroup will be discussed to illustrate their known structures and physiological roles (see Table 4).

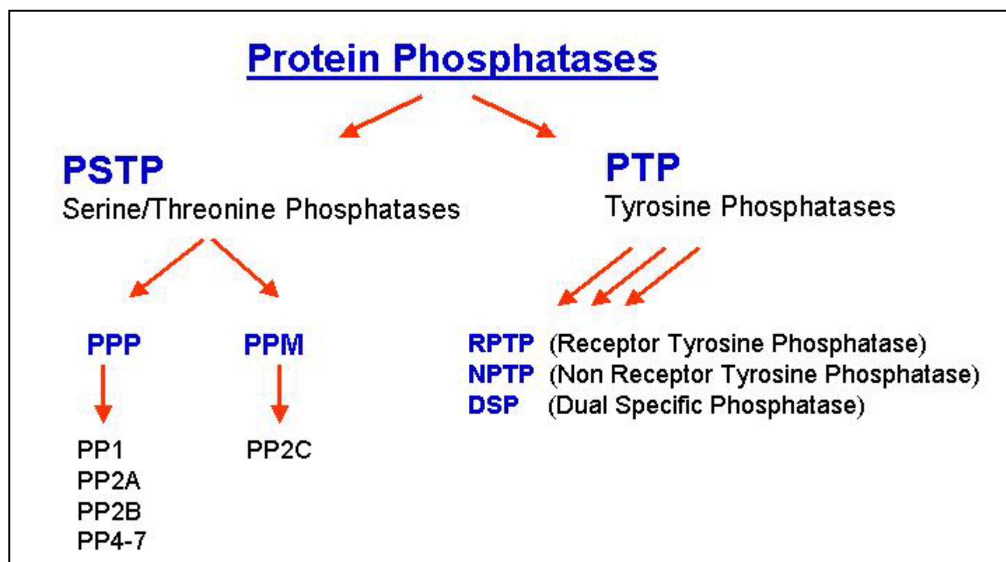


**Fig. 2:** Protein phosphatases are assigned to two classes, protein serine-threonine (PSTP) and protein tyrosine phosphatases (PTP). The PSTP class is further subdivided into the PPP and PPM families, and the PTP family consists of receptor- (RPTP), non receptor- (NPTP) and dual-specific protein tyrosine phosphatases (DSP).

## Protein Serine/Threonine Phosphatases (PSTP)

The major PSTPs were initially classified into two groups based on their ability to dephosphorylate the β subunit (type 1: PP1) or the α subunit (type 2: PP2A, PP2B and PP2C) of phosphorylase kinase. Another criterium was their sensitivity to inhibition by protein inhibitors 1 (I-1) and 2 (I-2) (type 1: sensitive, type 2: insensitive). The type 2 phosphatases were further characterized by their substrate specificity, divalent cation dependency for activity and mechanisms of regulation. A more recent classification into the *PPP* (PP1, PP2A, PP2B) and the *PPM* subfamilies (PP2C) is based on the sequence homology of their catalytic subunits (Fig.3 and Fig. 4). A growing number of minor human protein phosphatases (PP4, PP5, PP6 and PP7) have been identified recently that belong to the *PPP* subfamily.

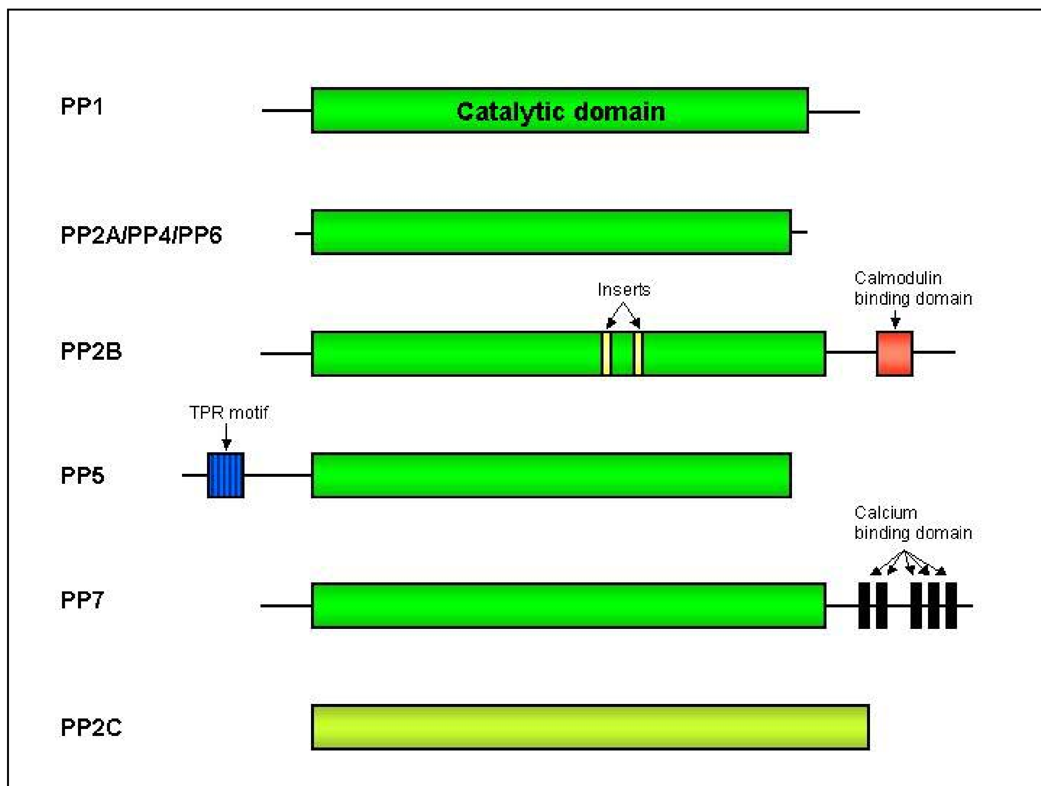


**Fig. 3:** The protein serine-threonine phosphatases are classified into the PPP (PP1, PP2A, PP2B, PP4-7) and the PPM (PP2C) subfamilies based on the sequence homology of their catalytic subunits.

## *PPP* Subfamily

**Protein phosphatase 1** (PP1 or *PPP1*) is an iron-zinc metalloenzyme present in the cytoplasm and the nucleus of eucaryotic cells (reviewed in (Ceulemans and Bollen, 2004; Cohen, 2002; Wera and Hemmings, 1995)). PP1 is involved in a variety of cellular processes, including dephosphorylation of several metabolic enzymes, regulation of glycogen metabolism (Suzuki *et al.*, 2001), muscle contraction (Carr *et al.*, 2002), $Ca^{2+}$-channels (Brown *et al.*, 2000), protein synthesis (Keller and Krude, 2000), cell division and meiosis (Bayliss et al., 2003; Margolis et al., 2003). Three different genes give rise to the four isoforms (α,β,γ1,γ2) encoding the 37-kDa catalytic subunit in mammalian cells. The three gene products share an amino acid sequence identity of >90% and are dispersed at different locations in the genome. The catalytic subunit (PP1c) exists as a heterodimer *in vivo* and has a distinct tissue distribution and subcellular localization. The crystal structures of PP1γ1 in complex with tungstate (Egloff et al., 1995) or PP1α in complex with microcystin (Goldberg et al., 1995) have been solved revealing important structural mechanisms of the regulation of PPP family members. More than 50 dimerization partners of PP1c have been identified to date that target a specific PP1 dimer to its restricted subcellular location and have distinct substrate specificities and diverse regulation. While only a small fraction of PP1 is inhibited by phosphorylation during the cell cycle, most forms of regulation and interaction are achieved through the regulatory subunits. This interaction occurs mainly through a short, conserved RVxF-binding motif initially identified in studies of the glycogen-targeting subunits (Cohen, 2002). A well-studied example is the dimer PP1G containing the PP1c subunit and a glycogen-binding G subunit (Stralfors *et al.*, 1985). This dimer regulates the dephosphorylation of glycogen phosphorylase and glycogen synthase. The G-subunit targets PP1 towards glycogen or sarcoplasmic reticulum and regulates cardiac muscle relaxation (Hubbard and Cohen, 1989; Hubbard *et al.*, 1990). The expression of PP1c in the brain plays a major role in neuronal development, signaling mechanisms and modulation of neuronal activity. The four isoforms of PP1c are expressed in different regions of the brain and are targeted to different neuronal cytoskeletal structures with high specificity. PP1c binds directly to

DARPP-32 (dopamine and adenosine 3', 5'-monophosphate-regulated phosphoprotein), an isoform of protein inhibitor-1 (I-1), and is involved in the PKA/DARPP-32/PP1 signaling cascade that regulates the functional status of neostriatal neurons (Yan *et al.*, 1999). In chromatin, PP1c is complexed with the RNA-binding, 39-kDa PP1 inhibitor NIPP-1 that was identified as a nuclear inhibitor of PP1c (Jagiello et al., 1997; Van Eynde et al., 1995). Phosphorylation of NIPP-1 by PKA disrupts the complex and activates PP1. This activating phosphorylation can be reversed by PP2A, suggesting that PP1 is regulated by PP2A (Elson, 1999). PP1 is also involved in regulating entry into M phase. The relevant mechanism responsible for inhibition of PP1 activity and successive prevention to enter M phase was not known until recently (Margolis et al., 2003) and will be described in more detail in the chapter "Cell Cycle Regulation by PP2A".

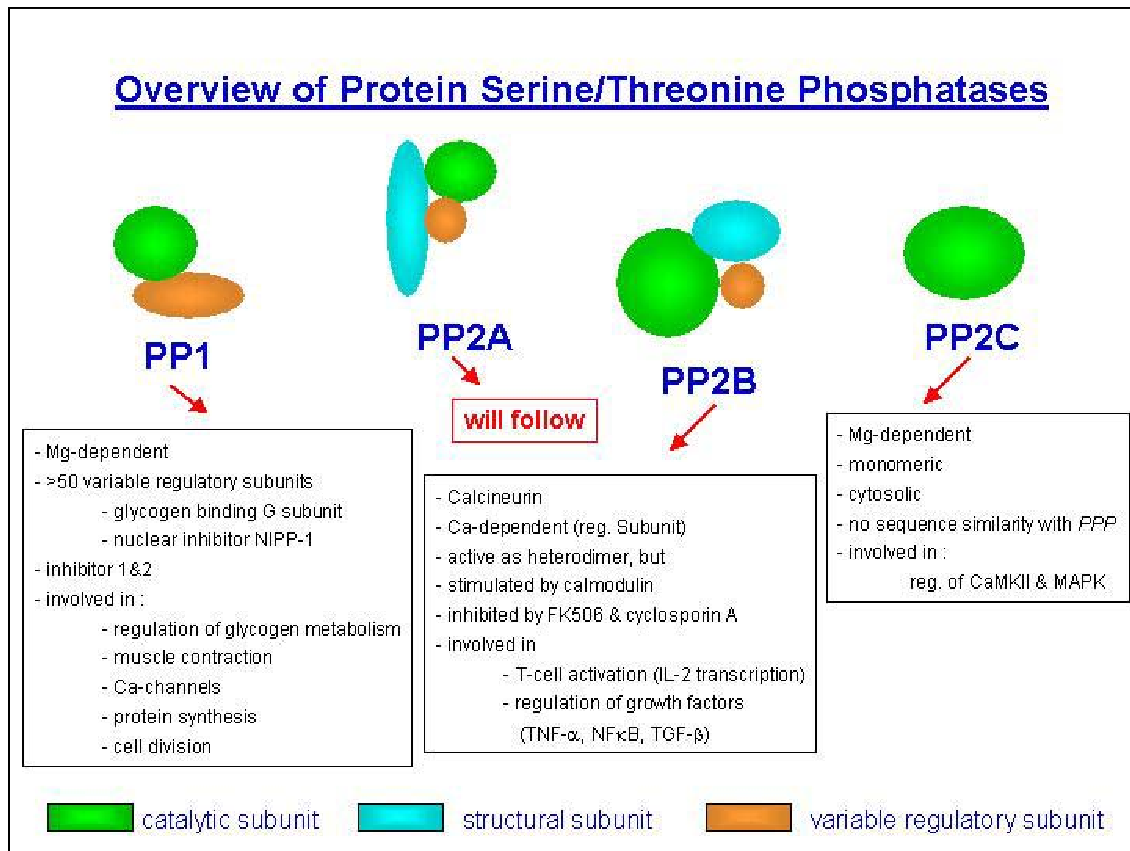


**Fig. 4:** Holoenzyme assembly of the four most abundant serine-threonine phosphatases.

**Protein phosphatase 2A**

Will be discussed in a later chapter (Structure and Regulation of PP2A).

**Protein phosphatase 2B** (PP2B, *PPP3* or calcineurin) is a $Ca^{2+}$-dependent PSTP with a narrower substrate specificity than PP1, PP2A and PP2C. This calmodulin-stimulated phosphatase is abundant in the brain (1% of total brain protein) but is also present in other tissues and is highly conserved between species. It is the only PSTP under $Ca^{2+}$/calmodulin control and, therefore, important for coupling $Ca^{2+}$ signals to cell responses (Klee *et al.*, 1998). PP2B is active as a heterodimer containing a 60-kDa catalytic subunit (calcineurin A) and a 19-kDa $Ca^{2+}$-binding regulatory subunit (calcineurin B). Three genes producing alternative splice variants encode six isoforms of the catalytic subunit (α1, α2, β1, β2, β3, γ) that share an amino acid sequence identity of 80% and also a high similarity with the catalytic subunits of PP1 and PP2A. The isoforms differ mainly at the C-terminus, which may explain their different functions, since the C-terminus is involved in the regulation of activity in the absence of $Ca^{2+}$ and calmodulin. The α and β isoforms are expressed in all tissues, with the highest level in the brain, whereas γ is expressed specifically in testis. All β isoforms contain a proline-rich region with 11 consecutive proline residues close to the N-terminus. The crystal structure identifies PP2B as an iron-zinc metallo-enzyme, since the catalytic subunit contains a binuclear metal center (Egloff *et al.*, 1995; Kissinger *et al.*, 1995). PP1 and PP2A are also metallo-enzymes with two metal atoms at the active site. Calcineurin B is encoded by two different genes giving rise to three isoforms: α1 is expressed in the brain as well as in other tissues, while α2 and β are testis specific. On the basis of the four $Ca^{2+}$- binding domains, the regulatory subunit was originally identified as 'EF-hand' $Ca^{2+}$-binding protein. The regulatory subunit also shares 35% sequence identity with calmodulin, which increases the activity of PP2B 20-fold when complexed with the catalytic subunit to form a heterotrimer. PP2B regulates the function of growth factors like interleukin-2, TNFα, NF-κB and TGFβ. It also promotes binding of hyperphosphorylated tau to the microtubule and inhibits the release of neurotransmitters and desensitizes postsynaptic NMDA receptor-coupled $Ca^{2+}$-channels in neuronal cells (Lieberman and Mody, 1994).

Activation of the T-cell receptor by PP2B drives transcription of IL-2 and, subsequently, T-cell proliferation. The two pathways necessary for transactivation of the IL-2 gene can be activated by $Ca^{2+}$ ionophores and phorbol esters (Nolan, 1994). The $Ca^{2+}$- dependent pathway is mediated via activation of PP2B, which leads to dephosphorylation of the transcription factor NF-AT. Upon dephosphorylation, NF-AT is translocated to the nucleus, where it binds and activates the IL-2 promoter (Crabtree and Olson, 2002). PP2B is a target for two immunosuppressive drugs, cyclosporine A and FK506. Both immunosuppressants block T-cell activation and suppress cardiac hypertrophy when bound to PP2B as complexes with the immunophilin proteins cyclophilin and FKBP12, respectively. Inhibition of PP2B activity blocks NF-AT activation and leads to immunosuppression (Molkentin *et al.*, 1998).

**Protein phosphatase 4** (PP4 or *PPP4*) is found in the cytoplasm and to a higher extent in the nucleus of all mammalian cells (Brewis and Cohen, 1992). PP4 associates with centrosomes, where it may participate in the initiation of microtubule growth and organization. Analysis of the homologue in *C. elegans* by RNA-mediated interference showed that PPP4c is also essential for formation of the mitotic spindle in mitosis and is required for sperm meiosis (Sumiyoshi *et al.*, 2002). The 35-kDa catalytic subunit shows 65% identity to PP2A and a 45% identity to PP1. Furthermore, the human catalytic subunit is 100% identical to the mouse protein sequence and 94% identical to the *Drosophila* protein. This high degree of sequence conservation suggests that PP4 performs a critical function in the cell.

PPP4 exists as high molecular mass complexes of 450-600 kDa, and two putative regulatory subunits have been identified to date: PPP1R1 (Kloeker and Wadzinski, 1999) and R2 (Hastie *et al.*, 2000). The 105-kDa regulatory subunit PPP4R1 contains 13 non-identical repeats similar to the 15 HEAT-repeats of the regulatory PR65/A subunit of PP2A. Although the structure of the PPP4 dimer is similar to the core AC unit of PP2A, the catalytic subunit of PP4 is unable to form a complex with the PR65/A subunit.

The 50-kDa PP1R2 subunit targets the phosphatase to the centrosomes, suggesting that PPP4R2 also regulates the activity of PPP4c at centrosomal microtubule organizing centers (Hastie *et al.*, 2000). Two novel `variable' regulatory subunit(s) Gemin3 and/or

Gemin4 have been identified that are components of the SMN complex. These two proteins were identified previously as components of the Survival of Motor Neurons (SMN) protein complex, which is functionally defective in the hereditary disorder spinal muscular atrophy (Hastie et al., 2000; Melki, 1997).

One known function of PP4 is the association with and activation of the two transcription factors c-Rel and NF-κB (Hu *et al.*, 1998). Similar to PP2A, PP4 forms a phosphorylation-independent complex with the α4 phosphoprotein. This complex is believed to be involved in a rapamycin-sensitive pathway that may regulate translation in response to nutrient availability (Chen *et al.*, 1998; Kloeker *et al.*, 2003).

**Protein phosphatase 5** (PP5 or *PPP5*) is a PSTP abundant in the nucleus and cytoplasm of mammalian, *Drosophila* and yeast cells (Chen *et al.*, 1994b). The 58-kDa catalytic subunit is related to that of PP1, PP2A and PP2B but has an N-terminal extension of 200 amino acids that serves both regulatory and targeting functions (reviewed in (Chinkers, 2001). This extension contains three tetratrico-peptide repeat (TPR) motifs that have also been found in proteins required for mitosis, transcription and mRNA splicing (Goebl and Yanagida, 1991; Lamb *et al.*, 1995). The repeats are involved in protein-protein interactions by forming a scaffold-like structure and are thought to target PP5 to its site of action. In addition, the TPR repeats are responsible for stimulating the phosphatase activity >25-fold by poly-unsaturated fatty acids such as arachidonic acid (Chen and Cohen, 1997; Sinclair et al., 1999; Skinner et al., 1997).

PP5 is involved in the regulation of ribosomal RNA transcription by regulating RNA polymerase II activity in the nucleus. This enzyme also promotes cellular proliferation by binding to Hsp90 in the glucocorticoid/Hsp90/p23-heterocomplex and inhibiting and/or activating glucocorticoid-induced signaling pathways (Chen *et al.*, 1996; Zuo *et al.*, 1999). Putative roles for PP5 in cell cycle regulation (Chinkers, 2001) include promoting progression into S-phase by dephosphorylating p53 and regulating progression through mitosis by binding to CDC16 and CDC27 of the anaphase-promoting complex (APC). In view of these functions, it is assumed that PP5 could have an effect on the development of cancer cells. PP5 has been suggested to be involved in dephosphorylation of tau protein *in vitro* and it is assumed that that PP5 can also dephosphorylate tau *in vivo*, since a small

pool of PP5 is also associated with microtubules in PC12 cells and in neurons of postmortem human brain tissue. These results suggest that PP5 might be involved in the molecular pathogenesis of Alzheimer's disease (Gong et al., 2004; Liu et al., 2002a). PP5 interacts with active forms of Galpha(12) and Galpha(13) through its TPR domain and its phosphatase activity gets activated about 2.5-fold. In addition, the active form of Galpha(12) translocates PP5 to the cell periphery and colocalizes with PP5 proposing a new signaling pathway of G(12) family G proteins (Yamaguchi et al., 2002).

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**Protein phosphatase 6** (PP6 or *PPP6*) is a PP2A-like PSTP initially identified in yeast cells. The PP6 35-kDa catalytic subunit is highly conserved between species and shares 57% amino acid sequence identity with PP2A. PP6 is expressed in testis, heart and skeletal muscle in humans. PP6 interacts, like PP2A and PP4, with the α4 protein, suggesting that these phosphatases influence the mammalian rapamycin-sensitive pathway mediated by mTOR (Chen *et al.*, 1998). PP6 has been identified in a complex with spliceosomal small nuclear ribonucleoproteins in lymphocyte extracts and is regulated by IL-2 in peripheral blood T cells, suggesting that PP6 is a component of a signaling pathway regulating cell cycle progression in response to IL-2 receptor stimulation (Filali et al., 1999).

**Protein phosphatase 7** (PPEF, PP7 or *PPP7*) is abundant in the retina and shows 35% similarity to other PSTPs. The 75-kDa enzyme, exclusively detected in retina and retinal-derived retinoblastoma cells, has unique N- and C-terminal regions. The C-terminal region contains five $Ca^{2+}$-binding sites (EF-hand motifs) but its structure is distinct from that of PP2B. The function of the N-terminal domain is not yet known. Recombinant PP7 is $Mg^{2+}$-dependent and activated by calcium through the EF-hand motifs. It is assumed that PP7 is involved in a genetic disorder called retinitis pigmentosa (RP) that results in degeneration of photoreceptor cells (Huang and Honkanen, 1998).

## *PPM* Subfamily

**Protein phosphatase 2C** (PP2C or *PPM1*) is a monomeric PSTP that does not form complexes with regulatory subunits. It shares sequence similarity with the other phosphatases over a limited 80-amino-acid region. Thus, it is assumed that the *PPP* and the *PPM* phosphatases are derived from different ancestral genes and may represent an example of convergent evolution in enzyme structure/function.

Encoded by at least 10 different genes in mammalian cells, the PP2C isoforms share 20-76% sequence identity. In addition, two splice variants of PP2Cα and six splice variants of PP2Cβ have been identified. All 10 distinct PP2C gene products, including PP2Cη, share six conserved motifs and have $Mg^{2+}$- and/or $Mn^{2+}$-dependent protein phosphatase activities against artificial substrates *in vitro* (Komaki *et al.*, 2003). The catalytic subunits of PP2C α, β and γ are $Mg^{2+}$ dependent, while the δ isoform is inhibited by $Mg^{2+}$. Unlike PP2Cβ1, the β2 subtype is not widely expressed but is specifically found in brain and heart. PP2Cγ is mostly present in skeletal muscle, heart and testis. It is distinguishable from the other isoforms by its 54-residue acidic domain, 75% of which are glutamate and aspartate residues. The PP2Cδ isoform shares 30% amino acid sequence identity to the other PP2C isoforms but lacks 90-amino-acid domains at the C-terminal sequence usually conserved in other PP2C isoforms.

PP2Cε is composed of 303 amino acids, and the overall similarity of amino acid sequence between PP2Cε and PP2Cα is 26%. PP2Cε inhibits the IL-1- and TAK1-induced activation of the mitogen-activated protein kinase kinase 4 (MKK4)-c-Jun N-terminal kinase or MKK3-p38 signaling pathway. This suggests that, in the absence of an IL-1-induced signal, PP2Cε helps maintain the TGFβ-activated kinase 1 (TAK1) signaling pathway in an inactive state by associating with and dephosphorylating TAK1 (Li et al., 2003b).

PP2Cζ, which is composed of 507 amino acids, has a unique N-terminal region and is specifically expressed in testicular germ cells. The overall similarity of the amino acid sequence between PP2Cζ and PP2Cα is 22%. PP2Cζ is able to associate with ubiquitin conjugating enzyme 9 (UBC9) and the association is enhanced by co-expression of small ubiquitin-related modifier-1 (SUMO-1), suggesting that PP2Cζ exhibits its specific role

through its SUMO-induced recruitment to UBC9 (Kashiwaba *et al.*, 2003). Although little is known about the regulation of PP2C, its activity is stimulated by unsaturated fatty acids. PP2C is able to dephosphorylate DARPP-32 at Ser137, which then no longer inhibits the dephosphorylation of Thr34, the regulatory site for inhibiting activity of DARPP-32 by PP2B. This process activates PP1, which is inhibited by Thr34 phosphorylation of DARPP-32 (Huang *et al.*, 1999). Calcium-calmodulin kinase II (CaMKII) is possibly a neuronal substrate for PP2C. It is dephosphorylated by PP2C at its autophosphorylation site (Fukunaga *et al.*, 1993).

**Protein Tyrosine Phosphatases (PTP)**

The protein tyrosine phosphatases are intracellular and integral membrane phosphatases that dephosphorylate protein tyrosine residues. Tyrosine phosphorylation is known to be a control mechanism for growth, differentiation, metabolism, cell cycle regulation and cytoskeletal function (reviewed in (Andersen *et al.*, 2001)). PTPs are abundant in mammalian and *Drosophila* cells and show positive and negative effects in various eucaryotic signal transduction pathways. The protein tyrosine phosphatases are classified into receptor (RPTP) or transmembrane PTPs, non-receptor (NPTP), also known as intracellular protein tyrosine phosphatases, and the dual-specific phosphatases (DSP). A further classification is based on their overall structure (Fig. 5). Since the identification of the first RPTP (CD45) and NPTP (PTP1B) in 1988, more than 100 PTPs have been cloned. Both groups of PTPs consist of at least one intracellular, conserved catalytic domain (~240 residues) harboring a consensus sequence ([I/V]HCxAGxxR[S/T]G), followed or preceded by a regulatory domain. The consensus PTP sequence is defined by these 11 amino acids, including the absolutely conserved cysteine residue necessary for phosphatase activity. PTPs dephosphorylate the autophosphorylated insulin receptor and epidermal growth factor receptor (EGF-R) *in vitro* as well as many other substrates. In addition, they function as "second messengers" of growth factor pathways and play an important role in neuronal development (reviewed in (Stoker and Dutta, 1998)).

**Receptor Tyrosine Phosphatase Subfamily (RPTP)**

RPTPs consist of an extracellular variable N-terminal domain, a transmembrane domain and an intracellular region. The intracellular variable region is followed by one or two catalytic phosphatase domains separated by 50-100 amino acids. The catalytic domain (D1) adjacent to the membrane provides the main phosphatase activity, while the second catalytic domain (D2) may be involved in the regulation of enzyme activation, protein-protein interaction, substrate specificity and presentation of substrates to the active catalytic domain. The RPTPs are classified based on structural differences in the extracellular domain. Six distinct groups of extracellular domains have been identified:

**Type I**, represented by the CD45 (PTPRC) family, is exclusively expressed in hematopoietic cells. It is heavily glycosylated and has a cysteine-rich region next to a fibronectin (FN III)-like region in the extracellular domain. CD45 dephosphorylates members of the Src-tyrosine kinase family and induces T-cell activation. Dimerized CD45 is inactive and subsequently inhibits T cell signaling. Mutation or deletion in the CD45 gene leads to severe combined immunodeficiency disease (SCID) in humans and T- and B-lymphocyte dysfunction (Kung *et al.*, 2000). CD45 can also function as a Janus kinase (JAK) tyrosine phosphatase that negatively regulates cytokine receptor signaling involved in the differentiation, proliferation, and antiviral immunity of haematopoietic cells (Irie-Sasaki et al., 2001; Irie-Sasaki et al., 2003).

**Type II** RPTPs have one to three extracellular immunoglobulin (Ig)-like domains, in addition to the two intracellular domains, followed by up to 10 fibronectin III (FNIII)-like domains. RPTPμ (PTPRM), κ, λ and ψ share an additional N-terminal meprin/A5/PTPmu (MAM) domain. Because of this pattern, the type II tyrosine phosphatases, like the neuronal-expressed LAR (PTPRF) (leukocyte antigen-related) and RPTPμ, are believed to function as cell-adhesion receptors, regulating tyrosine dephosphorylation in response to cell contact. An isoform of LAR has been identified as a receptor for the laminin/nidogen complex and may affect actin cytoskeleton structure, resulting in morphological changes in the cell (O'Grady *et al.*, 1998). It was suggested, that density-dependent regulation of LAR

expression is mediated by functional E-cadherin and may play a role in density-dependent contact inhibition by regulating tyrosine phosphorylation in E-cadherin complexes (Symons et al., 2002).

**Type III** phosphatase RPTPβ (PTPRB) contains only a single catalytic domain and an extracellular region with multiple FN III-like repeats. This extracellular domain functions as a ligand for neuronal receptor complexes, interleukin and growth hormones. The structurally related SAP-1 (PTPRH) consists of eight FN III-like repeats and multiple N-glycosylation sites. SAP-1 is mainly expressed in brain and liver, but not in pancreas or colon. In contrast, SAP-1 is highly expressed in colon and pancreas cancer cell lines and in a high percentage of surgically excised colorectal cancers (Matozaki *et al.*, 1994). SAP-1 induces apoptotic cell death by inhibition of cell survival signaling mediated by several kinases (PI 3-kinase, PKB, and ILK) and activation of a caspase-dependent proapoptotic pathway (Takada et al., 2002). In addition, SAP-1 plays also a potential role in hepatocarcinogenesis. SAP-1 expression is downregulated during the dedifferentiation of human hepatocellular carcinoma and therefore may play a causal role in disease progression (Nagano et al., 2003).

**Type IV** phosphatases consist of the two catalytic domains and a glycosylated extracellular region. In order to function as a receptor, the enzymes need to associate with proteins that have receptor-binding domains. Like other RPTPs, RPTPα (PTPRA) is able to form inactive hetero- and homodimers, which may be important for down-regulation of the phosphatase activity. RPTPα is assumed to dephosphorylate the Src proto-oncogene, thereby activating Src (Jiang *et al.*, 1999). Catalytically defective RPTPα shows reduced binding to Src, providing another potential checkpoint for physiological regulation of the Src-family pathway. RPTPε (PTPRE) plays a role in tumorigenesis in a manner consistent with its upregulation in mammary tumors induced by the oncogenes ras and neu (Elson, 1999).

**Type V** enzymes represented by RPTPζ (PTPRZ1) and RPTPγ (PTPRG) have a regulatory carbonic anhydrase (CA)-like region and a single FN III-like domain in

addition to the two catalytic domains. Similar to the type III phosphatases, RPTPζ functions as a ligand for neuronal receptor complexes. The CA-like domain and the FN III-like domain are assumed to be necessary for binding to the contactin-protein complexes. RPTPγ is believed to be a tumor suppressor gene, since it is located in a region frequently deleted in different types of renal and lung cancer (Panagopoulos *et al.*, 1996).

**Type VI** phosphatases are expressed in the brain and pancreas and contain a unique N-terminal extracellular domain with four cysteines and one intracellular catalytic domain. IA-2 (PTPRN) (islet cell antigen 512) and its homologue IA-2 beta (PTPRN2) are important autoantigens associated with insulin-dependent diabetes melitus (type 1 diabetes) (Bonifacio et al., 1998; Hanifi-Moghaddam et al., 2003).

**Non Receptor Tyrosine Phosphatase Subfamily (NPTP)**

The non-receptor tyrosine phosphatases are found in the cytosol and consist of a conserved catalytic domain followed by a variable regulatory domain. While the catalytic domain is responsible for dephosphorylation of the target protein, the regulatory domain is involved in modulation of activity, subcellular localization and interaction with other proteins and substrates. NPTPs are mostly involved in mitogenic signaling pathways, where they dephosphorylate proteins localized between the membrane and the cytoskeleton.

**PTP1B** (PTP1N), the prototype of cytoplasmic tyrosine phosphatases, has a regulatory domain that is necessary for localization to the endoplasmic reticulum (ER). Proteolytic cleavage of PTP1B leads to translocation of the catalytic domain from the membrane to the cytosol and subsequently to an increase in phosphatase activity. The crystal structure reveals, that the catalytic domain consists of a central twisted, mixed β sheet flanked by α helices. The conserved cysteinyl residue of the signature motif is located within a single loop at the base of a cleft on the protein surface (Barford et al., 1994). PTP1B was recently identified as a negative regulator of the insulin-signaling pathway, suggesting that

inhibitors of PTP1B support the treatment of type 2 diabetes (Harley and Levens, 2003; Kennedy, 1999).

**SHP1** (PTPN6) and SHP2 (PTPN11) phosphatases contain two Src homology-2 (SH2) domains N-terminal to the catalytic domain. These SH2 domains associate with phosphorylated tyrosine residues on target proteins and form active complexes. Elucidation of the crystal structure of SHP2 revealed how the two SH2 domains regulate the catalytic activity. The N-terminal SH2 domain acts as a conformational switch by binding and inhibiting the enzyme, or by binding phosphoproteins and activating the phosphatase. The second or C-terminal SH-2 domain is not involved in activation of the phosphatase, but contributes binding energy and specificity. SHP1 is highly expressed in hematopoietic cells, while SHP2 is expressed ubiquitously. Although the phosphatases share 55% sequence identity, they have distinct biological roles. SHP1 is a negative regulator of hematopoietic signaling pathways downstream of cytokine receptors, oligomeric receptors and receptor tyrosine kinase. SHP2 is involved in the MAP kinase (MAPK) signaling pathway. The autophosphorylated epidermal growth factor receptor (EGFR) activates the MAPK signaling pathway until it is dephosphorylated and subsequently deactivated by SHP2. MAPK in turn deactivates SHP2 by phosphorylation on threonine, thus closing an autoregulatory positive feedback loop. Interaction of EGFR and SHP2 in a growth-factor signaling pathway (Agazie and Hayman, 2003) is assumed to be involved in the development of defective cardiac semilunar valvulogenesis, an aortic valve disease (Chen *et al.*, 2000).

**PTP-PEST** (PTPN12) was found to be a PTP that may provide proline-rich binding sites in its PEST domain for SH3- or WW-domain containing proteins such as $p130^{Cas}$. The PEST motif is also assumed to be involved in protein degradation, since it has been identified in proteins with a short half-life. Dephosphorylation of $p130^{Cas}$ may be one of the physiological roles of PTP-PEST, controlling tyrosine-dependent signaling events and cell migration (Garton and Tonks, 1999). Serine phosphorylation of ShcA controls the ability of its phosphotyrosine-binding domain to bind PTP-PEST, which is responsible for the

dephosphorylation and down-regulation of ShcA after insulin stimulation (Faisal et al., 2002).

**PTPH1** (PTPN3) and PTP-MEG (PTPN4) have cytoskeleton-associated regulatory motifs that are also found in band 4.1, ezrin-, moesin-, radixin- and talin-directed binding of PTP to the interface between plasma membrane and cytoskeleton structures (Takeuchi *et al.*, 1994).

**Dual-Specific Phosphatase Subfamily (DSP)**

Dual-specific phosphatases are able to dephosphorylate tyrosine, as well as threonine and serine residues of phosphoproteins. The structure of these enzymes is more related to the PTPs, since they all exhibit the PTP fingerprint sequence containing the conserved cysteine residue.

**VH1** was the first identified DSP, encoded by the late H1 gene of the vaccinia virus, which dephosphorylates serine and tyrosine residues in a viral histone-like protein.
VHR (DUSP3), a VH1-related DSP, has been identified as a negative regulator of extracellular regulated kinases such as ERK1, ERK2 and JNK (Todd et al., 2002). VHR is constitutively expressed, localized to the nucleus (Todd *et al.*, 1999) and gets activated by ZAP70 kinase, a key component of the signaling machinery for the T cell antigen receptor (TCR) (Alonso et al., 2003). DUSP5 has been suggested to be a direct target of p53, in a novel mechanism by which p53 might negatively regulate cell-cycle progression by downregulating mitogen- or stress-activated protein kinases (Ueda et al., 2003).

**MKP-1** (DUSP1) is a VH1-related MAP kinase phosphatase involved in signaling of the mitogen-activated protein kinase. This is expressed in human skin fibroblasts and dephosphorylates the threonine and tyrosine residues of activated MAP kinases, thus inactivating them (Hirsch and Stork, 1997). In converse, activated ERK1/2 can trigger MKP-1 degradation via the ubiquitin-proteasome pathway, thus facilitating long-term activation of ERK1/2 against cytotoxicity (Lin et al., 2003).

**Cdc25** (CDC25C) with its three human isoforms (cell-division-cycle 25 A, B, C) and multiple splice variants is involved in the control of the cell cycle by dephosphorylating the threonine and tyrosine residues of cyclin-dependent kinases. *Cdc25A is* a potential human oncogene based on its ability to transform primary mouse embryo fibroblasts in cooperation with activated Ras or loss of *RB* (Galaktionov et al., 1995). In addition, *Cdc25A* and *Cdc25B* are overexpressed in a variety of human cancers (Broggini et al., 2000). *Cdc25A* is involved in the control of the G1/S transition, *Cdc25B* is active during $G_2$ phase, before *Cdc25C* is activated at the $G_2$/M transition) (Bulavin et al., 2003; Giles et al., 2003) (see also "Cell Cycle Regulation by PP2A" and (Margolis et al., 2003)). *Cdc25B* may trigger the activation of an auto-amplification loop required for entry into mitosis before being degraded (Nilsson and Hoffmann, 2000).

**PTEN** (phosphatase and tensin homologue deleted from chromosome ten) is a tumor suppressor gene, also called MMAC (mutated in multiple advanced cancers) or TEP-1 (TGF-β-regulated and epithelial cell-enriched phosphatase). It encodes a dual-specific protein phosphatase/lipid phosphatase that modulates signal transduction pathways involving lipid second messengers. Since PTEN dephosphorylates 3-phosphorylated phosphoinositides such as PtdIns-3,4,5-$P_3$, it is able to negatively regulate the activity of Akt/PKB and is involved in negative regulation of the modulation of cell migration. PTEN is one of the most common targets of mutation in human cancer, with a mutation frequency comparable to that of p53. PTEN loss of function results in formation of tumors in different tissues and is involved in, for example, glioblastoma, endometrial carcinoma and prostate cancer. Germ-line mutations are known in PTEN that cause three rare autosomal dominantly inherited cancer diseases showing benign tumors in which differentiation is normal but cells are not organized: Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndrome (Cantley and Neel, 1999; Sulis and Parsons, 2003).

***EPM2A***, a PTP with predicted dual-specificity phosphatase activity is involved in Lafora's disease (LD), an autosomal recessive form of progressive myoclonus epilepsy. Mutations identified in the *EPM2A* gene are predicted to cause deleterious effects in the

presumed protein (laforin), resulting in LD (Ianzano et al., 2004; Minassian et al., 1998). Recently, EPM2B a second gene associated with this disease was identified which encodes malin, a putative E3 ubiquitin ligase. Laforin together with malin, are suggested to operate in related pathways protecting against polyglucosan accumulation and epilepsy (Chan et al., 2003).

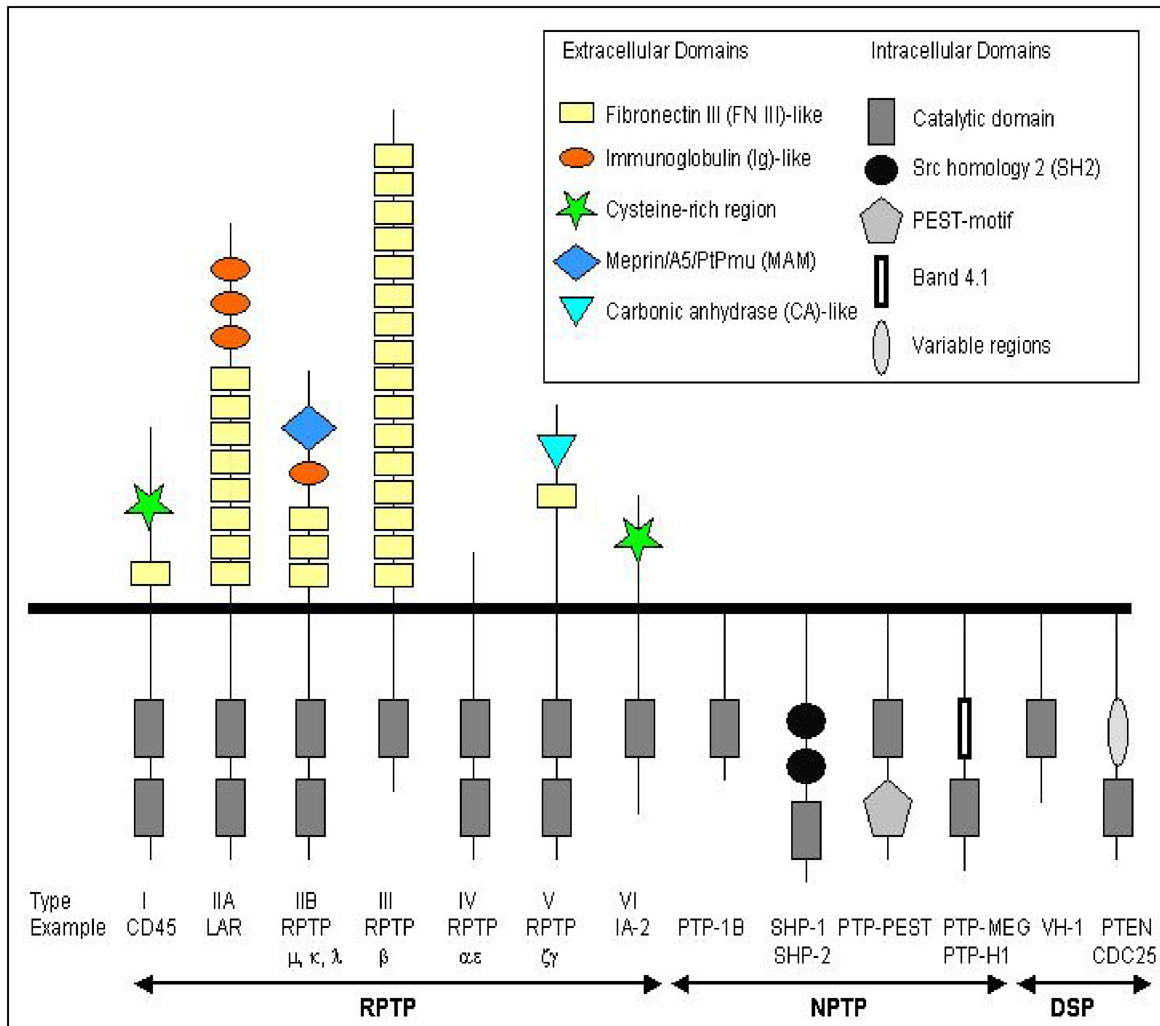


**Fig. 5:** The protein tyrosine phosphatases are classified into receptor- (RPTP), nonreceptor- (NPTP) and into dual-specific phosphatases (DSP). A further classification is made based on their overall structure (adapted from (Andersen et al., 2001)).

## Structure and Regulation of PP2A

### Holoenzyme Formation

Protein phosphatase 2A (PP2A or *PPP2*) is a trimeric holoenzyme displaying the most diverse substrate specificity of all PSTPs identified so far. Present in the nucleus and cytoplasm of all eucaryotic cells, PP2A is involved in a large number of cellular processes, such as the regulation of signal transduction pathways, DNA replication and transcription, RNA splicing and translation, cell cycle progression, morphogenesis, (neuronal) development and transformation. PP2A consists of a core dimer consisting of the 36-kDa catalytic subunit (C) complexed *in vivo* with the scaffold regulatory subunit (PR65/A).

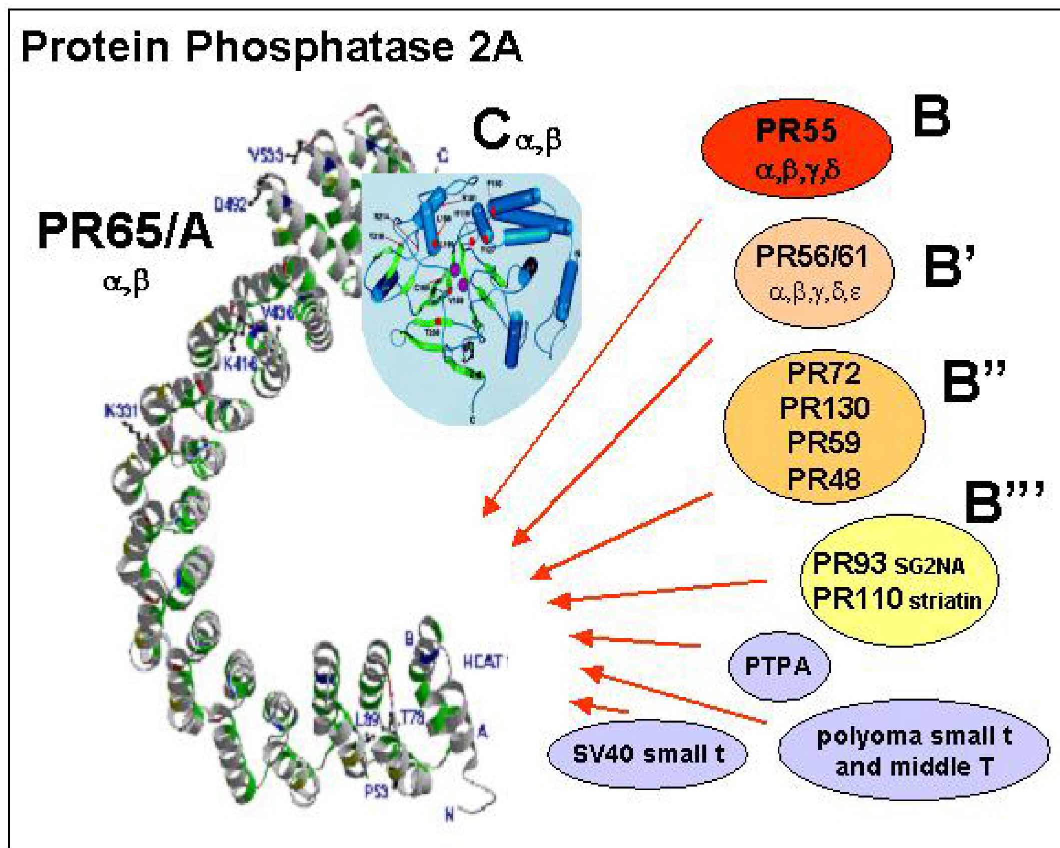


**Fig. 6:** Holoenzyme assembly of PP2A. The catalytic C subunit (Evans et al., 1999) and the structural A subunit (Groves et al., 1999) form the core dimer of the phosphatase. Any of the variable regulatory subunits bind to the core dimer in order to activate or inhibit (viral proteins) the phosphatase.

This dimer associates with any one of the second regulatory subunits (PR55/B, PR61/B' PR72/B" and PR93/110/B''') to form heterotrimeric holoenzyme complexes (Fig.6). The question of whether the dimer exists *in vivo* has not yet been answered satisfactorily, although it was reported to have been purified from various tissues. On the one hand it was shown that the dimer was generated during purification of the trimer due to dissociation or proteolysis of the B subunit, whereas others show the presence of the dimer in an early step of the purification (Cayla et al., 1990; Cohen, 1989; Depaoli-Roach et al., 1994). However, the level of AC dimer has been estimated to be around 30% of all cellular PP2A (Kremmer *et al.*, 1997).

**Catalytic C Subunits**

The two closely related genes of the catalytic subunit Cα and Cβ are ubiquitously expressed and share 98% sequence identity to each other and about 40% identity to PP1C (Arino et al., 1988; Green et al., 1987; Stone et al., 1988). Interestingly, PP2ACα is about 10 times more abundant than the β isoform (Khew-Goodall and Hemmings, 1988). Expression from the PP2ACα gene promoter is about 10-fold stronger than from the PP2ACβ promoter, which may account for the difference in protein levels (Khew-Goodall *et al.*, 1991). The two isoforms are encoded by two distinct genes (Khew-Goodall and Hemmings, 1988), localized to human chromosome 5q23-q31 and to 8p12-p11.2 (Jones *et al.*, 1993). The sequence of PP2A is highly conserved across evolution from plants to human (Arino et al., 1993; Cohen et al., 1990; Cormier et al., 1991; Kinoshita et al., 1990; MacKintosh et al., 1990; Orgad et al., 1990; Sneddon et al., 1990; Van Hoof et al., 1995).

**Regulatory PR65/A Subunits**

The two PR65/A isoforms (α and β) share 86% sequence identity and have an unusual structure of 15 pairs of antiparallel helical HEAT repeats (huntingtin/elongation/A subunit/TOR) (Hemmings et al., 1990). This motif consists of 39-41 amino acid sequences forming superimposed α-helices that elongate into a hook-like structure with a scaffolding function for other interacting proteins (Andrade and Bork, 1995; Groves *et al.*, 1999). The catalytic C subunit binds to C-terminal repeats 11 - 15 and regulatory B

subunits bind to N-terminal repeats 1 – 10 (Ruediger et al., 1994; Ruediger et al., 1992). Comparable to the catalytic subunit, both isoforms are ubiquitously expressed (Hemmings *et al.*, 1990) and with the exception of *Xenopus laevis* oocytes, PR65/Aα seems to be more abundant than the β isoform (Hendrix *et al.*, 1993). Recent investigations identified somatic alterations in the human PR65/Aβ sequence in 15% of primary lung and colon tumor-derived cell lines and one deletion mutation in PR65/Aβ was shown to restrict binding of PR65/Aβ to the catalytic subunit (Wang *et al.*, 1998). Because of the suggestion that PP2A acts as a tumor suppressor gene, further implications of the above results will be discussed in the chapter "PP2A and disease".

**Variable B Regulatory Subunits**

**PR55/B**

The four unrelated and variable regulatory subunits (B, B', B'', B''') are encoded by 14 distinct genes and have been described in mammalian cells (Barford, 1996; Millward *et al.*, 1999; Strack *et al.*, 1999; Yan *et al.*, 2000). The B regulatory subunits are not only striking because of their high subunit diversity, but also for their lack of sequence similarity to each other and their ability to interact with similar PR65/A regions. They are also responsible for substrate specificity and subcellular localization of PP2A and are involved in developmental regulation, cell fate determination, complex formation and cell division. Four isoforms with a sequence identity of >90% to each other encode the family of mammalian PR55/B. They display a tissue-specific distribution: PR55/Bα is present in all tissues, Bβ is expressed in brain and (mouse) testis, Bγ is brain specific and Bδ is a newly identified isoform present in all tissues. (Healy et al., 1991; Mayer et al., 1991; Schmidt et al., 2002; Strack et al., 1999; Strack et al., 1998; Zolnierowicz et al., 1994). Two splice variants of the mouse PR55/Bβ have been identified, PR55/Bβ.1 and PR55/Bβ.2, encoding N-terminally spliced forms that preserve the ORF and result in a protein in which the first 23 amino acids are replaced by five and 26 novel amino acids, respectively (Schmidt *et al.*, 2002). The diversity, developmental regulation and distribution of the regulatory B subunits will be discussed in the results section. All PR55/B regulatory subunits were identified as WD-40 repeat motif proteins (Fig. 7). One

repeat of this structural motif consists of 40-60 minimally conserved amino acids typically between GH and WD dipeptides. The propeller-like structure is formed of four stranded antiparallel β-sheets and at least four repeats are necessary to form a circular structure. Depending on the stringency of the parameters, the B regulatory subunits contain 5-7 of this imperfect repeat in mouse and human. All WD-40 proteins show this structural relationship, but the functional relationship remains unclear, although it is assumed that the motifs serve as docking sites for other interacting proteins. (Schmidt et al., 2002; Smith et al., 1999). In this regard, PR55/Bα and PR55/Bβ have been shown to interact with the cytoplasmic domain of TGF-β receptors and to be a direct target for their kinase activity (Griswold-Prenner *et al.*, 1998). The TGF-β receptor II interacting protein-1 (TRIP-1), a protein largely composed of WD-40 repeats, also associates with the related type II TGF-β receptors (Chen *et al.*, 1995). This suggests that binding of both PR55/B and TRIP-1 to the TGF-β receptors is mediated by their WD-40 repeat motifs.

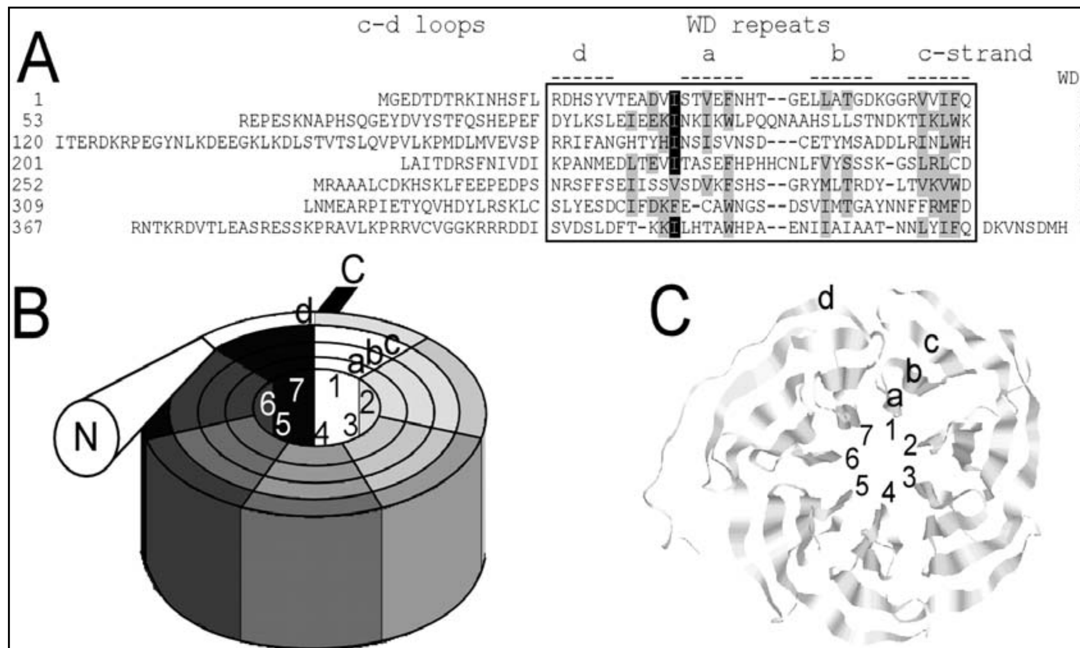


**Fig. 7**: Structure prediction of B-family regulatory subunits. *A*, the amino acid sequence of Bγ was aligned according to boundaries of the seven WD repeats and component β-strands (*d* and *a-c*) provided by the Pfam web application. Sequence conservation of WD repeats are *gray* and *black shading*. *B*, β-strand arrangement of the α-propeller fold. *C*, ribbon view of the Bγ subunit model based on the Gβ1 crystal structure (taken from (Strack *et al.*, 2002)).

**PR61/B’**

Five genes encoding isoforms of the PR61 family (B' or B56) have been cloned so far (α, β, γ, δ, ε) and these are localized on human chromosomes 1q41, 11q12, 3p21, 6p21.1 and 7p11.2-p12, respectively. These five isoforms undergo alternative splicing to generate as many as 11 isoforms (Andjelkovic et al., 1996b; Csortos et al., 1996; McCright et al., 1996a; McCright et al., 1996b; McCright and Virshup, 1995; Tanabe et al., 1996; Tehrani et al., 1996). The B’ subunits share an 80% sequence identity in the core domain, whereas the N- and C-termini are less conserved. Similar to the PR55/B subunits, it is assumed that the conserved region serves as a binding site for PP2A holoenzyme formation, whereas the divergent N- and C-termini are involved in the regulation of substrate specificity and subcellular localization. This notion is supported by the fact that PR61/B’α, PR61/B’β and PR61/B’ε are found in the cytoplasm, whereas PR61/B’γ is localized to the nucleus and PR61/B’δ is found in both cytoplasm and nucleus. In addition, all isoforms show a tissue-specific expression pattern and are all phosphoproteins, with the exception of PR61/B’γ1 (Csortos et al., 1996; McCright et al., 1996b; McCright and Virshup, 1995; Tehrani et al., 1996).

**PR72/B”**

The B'' subunits are encoded by a gene generating two isoforms (PR72/B” and PR130/B”) by alternative splicing. PR130/B” is ubiquitously expressed, whereas PR72/B” expression is restricted to heart and muscle (Hendrix *et al.*, 1993). PR72/B” contains a potential nuclear localization signal that may account for the presence of PP2A in the nucleus. A related gene producing PR59/B” and a third gene encoding PR48/B” have been identified by yeast two-hybrid screening. PR59/B” shares 56% sequence identity and plays a role in cell cycle control by dephosphorylating p107, a pocket protein of the retinoblastoma family (Voorhoeve *et al.*, 1999). It is ubiquitously expressed except in muscle. PR48 shares sequence identity of 68% with PR59 and is highly homologous to PR72. PR48 is localized to the nucleus and inhibits cell cycle progression by dephosphorylating cdc6. This suggests that the selective interaction between a PR48-containing PP2A trimer and cdc6 controls initiation of DNA replication (Farhana *et al.*, 2000). Interestingly, analysis

of the human EST-database and human PR48 gene structure revealed that the human PR48 clone published is incomplete. Instead, it is proposed that the gene encodes a protein of 70 kb rather then 48 kb and should be renamed PR70/B" (Farhana *et al.*, 2000).

**PR110/B'''**

Striatin (PR110) and S/G2 nuclear autoantigen (SG2NA; PR93) have been identified as new members of a putative third B regulatory subunit family (B'''), based on a conserved epitope shared with the B′ subunits. Both proteins were identified as WD-40 repeat proteins, but have no sequence homology to the PR55/B subunits. Striatin is localized to the postsynaptic densities of neural dendrites and SG2NA is nuclear. Other unknown proteins have been identified in the corresponding trimers, which leads to the assumption that both function as a scaffold for other interacting proteins (Moreno *et al.*, 2000).

**Posttranslational Modifications**

Reversible covalent modification of proteins is an important step in cellular signaling. The list of posttranslational modifications includes phosphorylation, acetylation, lipid modifications, glycosylation, and methylation. These modifications are involved in a variety of molecular functions, but are especially important in regulating protein-protein interactions and formation of signaling complexes.

**Phosphorylation**

The catalytic subunit undergoes reversible phosphorylation. The phosphorylation occurs on $Tyr^{307}$, which is located in the conserved C-terminal part of PP2AC, and results in inactivation of the phosphatase. Okadaic acid (OA) enhances tyrosine phosphorylation, suggesting that PP2A can rapidly re-activate itself by auto-dephosphorylation.

This also indicates that PP2A can act as a phosphotyrosine phosphatase.

*In vitro*, the catalytic subunit of PP2A can be phosphorylated by the tyrosine kinases $pp60^{v\text{-}src}$, $pp56^{lck}$, EGFR and insulin receptor (Chen *et al.*, 1992). *In vivo*, tyrosine phosphorylation was detected in activated human T cells and in fibroblasts overexpressing $pp60^{v\text{-}src}$. In addition, stimulation with growth factors such as EGF or serum (Chen *et al.*,

1994a), interleukin-1 or TNFα (Guy *et al.*, 1995), and insulin (Begum and Ragolia, 1996; Begum and Ragolia, 1999; Srinivasan and Begum, 1994{Begum, 1996 #289) also induced inactivation of PP2A by tyrosine phosphorylation. This interesting *in vivo* regulation model places PP2A in an important position to accelerate the transmission through signaling cascades. In addition, PP2AC undergoes phosphorylation on an unidentified threonine residue in response to autophosphorylation-activated protein kinase (Guo and Damuni, 1993). This phosphorylation leads to the inactivation of both the phosphoserine/threonine (Guo and Damuni, 1993) and the phosphotyrosine phosphatase activities of PP2A (Damuni *et al.*, 1994). The physiological implications of the inactivation of PP2A have to be further investigated. In addition, members of the PR61/B' family can be phosphorylated. PKR (double-stranded-RNA-dependent protein kinase) interacts with and phosphorylates PR61/B'α and increases the activity of the PR61/B'α-containing trimer towards PKC-phosphorylated myelin basic protein and PKR-phosphorylated eIF2α *in vitro* (Xu and Williams, 2000). Phosphorylation of PR61/B'δ by PKA *in vitro* does not affect association with the core dimer, but changes the substrate specificity of the phosphatase (Usui *et al.*, 1998).

**Methylation**

Taking phosphorylation as the most abundant posttranslational modification of proteins, several of the less prevalent modifications, including methylation, also play important roles in signaling events (Fig. 8) (reviewed in (Mumby, 2001)). Not only the small GTPases like Ras, Rac, Rho, and Cdc42 but also the γ subunit of heterotrimeric GTP-binding proteins (G proteins) and the catalytic subunit of PP2A are methylated signaling molecules.

The six C-terminal residues (TPDYFL) are conserved in all PP2A catalytic subunits and are important for interaction of the core dimer with the regulatory subunits. The catalytic subunit of PP2A is carboxymethylated by a unique methyltransferase (PPMT) on the C-terminal $Leu^{309}$ residue that is specific for the catalytic subunits of PP2A, PP4, and PP6 (Kloeker and Wadzinski, 1999; Lee and Stock, 1993{Xie, 1994 #277; Xie and Clarke, 1994). The methylation is reversible and can be removed by a specific methylesterase (PPME) (Lee *et al.*, 1996). PPMT and PPME appear to selectively interact with and

reversibly methylate the AC dimer and not the free catalytic subunit. The regulatory subunits are not found in complexes with PPME and the core dimer but, in contrast, they compete with PPME and protect the holoenzymes from demethylation (Tolstykh *et al.*, 2000). Methylation of the PP2A catalytic subunit selectively enhances association of the core dimer with the PR55/B and PR61/B' regulatory subunits (Millward et al., 1999{Virshup, 2000 #281; Virshup, 2000), but it does not directly alter the activity of the PP2A catalytic subunit (Tolstykh *et al.*, 2000). The binding of other regulatory subunits like PR72/B", striatin, SG2NA or polyomavirus middle T tumor antigen to the core dimer are unaffected (De Baere *et al.*, 1999; Yu *et al.*, 2001). The different methylation dependencies of interacting proteins have important consequences for the equilibrium between core dimer and various holoenzymes, such as altered targeting of PP2A and subsequent protein dephosphorylation.

Increased methylation of the AC dimer would favor association with methylation-dependent proteins and decrease the formation of complexes with methylation-independent interacting proteins.

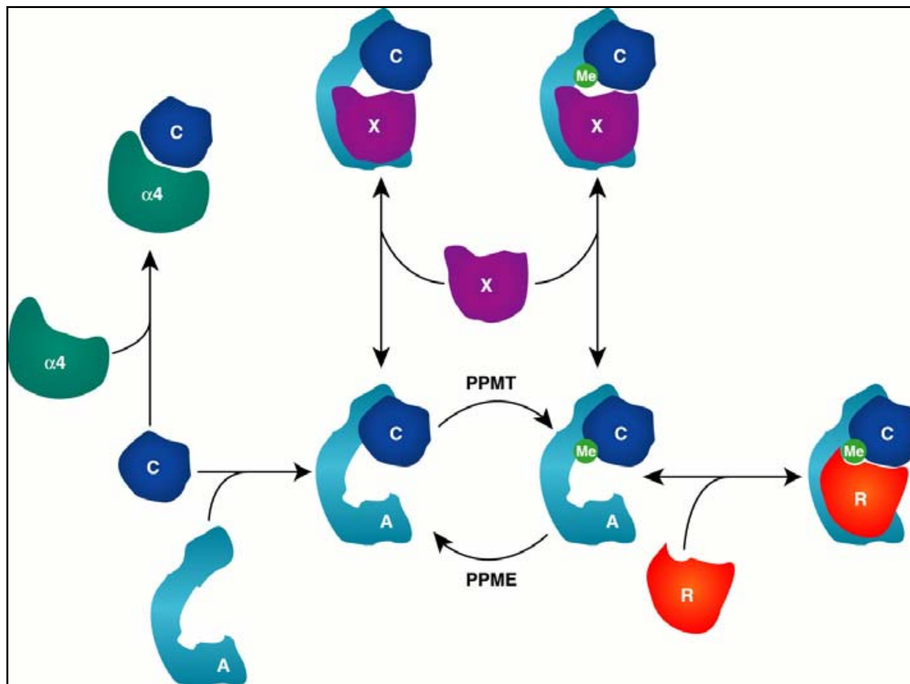


**Fig. 8:** Reversible methylation regulates the assembly and distribution of PP2A holoenzymes. The core dimer is methylated (Me) by a specific protein phosphatase methyltransferase (PPMT). The AC dimer is demethylated by a specific protein phosphatase methylesterase (PPME). (taken from (Mumby, 2001)).

**Protein Phosphatase Inhibitor Okadaic Acid**

The naturally occurring phosphatase inhibitor OA has become an essential tool for the investigation of protein phosphatases in signaling events *in vivo* (Fernandez et al., 2002). This potent tumor promoter is a $C_{38}$ polyether fatty acid produced by marine dinoflagellates and the causative agent of diarrhoetic shellfish poisoning (Bialojan and Takai, 1988). OA induces various biological effects *in vivo*, including promotion of tumor growth (Suganuma *et al.*, 1988), prolonged smooth muscle contraction (Shibata *et al.*, 1982) and promotion of genomic instability (Kohno and Uchida, 1987; Nagao et al., 1995; Tohda et al., 1993). Malignant transformation and cell growth is found to be both promoted and inhibited, depending on the system used (Katoh *et al.*, 1990). The variety of effects caused by OA is presumably a result of the down-regulation of OA-sensitive protein phosphatases. Treatment of SCC-25 carcinoma cells with OA enhanced the expression of mRNAs and proteins of both Fas receptor and Fas ligand. OA treatment did not only lead to translocation of NFkB from the cytosol to the nucleus, its levels also increased, whereas the amount of IkB-α decreased. This suggests that NF-kB activated at early stages by OA stimulated the promoter activity of Fas receptor in the cells leading to apoptosis (Fujita et al., 2004). Another apoptotic effect of was shown in HL-60 cells, where OA induces bcl-2 mRNA destabilization which is associated with decreased binding of trans-acting factors to the AU-rich element (ARE) (Sengupta et al., 2003). Inhibition of PP2A by OA in metabolically competent rat brain slices induced a dramatic increase in the phosphorylation/activation of ERK1/2, MEK1/2, and p70 S6 kinase as well as the phosphorylation of tau at several sites (Pei et al., 2003). In addition, several other phosphatase inhibitors have been identified and described, including calyculin A (Ishihara *et al.*, 1989), microcystin-LR (Honkanen *et al.*, 1990), tautomycin (MacKintosh and Klumpp, 1990), nodularin (Honkanen *et al.*, 1991), cantharidin (Li and Casida, 1992). Treatment of cells with OA leads to concentration-dependent inhibition of serine/threonine protein phosphatases and often results in apoptosis. While PP2A is inhibited most efficiently ($K_i$ 0.2 nM), PP1 is 100-fold less sensitive to OA *in vitro*. The effect on PP2B is even lower and PP2C is insensitive to the treatment. Since OA does not

penetrate cell membranes rapidly to accumulate on the catalytic subunit of the phosphatases, it is very difficult to control the actual concentration of the compound *in vivo*. However, conditions for the selective inhibition of PP2A in cells have been established and 1 μM OA applied to the living cell is sufficient to specifically block PP2A activity (Favre *et al.*, 1997). We have to consider that less-abundant protein phosphatases like PP4, PP5 and PP6 are as sensitive to OA as PP2A and, thus, that the cellular effects of OA can no longer be entirely attributed to PP2A inhibition. On the other hand, the physiological role of these novel phosphatases is still unclear and they represent only a minor fraction of total cellular phosphatase activity (Chen et al., 1998; Fernandez et al., 2002; Usui et al., 1998). The use of OA as specific inhibitor has become interesting in terms of drug development (Honkanen and Golden, 2002; McCluskey et al., 2002). Unfortunately, OA induces tumor formation in a mouse skin two-stage model (Suganuma et al., 1988), which almost immediately disqualifies OA for therapeutical use in patients.

## Biological Role of PP2A

### Cell Cycle Regulation by PP2A

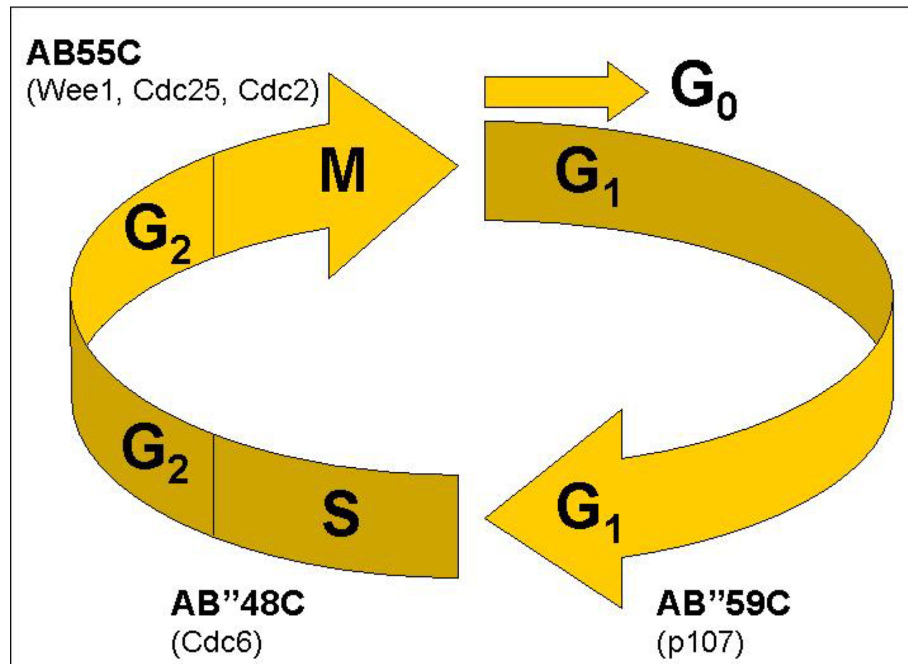


**Fig. 9:** Holoenzymes of PP2A involved in the regulation of cell cycle. Substrates of different PP2A holoenzymes are indicated in brackets (adapted from (Zolnierowicz, 2000)).

Cell cycle progression is regulated by the activity of Cdc/cyclin (or CDK/cyclin) complexes that are controlled by reversible phosphorylation. PP2A plays both a negative and a positive role in the regulation of cell cycle progression, especially during mitosis, due to the multiplicity and substrate specificity of the trimeric holoenzyme (Fig. 9). For example, a mutation in yeast PR55/B, displays a cold-sensitive phenotype with a defect in cell septation and separation (Healy *et al.*, 1991). Further analysis of this mutant revealed that the PR55/B subunit is required for a kinetochore/spindle checkpoint (Wang and Burke, 1997). In *Drosophila*, the absence of the PR55/B protein results in an abnormal sister chromatid separation called abnormal anaphase resolution (*aar*) (Mayer-Jaekel *et al.*, 1993).

PP2A is reported to play a negative role in the transition from G2 to M phase (Fig. 10), which is controlled by the CDC2–cyclin B complex also called MPF (M-phase-promoting factor)(reviewed in (Millward *et al.*, 1999)). MPF phosphorylates specific substrates, such as histone H1, lamins, vimentin, cyclins and microtubule-associated proteins. This explains the initiation of mitotic processes such as nuclear breakdown, chromosome condensation and spindle formation. The kinase activity of the complex is absolutely dependent on the phosphorylation of Thr-161 on CDC2, whereas phosphorylation of two additional sites, Thr-14 and Tyr-15, inhibits the activity of this enzyme. PP2A can regulate the activity of CDC2 and related kinases by at least three different mechanisms.
(i) PP2A and KAP (CDK-associated protein phosphatase) have been suggested to be active phosphatases at Thr-161 of CDC2, although it is more likely that PP2A inhibits the pathway rather than being involved in the direct dephosphorylation of Thr-161 (Lee *et al.*, 1994b; Poon and Hunter, 1995). (ii) PP2A can inactivate CDC2 indirectly by both the activation of WEE1 kinase, which phosphorylates CDC2 at Tyr-15 (Borgne and Meijer, 1996; Mueller et al., 1995), and (iii) by the inactivation of dual-specificity phosphatase CDC25, which removes phosphate from both Thr-14 and Tyr-15 of CDC2 (Clarke *et al.*, 1993). In addition, PP2A or PP-1 may also be involved in exit from mitosis, since cyclin degradation and the subsequent inactivation of MPF (by dephosphorylation of Thr-161) at the metaphase/anaphase transition are affected by an OA-sensitive PPase (Felix *et al.*, 1990; Yamashita *et al.*, 1990). The regulatory functions of PP2A and the important protein kinases involved at the G2/M transition are summarized in Figure 10. In order to achieve these functions, PP2A has to be inactivated for progression into mitosis and reactivated at the exit from mitosis. As mentioned above, PP2A and probably PP1 play a role in maintaining G2 arrest by preventing activation of MPF complex. The transcription factor HOX-11 was reported to interact with and inhibit PP2Ac and PP1c (see also Table 3). Expression of HOX-11 inhibits PP2Ac, disrupts G2 checkpoint and allows cells to proceed inappropriately through M phase. This is suggested to promote genomic instability and oncogenesis (Kawabe et al., 1997). A recent report shows, that PP1 is required for dephosphorylation of the Cdc2-directed phosphatase Cdc25 at Ser287 (of *Xenopus* Cdc25; Ser216 of human Cdc25C), a site that suppresses Cdc25 during interphase. Moreover, PP1 recognizes Cdc25 directly by interacting with a PP1-binding

motif in the Cdc25 N-terminus. They also show that dissociation of 14-3-3 protein from Cdc25 upon entry into mitosis leads to Ser287 dephosphorylation in an phosphatase-independent pathway (Margolis et al., 2003).

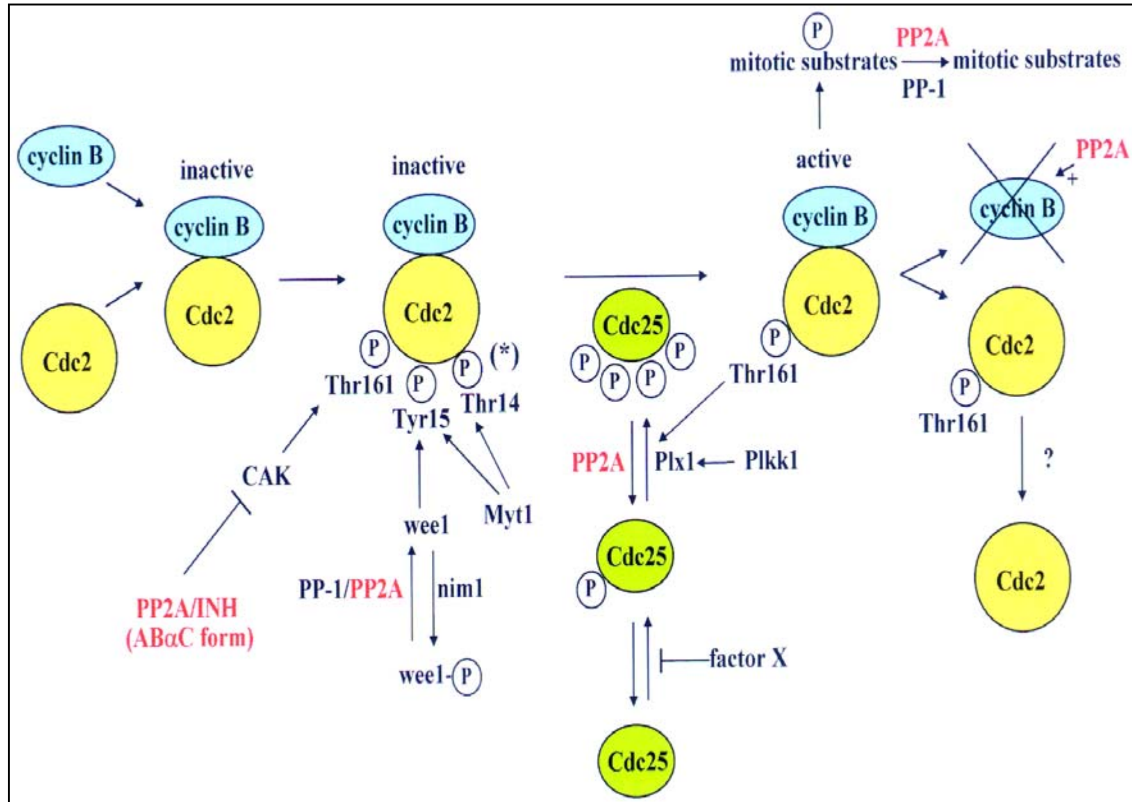


**Fig. 10:** PP2A and regulation of the G2/M transition.
The most important protein kinases and protein phosphatases implicated in the activation of MPF (Cdc2/cyclin), governing the G2/M transition, are depicted. In early G2, PP2A (ABαC) is required to keep MPF in its inactive precursor form by inhibiting the activities of both CAK and Wee1. PP2A also inhibits complete Cdc25 phosphorylation (and activation) by counteracting the Plx1 kinase. Finally, PP2A is also positively implicated in exit from mitosis through its role in cyclin B destruction and by dephosphorylating specific mitotic substrates of activated MPF (taken from (Janssens and Goris, 2001)).

Since different functions of PP2A are exerted by its regulatory subunits, it is not surprising that two members of the PR72/B" family, namely PR48 and PR59, have been involved in the dephosphorylation of cell cycle regulators. PR59/B" specifically targets p107, a retinoblastoma protein (pRB)-related protein, for dephosphorylation by PP2A and inhibits cell proliferation by causing cells to accumulate in G1 (Voorhoeve *et al.*, 1999). Dephosphorylation of p107 in late G1 phase inhibits the release and the activation of E2F transcription factors. PR48 targets PP2A to dephosphorylate CDC6 and blocks cell cycle progression, causing a G(1) arrest. Phosphorylation of CDC6 by CDK2 is required for initiation of DNA replication and the export of CDC6 from the nucleus. Since PR48 is localized to the nucleus, the role of a PR48-containing trimer could be to maintain levels of dephosphorylated CDC6 until replication is triggered by CDK2 (Yan *et al.*, 2000). In contrast, PP2A appears to have activating effects during the early phases of G1 by affecting the activity of G1-specific CDK complexes. In particular, the inhibition of PP2A activity results in decreased expression of cyclin D2, cyclin E, and cyclin A, which are essential regulatory subunits of the respective CDK complexes. The unavailability of cyclins results in severely impaired CDK activity and the cells become arrested in the G1 phase of the cell cycle (Schonthal and Feramisco, 1993; Yan and Mumby, 1999). This positive function of PP2A obviously contrasts with its well-established negative role during the G2/M transition, but could be due to the actions of distinct PP2A holoenzymes that are targeted at different G1 versus G2/M phase substrates.

**PP2A and Apoptosis**

Apoptosis is an active intracellular signal transduction pathway by which the cell regulates its own death. It is required for the elimination of harmful cells and for cell turnover during embryonic development and tissue homeostasis. Dysfunction of the tightly regulated apoptotic signal transduction pathway can lead to cancer, auto-immune diseases, and neurodegenerative disorders (Bratton and Cohen, 2001; Rathmell and Thompson, 2002). Apoptosis is regulated by reversible phosphorylation of apoptotic signaling proteins and is, therefore, controlled by the activity of protein kinases and protein phosphatases. PP2A plays an important role the regulation of apoptosis by its interaction with caspase-3 (Allan et al., 2003; Santoro et al., 1998), Bcl2-family (Deng *et al.*, 1998; Ruvolo *et al.*, 1999) and adenovirus E4orf4 protein (Kleinberger and Shenk, 1993) (Marcellus et al., 2000; Shtrichman et al., 1999; Shtrichman et al., 2000).

Caspase-1 and caspase-3 are two important factors involved in apoptosis (Fig. 11). Caspase-3 is not only responsible for the cleavage of enzymes involved in DNA repair, such as poly (ADP-ribose) polymerase and DNA-dependent protein kinase (DNA-PK), but is also an interaction partner of the PR65/A subunit of PP2A (Santoro *et al.*, 1998). Cleaving of PR65/A by caspase-3 results in increased PP2A activity of the catalytic subunit, which is measured by decreased phosphorylation of MAPK and correlates with the commitment of cells to apoptosis by specific activation of caspase-3 (Allan et al., 2003; Santoro et al., 1998). However, the physiological targets of this free pro-apoptotic PP2A catalytic subunit are not known. As already shown for the PP2A-α4 complex in the TOR signal transduction pathway (Chung *et al.*, 1999), it might be possible that the free catalytic subunit associates with other regulatory proteins altering the PP2A activity towards specific substrates.

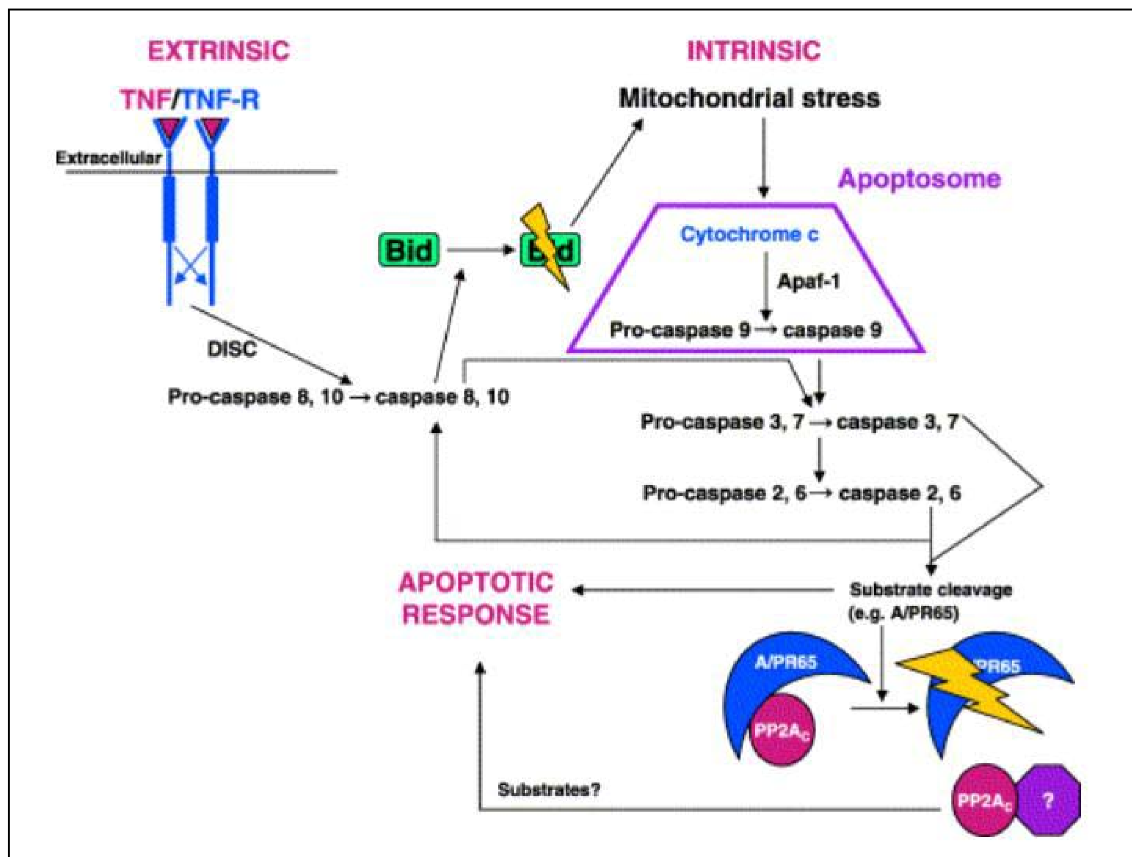


**Fig. 11.** Schematic presentation of the caspase cascades activated by apoptotic signals. In the extrinsic apoptotic pathway, ligand binding to death receptors induce the formation of a Death Inducing Signaling Complex (DISC), leading to the activation of initiator caspases 8 or 10. These caspases cleave the pro-apoptotic Bcl-2 family member Bid, activating the intrinsic pathway or directly cleave and activate the effector caspases 3 and 7. In the intrinsic signaling pathway, apoptotic signals sensed by the Bcl-2 family at the mitochondrial membrane result in the release of cytochrome *c*. Cytochrome *c* and ATP bind to Apaf-1, eliciting the recruitment of pro-caspase 9 in this apoptosome complex, causing its activation. Both initiator caspases of the intrinsic and extrinsic signaling pathway cleave and subsequently activate effector caspases, responsible for auto-amplification of the cascade as well as execution of the apoptotic response by cleaving cellular substrates involved in the apoptotic morphology changes of the cell. One of the cellular substrates for effector caspase 3 is shown to be the A/PR65 subunit of PP2A. Degradation of this regulatory PP2A subunit alters the PP2A activity of the catalytic subunit (whether or not associated with a specific regulatory protein) towards unidentified substrates, resulting in the promotion of the apoptotic response (taken from (Van Hoof and Goris, 2003)).

The Bcl-2 family consists of pro- and anti-apoptotic proteins localized at the mitochondrial outer membrane (Fig.12). Bad is a pro-apoptotic member whose function is regulated by reversible phosphorylation. Bad gets phosphorylated at Ser-112, Ser-136

and/or Ser-155 by different pro-survival kinases such as PKA and PKB, which mediate binding of Bad to 14-3-3 proteins. This interaction confines Bad to the cytosol, altering translocation to the mitochondrial membrane. Bad cannot heterodimerize with Bcl-2 and fails to inhibit the anti-apoptotic protein. In the absence of survival stimuli, Bad is dephosphorylated by PP2A and leads to inhibition of Bcl-2, leading to apoptotic cell death (Datta *et al.*, 2000; Tzivion and Avruch, 2002). The activity of the anti-apoptotic protein Bcl-2 is regulated by phosphorylation on Ser70. This phosphorylation is required for the apoptosis-suppressing ability and can be reversed by PR61/B' containing PP2A (Deng *et al.*, 1998). Since PP2A activates pro-apoptotic (Bad) and inhibits anti-apoptotic proteins (Bcl-2) of the Bcl-2 family, it is generally assumed that PP2A has a positive regulatory function in apoptosis. Nevertheless, this pro-apoptotic function of PP2A stands in contrast to results obtained from RNAi knockdown experiments in *Drosophila*, which show that a PR61/B' containing PP2A is inhibitory for apoptosis (Li *et al.*, 2002; Silverstein *et al.*, 2002). This suggests different regulatory mechanisms and substrates in the Drosophila apoptotic signaling pathway and that of mammals.

In this context, interaction of PP2A with other proteins modulates its activity towards specific substrates in apoptosis. Cyclin G interacts with PP2A and recruits it to dephosphorylate the G-binding partner Mdm2 at $Thr^{216}$ (Okamoto *et al.*, 1996; Okamoto *et al.*, 2002). Mdm2 is known to be a negative regulator of p53, inducing cyclin G transcription. Dephosphorylation of this Mdm2 site by cyclin G1-directed PP2A leads to Mdm2 activation followed by degradation of p53 (Okamoto *et al.*, 2002). This might explain the anti-apoptotic effect of B'/PR61 containing PP2A in *Drosophila.* It is clear that the diversity of the incoming signals will ultimately alter the balance between pro-survival and pro-apoptotic signaling. PR55/Bβ2 has been reported to colocalize with mitochondria in neuronal PC12 cells due to its subcellular targeting signal containing N-terminus. Inducible or transient expression of PR55/Bβ2 specifically accelerates apoptosis in response to growth factor deprivation, suggesting that alternative splicing of a mitochondrial localization signal generates a PP2A holoenzyme involved in neuronal survival signaling (Dagda et al., 2003).

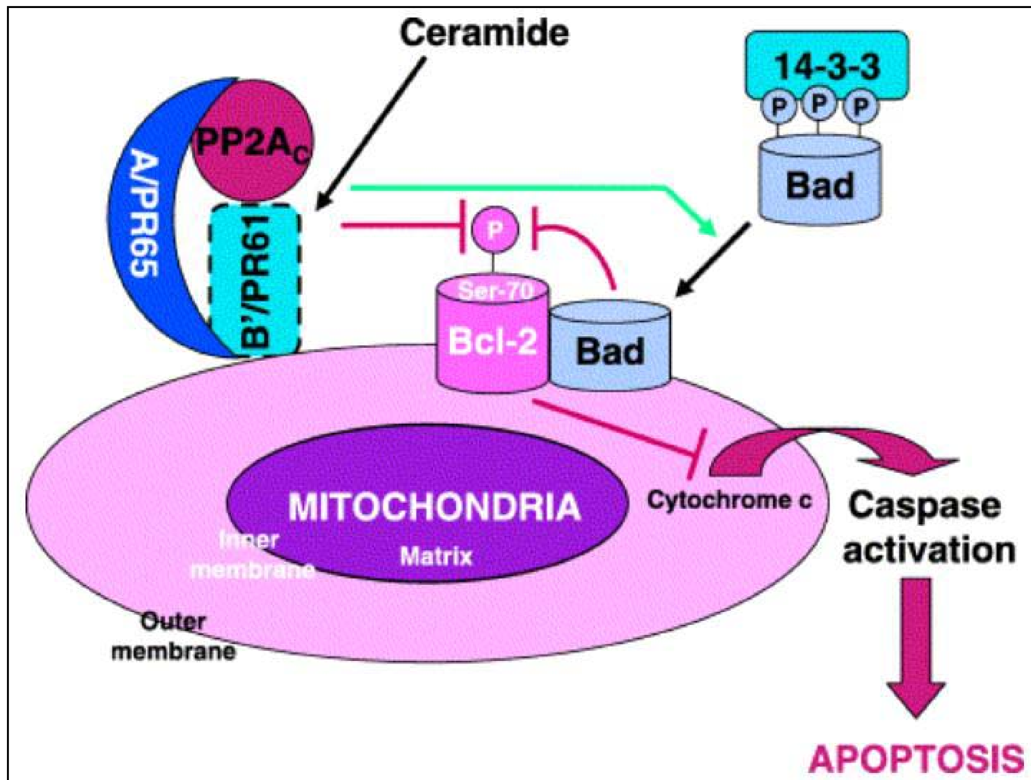


**Fig. 12:** Regulatory role of PP2A in the apoptotic signal transduction pathway upstream of the Bcl-2 family. Ceramide induces the expression and translocation of the B'/PR61 subunit, resulting in the assembly of a mitochondrial PP2A trimer dephosphorylating and inactivating the anti-apoptotic Bcl-2. PP2A is also a Bad phosphatase, keeping this pro-apoptotic protein in a dephosphorylated and mitochondrial located state, where it is functional in inhibiting Bcl-2. The net result is the release of cytochrome c, required for the apoptotic response. The composition of the Bad PP2A trimer is not yet defined (taken from (Van Hoof and Goris, 2003)).

A cell that is infected with a virus undergoes apoptosis due to its defense mechanism. In contrast, viral infection can also inhibit this apoptotic response, driving cell proliferation and high yields of progeny. The virus inhibits apoptosis by encoding viral Bcl-2 and Caspase inhibitors. The virus will terminate infected cells in a later stage, allowing virus spread (Roulston et al., 1999; Teodoro and Branton, 1997). Several viral proteins such as adenovirus E4orf4 and simian virus small t antigen target PP2A to alter its activity, resulting in induction of apoptosis as a regulatory mechanism, enhancing virus spread (Fig. 13). The adenovirus E4orf4 protein interacts with PR55/Bα or through some of the PR61/B´ subunits and activates PP2A. However, only the interaction with PR55/Bα is

essential for the induction of apoptosis (Kleinberger and Shenk, 1993) (Marcellus et al., 2000; Shtrichman et al., 1999; Shtrichman et al., 2000). The PR61/B' regulatory subunit bound to E4orf4 might be involved in down-regulation of virus-stimulated transcription (Bondesson et al., 1996; Kleinberger and Shenk, 1993) and of alternative splicing (Kanopka *et al.*, 1998).

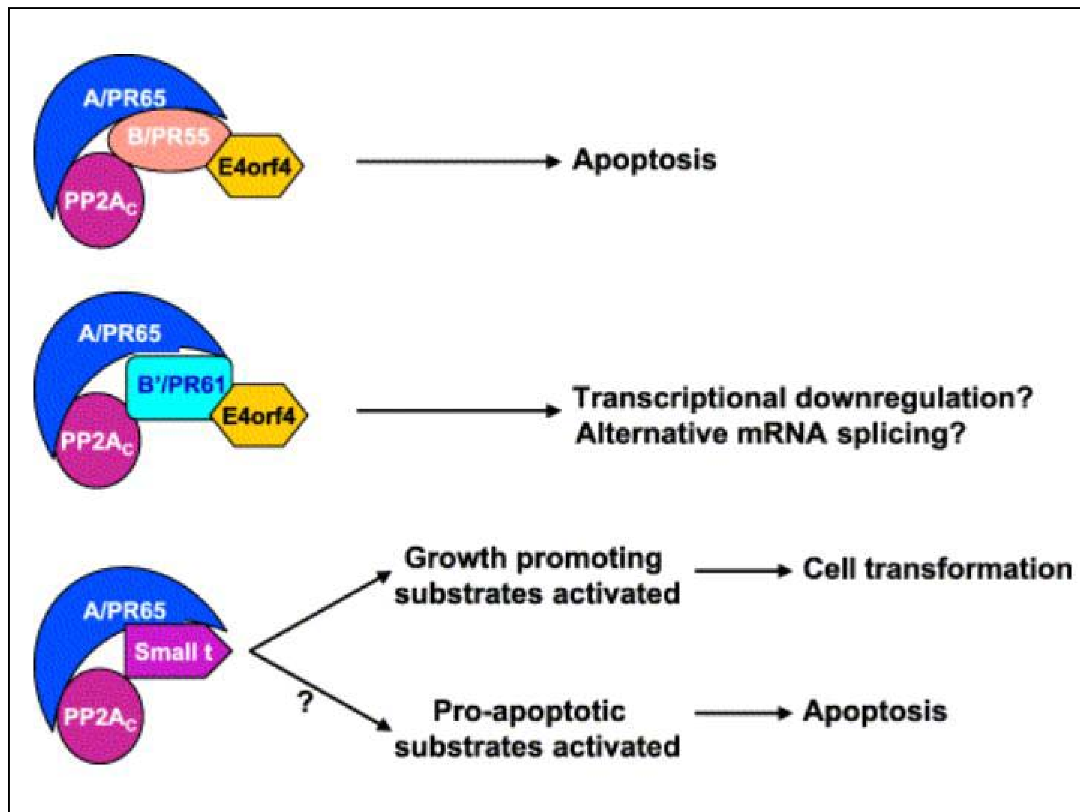


**Fig. 13:**. Interaction of viral proteins with different PP2A holoenzymes (taken from (Van Hoof and Goris, 2003)).

**PP2A and Yeast**

*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been used to investigate the biological role of PP2A. The catalytic subunits of *S. cerevisiae* are encoded by *PPH21* and *PPH22* (Ronne *et al.*, 1991), the structural PR65/A subunit by *TPD3* (van Zyl et al., 1992; van Zyl et al., 1989), and only two regulatory subunits by *CDC55* (PR55/B) and *RTS1* (PR61/B') (Healy et al., 1991; Shu et al., 1997; van Zyl et al., 1992). In addition to

the two catalytic subunits, there are three 2A-like phosphatases encoded by *PPH3, PPG1*, and *SIT4* (Ronne et al., 1991; Sneddon et al., 1990; Sutton et al., 1991).

Although *S. cerevisiae* has a much smaller set of possible PP2A heterotrimers, mutations in the above-mentioned five genes elicit complex pleiotropic phenotypes. Deletion of both catalytic subunits *PPH21* and *PPH22* generates cells that are temperature sensitive, have decreased growth rates, and exhibit cell wall and polarity defects, whereas single disruptions produce no mutant phenotype (Lin and Arndt, 1995; Ronne et al., 1991). Pph3 phosphatase activity is believed to be responsible for sustaining cell growth in the absence of PP2A. Deletion of *PPH3* in combination with *pph21Δ pph22Δ* is lethal (Ronne *et al.*, 1991), whereas inactivation of Pph3 alone is without any effect on cell growth (Hoffmann *et al.*, 1994). Sit4 normally associates with a family of related proteins termed Sap proteins (Luke *et al.*, 1996) and promotes progression through G1 via regulation of G1 cyclin production (Fernandez-Sarabia et al., 1992; Sutton et al., 1991). Yeast cells have another 2A-related phosphatase encoded by *PPG1*, whose inactivation reduces glycogen accumulation in yeast cells but does not affect cell growth (Posas *et al.*, 1993). PP2Ac and Sit4 have been found to form complexes with a phosphatase-associated protein termed Tap42 (Di Como and Arndt, 1996). Association with Tap42 prevents PP2Ac from interacting with Tpd3 and Cdc55, suggesting that Tap42 competes with Tpd3 and Cdc55 for PP2Ac binding (Jiang and Broach, 1999). The Tor-dependent phosphorylation of Tap42 seems to be important for the interaction of Tap42 with phosphatases, because inactivation of the Tor protein prevents formation of the Tap42-phosphatase complexes (Di Como and Arndt, 1996). The Tap42-phosphatase complexes have been demonstrated to play a major role in Tor-dependent phosphorylation of many factors downstream of the Tor proteins. Rapid dephosphorylation of these factors is found to accompany the dissociation of Tap42 from phosphatases (Bertram et al., 2000; Schmidt et al., 1998). This observation has led to the suggestion that Tap42 acts as a phosphatase inhibitor (Jacinto *et al.*, 2001). In a recent study it was shown, that the interaction between Tap42 and the catalytic subunits of PP2A is required for cell cycle-dependent distribution of actin. Further, PP2A activity might play a negative role in controlling the actin cytoskeleton and might be involved in regulation of the G2/M transition of the cell cycle (Wang and Jiang, 2003). Mutagenesis experiments and functional analysis of human PP2A in a yeast system

(Evans and Hemmings, 2000a; Evans and Hemmings, 2000b; Evans et al., 1999; Myles et al., 2001) reveal that the invariant C-terminal leucine-309, the site of regulatory methylation, is dispensable for protein function, whereas the L199P mutant protein is catalytically impaired despite binding to the yeast PR65/A subunit Tpd3p (Evans *et al.*, 1999). Also, mutations of other key residues such as Y127N (abolished PP2A function), H118N, D88N, V159E (all impaired catalytic activity), F232S, P240H (both temperature-sensitive impaired catalytic activity), Y307D (catalytically active, but impaired protein function), and T304D (impaired protein function) are important for PP2A function (Evans and Hemmings, 2000a).

Cells null for the structural A subunit *TPD3* are temperature sensitive, exhibit RNA-processing defects, and become multibudded at low temperatures (van Zyl et al., 1992; van Zyl et al., 1989). Mutations in the B regulatory subunit CDC55 result in highly elongated, multiply budded cells, which is an indication of delayed cytokinesis (Healy *et al.*, 1991). In addition, multiple roles in mitosis have been suggested for CDC55. For example, cdc55 mutants lack a functional kinetochore/spindle assembly checkpoint, whereas their cell cycle progression in response to DNA damage or an inhibitor of DNA synthesis is not affected (Minshull *et al.*, 1996; Wang and Burke, 1997). Instead of cyclin B destruction and sister chromatid separation, this defective spindle assembly checkpoint in cdc55 mutants allows inactivation of Cdc2-cyclin B by tyrosine phosphorylation (Minshull *et al.*, 1996). The other regulatory B' subunit Rts1p is necessary for regulating responses to a variety of stressful cellular conditions, for proper nucleus and spindle orientation, and for control of cyclin B2 degradation (Shu and Hallberg, 1995; Shu *et al.*, 1997). A novel mechanism was suggested to generate active PP2A in vivo. Deletion of the yeast phosphotyrosyl phosphatase activator (PTPA) homolog generated a PP2A catalytic subunit with a conformation different from the wild-type enzyme, as indicated by its altered substrate specificity, reduced protein stability, and metal dependence (Fellner et al., 2003).

## PP2A and *Drosophila*

The use of *Drosophila melanogaster* as a model organism has produced additional insights into the role of PP2A in developmental processes, cell cycle regulation and intracellular signaling. In contrast to the situation in yeast and mammalian cells, only PP2AC (Orgad *et al.*, 1990), the A/PR65 (Mayer-Jaekel *et al.*, 1992), the B/PR55 (Mayer-Jaekel *et al.*, 1993) and the B´/PR61 (Berry and Gehring, 2000) subunits have been cloned. With the exception of B´/PR61, all the subunits are encoded by a single gene. Ablation of either the catalytic subunit or PR65/A causes the disappearance of all PP2A subunits in an RNA interference approach. Also, targeting all four of the Drosophila PP2A regulatory subunits caused the disappearance of both the A and C subunits, indicating that only the trimer is stable in Drosophila cells. In addition, knockdown of C, A or PR55/B subunits increases insulin-mediated ERK activation, confirming that PP2A is a negative regulator of the MAPK pathway. In contrast to its known role in apoptosis, a C, A or PR61/B' subunit ablated form of PP2A reduces cell number and activates apoptosis and apoptosis-related markers (Caspase-3) (see also chapter 'PP2A and apoptosis'). Unlike PP2A, knockdown of PP4 caused only a slight reduction in cell growth but had no effect on MAPK signaling or apoptosis. Depletion of PP5 had no effects on MAP kinase, cell growth, or apoptosis in Drosophila cells (Avdi *et al.*, 2002; Silverstein *et al.*, 2002). Mutants of the PP2A catalytic subunit die in embryogenesis around the time of cellularization, exhibiting overcondensed chromatin and a block in mitosis between prophase and the initiation of anaphase. The fact that they also possess multiple centrosomes with disorganized, elongated arrays of microtubules suggests that PP2A is required for the attachment of microtubules to chromosomal DNA at the kinetochore and the proper initiation of anaphase (Snaith *et al.*, 1996). Moreover, mutation of PP2AC stimulates signaling from Ras1 but impairs signaling from Raf, suggesting that PP2A regulates the Ras1 cascade both negatively and positively, by dephosphorylating factors that function at different steps in the cascade (Wassarman *et al.*, 1996).

A cell cycle function of PP2A is suggested not only by the fact that levels of the C and A subunit of PP2A change during development in a tissue- and time-specific manner (Mayer-Jaekel *et al.*, 1992). Mitotic defects are shown by two Drosophila mutants, termed

aar1 (abnormal anaphase resolution) and twinsP (Fig. 14). Both mutations are defective in the gene encoding B/PR55. aar1 mutants show intact lagging chromatids separated from their sisters but remaining at the position formerly occupied by the metaphase plate. In addition, they display anaphase figures that show bridging chromatin with two centromeric regions (Gomes *et al.*, 1993). These defects can be rescued by re-introducing one allele of full-length PR55/B (Mayer-Jaekel *et al.*, 1993). The second mutation twinsP contains morphologically abnormal imaginal discs where part of the wing imaginal disc is duplicated in a mirror-image fashion (Uemura *et al.*, 1993).

These data suggest that a PR55/B-containing trimer directs substrate specificity and is involved in cell fate determination and cell cycle regulation. This role in cell cycle regulation is indicated by the fact that increase in the severity of the mutation correlates with a decrease in phosphatase activity towards $p34^{cdc2}$ phosphorylated proteins (Mayer-Jaekel *et al.*, 1994). In addition, mutations in PR55/B suppress a mks mutant-dependent metaphase arrest and permit an alternative means of initiating anaphase. It also suggests that the anaphase-promoting complex (APC) is normally required to inactivate wild-type PR55/B (Deak *et al.*, 2003).

The Drosophila PR61/B’ regulatory subunit termed widerborst (wdb) is suggested to be involved in organizing tissue polarity proteins into proximal and distal cortical domains, thus determining wing hair orientation. It is also needed to generate the polarized membrane outgrowth that becomes the wing hair (Hannus *et al.*, 2002).

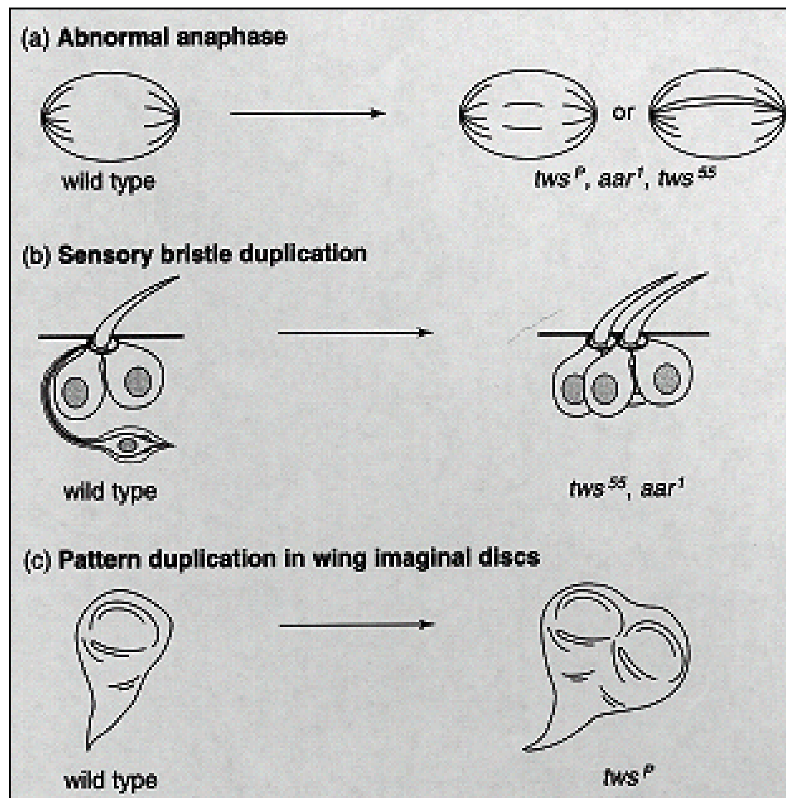


**Fig. 14:** Drosophila PR55/B mutant defects (taken from (Mayer-Jaekel and Hemmings, 1995))

## PP2A Mutant Mice

Since PP2A plays an important role in a diverse set of cellular functions, it is expected that deregulation of this phosphatase will have severe consequences on different organisms. In this context, it has been shown that mice with a disrupted Cα subunit of PP2A die at embryonic day 5.5-6.5. Despite the fact that Cα shares a sequence identity of 97% and that total levels of PP2A catalytic subunit are comparable with those in wild-type embryos, Cβ is not able to compensate for the loss of Cα after E6.5. Degenerated embryos can be recovered even at embryonic day 13.5, indicating that, although embryonic tissue is still capable of proliferating, normal differentiation is significantly impaired. The primary germ layers ectoderm and endoderm are formed, but mesoderm formation is absent in degenerating knockout embryos (Gotz *et al.*, 1998). The functional

difference between PP2ACα and PP2ACβ may be explained by their distinct subcellular localizations in the early embryo: while Cα was found predominantly in the plasma membrane, Cβ was localized mainly within the cytoplasm and the nucleus (Gotz *et al.*, 2000). Cα is in a stabilizing complex containing E-Cadherin and β-catenin, whereas E-cadherin and β-catenin in the knockout embryos are redistributed to the cytoplasm, resulting in degradation of β-catenin in both the presence and the absence of a Wnt signal (Gotz *et al.*, 1998) (Gotz *et al.*, 2000). This might be an indication that embryonic lethality results from defects in cell adhesion caused by insufficient levels of membrane-associated E-cadherin and β-catenin (Zolnierowicz and Bollen, 2000), which is in agreement with a proposed role of PP2A in Wnt/β-catenin signaling (Hsu et al., 1999; Ikeda et al., 2000; Seeling et al., 1999).

No reports of PP2A regulatory subunit knockout in mouse have been published so far. Attempts in our laboratory and in the group of our collaborator J. Goetz from the University of Zurich have not been successful so far. Splice variants with variable first exons more than 50 kb apart from each other and the possibility of splicing out targeted regions had significant consequences for the design of the targeting strategy (Schmidt *et al.*, 2002). So far, only PR61/B'γ has been expressed in transgenic mice, which die neonatally and lack normal lung structure (Everett *et al.*, 2002). β-catenin is absent in PR61/B'γ transgenic embryos, suggesting a role for PR61/B'γ in Wnt signaling during lung airway morphogenesis.

Two dominant negative transgenic mouse lines have been established with mutations in the catalytic subunit. One carries a mutation in the catalytic site (L199P) and is catalytically impaired, probably due to disruption of metal- or substrate-binding implicated in catalytic function rather than due to a disturbed subunit interaction through misfolding. The reduced activity is associated with altered compartmentalization, hyperphosphorylation and ubiquitination of tau, resembling a key pathological finding in Alzheimer's disease (AD) (Kins *et al.*, 2001). In addition, reduced PP2Ac activity in L199P transgenic mice causes the activation of ERK and JNK, which results in nuclear accumulation of their substrates, Elk-1 and c-Jun. This suggests that PP2A is a negative regulator of the ERK and JNK signaling pathways *in vivo* (Kins et al., 2003). The second mutation (L309A) is located at the very C-terminus of Cα and inhibits PP2A activity by

preventing the binding of the PR55/B regulatory subunits to the PP2A core dimer. Similar to the catalytic mutation, tau protein is hyperphosphorylated and translocated to the somatodentritic domain of neurons, suggesting a role for PR55/B in the tau pathogenesis of AD (Gotz and Schild, 2003).

Transgenic mice were generated expressing high levels of a dominant negative mutant of the PR65/A subunit in heart, skeletal muscle, and smooth muscle. The mutant PR65/A carrying a deletion of repeat 5 competes with the endogenous A subunit for binding the C subunit, but does not bind B subunits. The generated transgenic mouse has an increased ratio of the core enzyme relative to the holoenzyme in the heart. Already at day 1 after birth, transgenic mice have an increased heart-to-body weight ratio that persists throughout life. End-diastolic and end-systolic dimensions are increased and fractional shortening is decreased. In addition, the thickness of the septum and of the left ventricular posterior wall is significantly reduced. This phenotype is considered to be a form of dilated cardiomyopathy that frequently leads to premature death. (Brewis *et al.*, 2000).

**PP2A Substrates and Complex Formation**

PP2A regulates many cellular processes by reversing the actions of protein kinases. In addition, many kinases are regulated by reversible phosphorylation and turn out to be substrates or form stable complexes with protein phosphatases. PP2A appears to be the major kinase phosphatase in eucaryotic cells that down regulates activated protein kinases. Thus, PP2A is likely to play an important role in determining the activation kinetics of protein kinase cascades. In contrast, the activity of the PP2A and other phosphatases can be regulated by kinases as well, which would lead, in the case of PP2A, to inhibition. More than 30 protein kinases are known to be regulated by the action of PP2A (Table 2) and many of them form stable complexes *in vitro* (Table 3) and some *in vivo*. Most of the kinases regulated by PP2A belong to distinct families of protein kinases, such as the AGC family (PKA, PKB, PKC, p70S6K), the CMGC family (ERK, MAPK, CDK) and the CAMK family (CaMK, IkK). With some exceptions (CK1, Raf-1, GSK-3, MST1, Wee1), all protein kinases get inactivated upon PP2A-mediated dephosphorylation (reviewed in (Millward *et al.*, 1999)).

For example, PKB is stimulated in cells upon treatment with PP2A-specific inhibitors OA (Andjelkovic *et al.*, 1996a) and calyculin A (Meier *et al.*, 1998), indicating its negative regulation by PP2A. PKCα is dephosphorylated by a B/PR55-containing trimer *in vitro* (Ricciarelli and Azzi, 1998) and in cell extracts by a membrane-bound, B/PR55-containing PP2A trimer (Hansra *et al.*, 1996). Similar to PKB, MEK1 and ERK-family kinases are activated after treatment of cells with OA (Gause *et al.*, 1993; Sonoda *et al.*, 1997) and dephosphorylated by PP2A *in vitro* (Anderson et al., 1990; Gomez and Cohen, 1991; Haccard et al., 1990).

CyclinB-Cdc2 is dephosphorylated by PP2A, since it was shown that it is activated *in vivo* by OA and other PP2A-specific inhibitors (Lee *et al.*, 1991a). As mentioned above, some of the substrates of PP2A also form stable complexes with different subunits of the PP2A trimer. For example, CKIIa the catalytic subunit of CK2, forms a complex with the PP2A core enzyme via the C subunit, and stimulates PP2AC activity towards Raf-phosphorylated MEK1 (Heriche *et al.*, 1997). Expression of activated Raf results in disruption of the CKIIa-PP2A association (Lebrin *et al.*, 1999), which may be a necessary step for maximal activation of the MAPK pathway by Raf. A complex between $Ca^{2+}$/calmodulin-dependent kinase IV (CaMKIV) and PP2A dephosphorylates and inactivates a kinase, acting as a negative regulator of CREB-mediated transcription in Jurkat T cells (Westphal *et al.*, 1998).

In rat brain extracts, PAK1, PAK3 (Westphal *et al.*, 1999), as well as p70S6 kinase (Peterson *et al.*, 1999; Westphal *et al.*, 1999) form complexes with PP2A. In addition, dephosphorylation of p70S6 kinase by purified PP2A leads to inactivation of the kinase (Ballou *et al.*, 1988). Another PP2A-interacting kinase is the Janus kinase JAK2, which associates transiently with PP2A, PI-3K, and Yes (Src family member), upon interleukin-11 stimulation of adipocytes (Fuhrer and Yang, 1996).

The IkB kinase complex (catalytic IKKα and β subunit and regulatory IKKγ subunit) phosphorylates IkB, an inhibitory subunit of NF-kB, and targets it for polyubiquitination and proteasome-mediated degradation. IKKα is activated upon exposure of cells to OA and is inactivated by PP2A *in vitro* (DiDonato *et al.*, 1997). IKKγ binds Tax (transactivator/oncoprotein of HTLV-I) and PP2A, and forms a stable ternary complex via a tripartite protein-protein interaction. Tax-mediated constitutive IKK activation is

due in part to a direct interaction between Tax and IKKγ (Chu et al., 1998; Chu et al., 1999; Good and Sun, 1996; Jin et al., 1999; Sun and Ballard, 1999; Sun et al., 1994; Xiao and Sun, 2000). It is assumed that IKKγ-associated PP2A is responsible for the rapid deactivation of IKK, and inhibition of PP2A by Tax in the context of IKK-PP2A-Tax ternary complex leads to constitutive IKK and NF-kappa B activation (Fu *et al.*, 2003).

PP2A interacts directly with axin and GSK3 in the β-catenin-containing adenomateus polyposis coli (APC)-complex (Fig. 15). The APC protein is mutated in over 80% of sporadic colon cancers and it is thought that the PP2A-APC complex plays a role in the turnover of the β-catenin protein, whose stabilization itself plays an important role in the development of cancer (Polakis, 2000). The PR61/B'-containing PP2A trimer functions as scaffold protein for axin, GSK3 and β-catenin assembly (Seeling *et al.*, 1999). Within this complex, GSK3 is constitutively active and phosphorylates β-catenin, which is promoted by axin. The inactive PP2A is not able to dephosphorylate its substrates due to inhibitory regulation of catalytic PP2A activity by PR61/B' (Ratcliffe *et al.*, 2000). This leads to β-catenin degradation and inhibits the Wnt signaling pathway (Hsu *et al.*, 1999). In the presence of a Wnt signal, Dsh inhibits GSK3, which leads to accumulation of unphosphorylated β-catenin, axin and APC. Activated PP2A complexed to axin may contribute directly to this state by dephosphorylating GSK3-phosphorylated members of the complex. β-catenin can then translocate to the nucleus and activate Wnt target genes (Kikuchi, 2000). PP2A complexed to axin can directly dephosphorylate GSK3-phosphorylated APC and axin (Ikeda *et al.*, 2000).

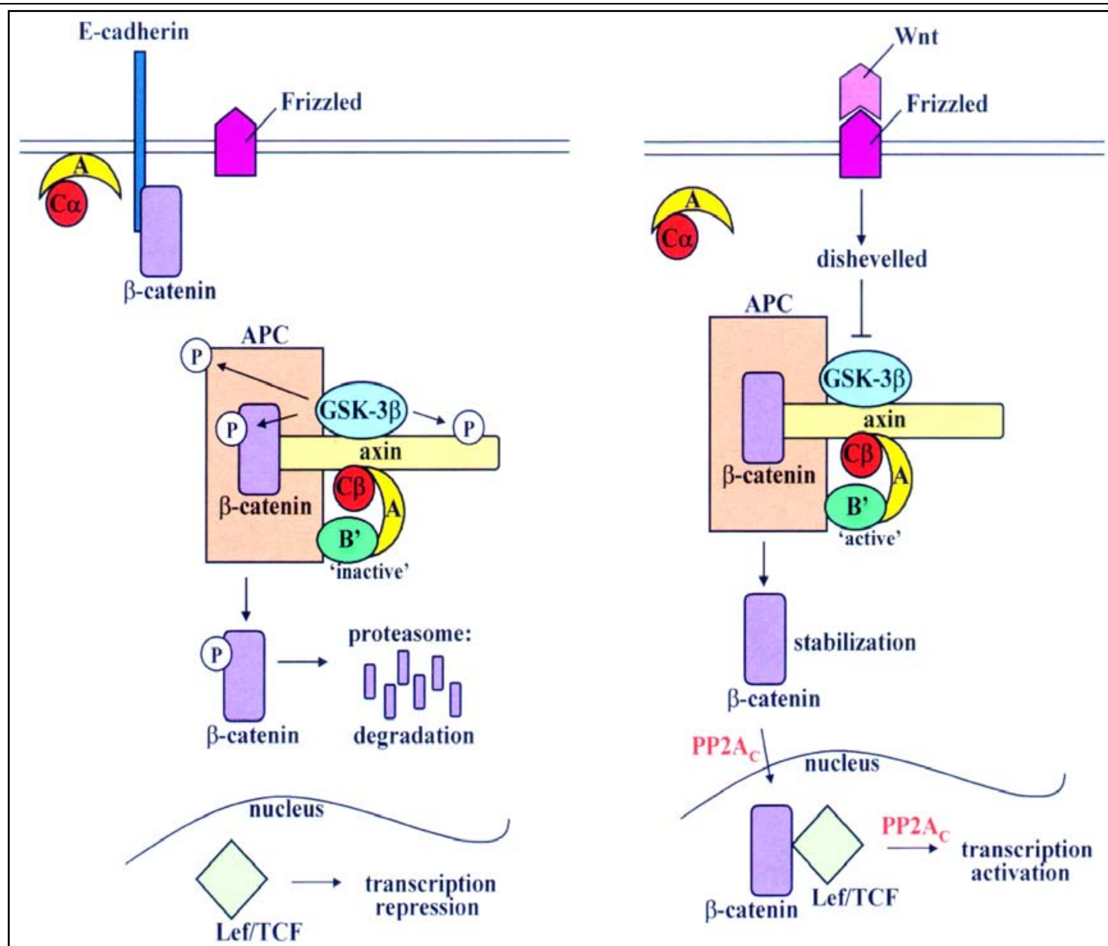


**Fig. 15:** In the absence of a Wnt signal, β -catenin is present in two distinct complexes. One complex is located at the plasma membrane, where $PP2A_{C\alpha}$ stabilizes the β-catenin–E-cadherin complex, which itself mediates interactions with the actin cytoskeleton. The other complex is located in the cytoplasm and contains axin, APC, GSK-3b and PP2A (AB´Cβ). Within this complex, GSK-3β is thought to be constitutively active, resulting in the phosphorylation of β-catenin, APC and axin. In this case, the associated PP2A activity may not be high enough to counteract GSK-3β-mediated phosphorylation. This may be achieved by negative regulation of $PP2A_C$ activity by B´/PR61 – hence the PP2A AB´C trimer is denoted 'inactive'. Phosphorylated β-catenin is unstable, becomes ubiquitinated and is eventually degraded by proteasomes. In the presence of a Wnt ligand, GSK-3β activity in the APC–β-catenin–axin–GSK-3β–PP2A complex is blocked by Disheveled, resulting in the accumulation of unphosphorylated axin, APC and β-catenin. PP2A may contribute to this state by directly dephosphorylating APC and axin, and possibly β-catenin. This implies that PP2A should be activated – or, alternatively, that the B´/PR61-mediated inhibition of $PP2A_C$ activity should be relieved. How exactly this is achieved is not clear. Unphosphorylated axin will be degraded specifically, leading to dissociation of unphosphorylated β-catenin from the complex and accumulation in the cytosol. After translocation to the nucleus, it can transactivate specific target genes. There is evidence that $PP2A_C$ is involved in this part of the pathway as well, either in the translocation of β-catenin to the nucleus or in the regulation of Lef/TCF transcriptional activity by β-catenin (taken from (Janssens and Goris, 2001)).

Integrins are shown to control PKCε phosphorylation by determining complex formation with PP2A and the upstream kinase PDK1. Complex formation between PKCε and PP2A is not constitutive, but seems to be highly regulated by changes in integrin engagement. The PP2A-induced loss of PKCε function results in attenuated interferon-γ (INF-γ)-induced phosphorylation of STAT1 downstream of JAK1/2. PKCε function and the IFN-γ response can be recovered by inhibition of PP2A if PDK1 is associated with PKCε in this complex. Therefore, PKCε is established as a point of convergence for the pathways operating downstream of the IFN-γ receptor and integrins (Ivaska *et al.*, 2003).

Recent studies reveal a role for PP2A in localization and activation of two proteins of the Ras–MAPK signaling pathway, Raf-1 and Kinase suppressor of RAS (Ksr) (Fig. 16). Using mass spectrometry, PP2A was identified as a component of the Ksr signaling complex in cell cultures and in brain lysates from mice. The PP2A core dimer is bound to Ksr under both stimulated and non-stimulated conditions, whereas the association of the regulatory B subunit with Ksr was dependent on growth factor stimulation (Ory *et al.*, 2003). Mutations in several PP2A subunit genes have been recovered in *Drosophila* and *C. elegans* genetic screens for components of the Ras pathway (Sieburth et al., 1999; Wassarman et al., 1996). PP2A is a positive effector of Ras signaling in *C. elegans*, while in *Drosophila*, PP2A has both positive and negative effects on the Ras pathway. These differences presumably reflect the multiplicity of targets that are affected by loss of PP2A activity. For example, MEK and ERK are likely candidates for the inhibitory effect of PP2A, given that both kinases can be dephosphorylated and inactivated by PP2A *in vitro* (Frost *et al.*, 1994; Zhou *et al.*, 2002) and that inhibition of PP2A leads to MEK and ERK activation *in vivo* (Alessi et al., 1995; Frost et al., 1994; Silverstein et al., 2002; Sontag et al., 1993). In conclusion, PP2A is a component of the KSR1 scaffolding complex and it positively regulates both KSR1 and Raf-1. Moreover, PP2A activity is required for KSR1 to promote ERK pathway activation. This defines a biochemical mechanism for PP2A acting as a positive effector of Ras signaling (reviewed in (Raabe and Rapp, 2003)).

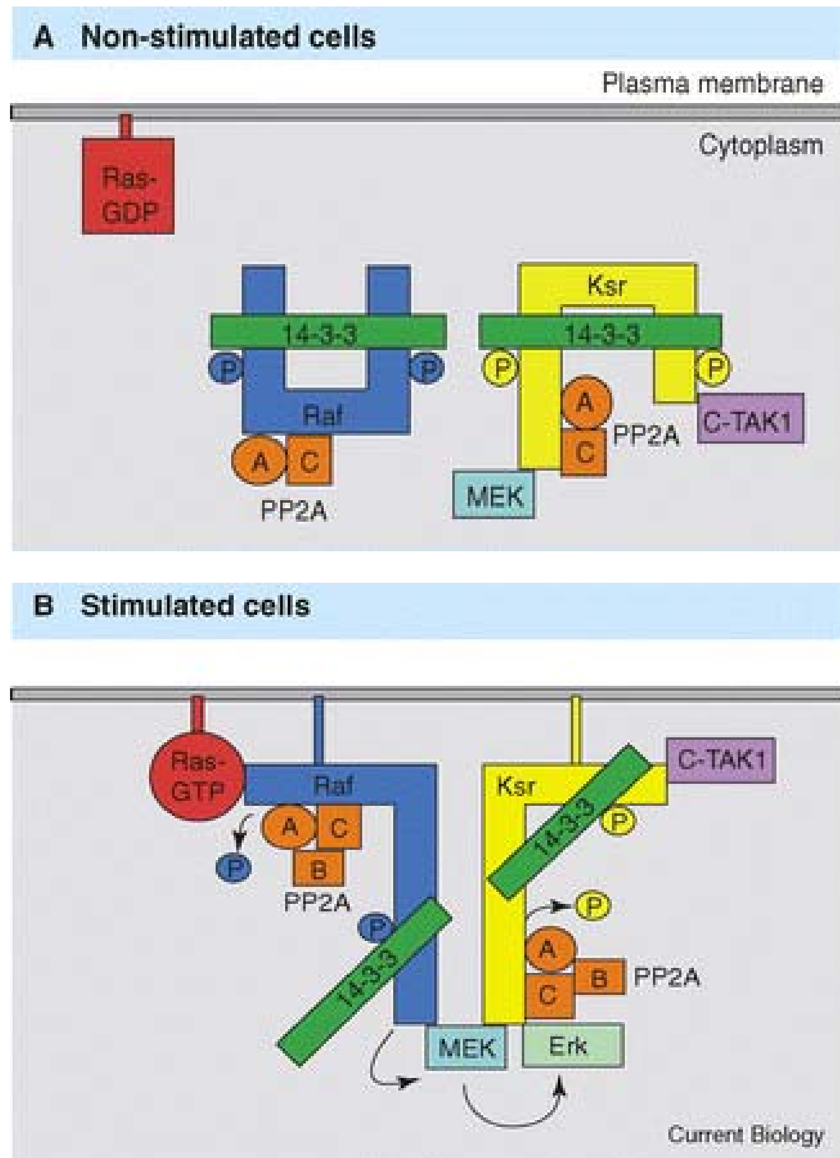


**Fig. 16:** Model for the regulation of Raf and Ksr by protein phosphatase 2A.
(A) In non-stimulated cells, binding of dimeric 14-3-3 to phosphorylated Raf (Ser259 and 621) and Ksr (Ser297 and 392) retains both complexes in the cytoplasm. Phosphorylation of Ksr at Ser297 and Ser392 is mediated by C-TAK1 whereas the kinase(s) that phosphorylate Ser 259 and 621 of Raf have not been fully elucidated. The PP2A core enzyme is bound to Raf and Ksr. In addition, inactive MEK is associated with Ksr. (B) Stimulation of cells by growth factors results in the assembly of the active PP2A holoenzyme leading to dephosphorylation of Ser259 in Raf and Ser392 in Ksr. Displacement of 14-3-3 from these sites facilitates the membrane recruitment of both proteins. A current model suggests that the transition from the inactive to the active, membrane-bound form of Raf also requires interaction with phospholipids, binding to Ras-GTP, several phosphorylation events and reassociation with 14-3-3. As a result, an active signaling complex of Raf, MEK and Erk is formed at the membrane (taken from (Raabe and Rapp, 2003)).

A novel regulatory mechanism has been suggested for microtubule-associated PP2A. The α4 protein interacts with the catalytic subunit of PP2A independent of its regulatory A and B subunits. The α4 protein is also able to complex with MID1, a phospho protein involved in the pathogenesis of BBB/G Opitz syndrome (OS) (Fig. 17). The MID1-α4 complex is suggested to function as an E3-ubiquitin ligase for degrading the catalytic subunit of PP2A, leaving as-yet-undefined proteins bound to microtubules in their phosphorylated state. Mutated MID1, as frequently found in OS, loses its ability to bind to the microtubules and can no longer provide the scaffold for the ubiquitination complex. PP2Ac assembles with its regulatory subunits and dephosphorylates the unknown phosphoproteins (Trockenbacher et al., 2001) (reviewed in (Schweiger and Schneider, 2003)).

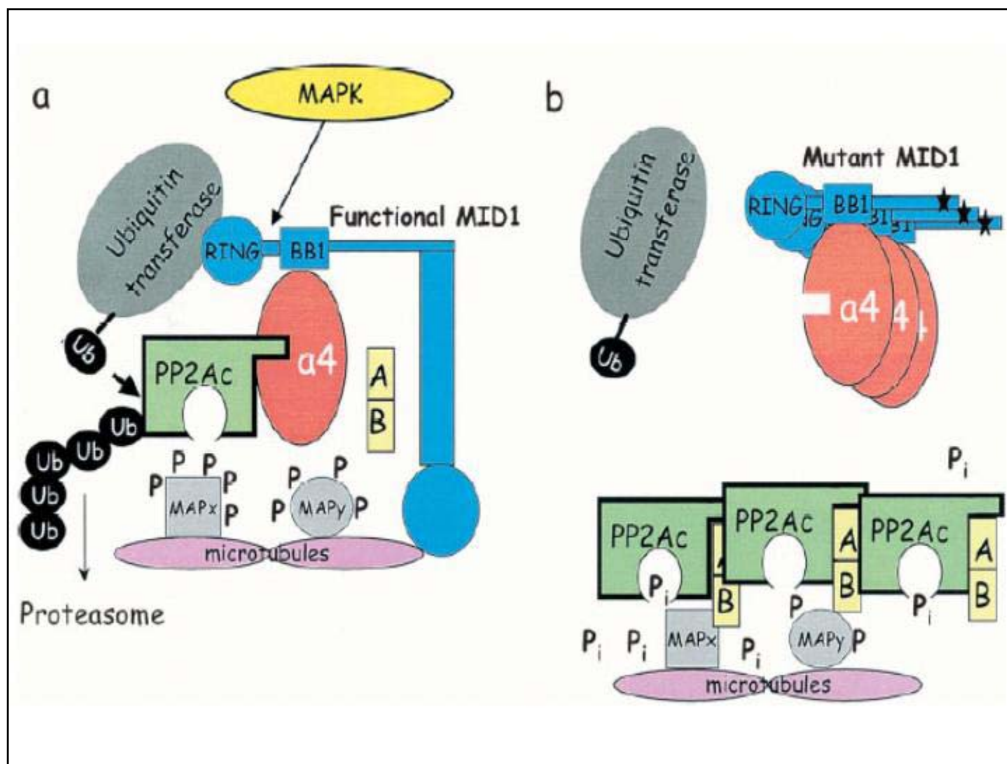


**Fig. 17:** Model of the MID1-mediated ubiquitin-dependent regulation of PP2A (a) and its disruption in BBB/G Opitz syndrome (b). a: MAPK triggers microtubule association of MID1 via phosphorylation of Ser96. Functional MID1 binds to α4 and MID1–α4 displaces the A and B regulatory subunits from microtubule-associated PP2Ac. A postulated ubiquitin transferase unit bound to the RING finger of MID1 transfers ubiquitin onto PP2Ac, which, in its polyubiquitinated state, will be degraded by the

proteasome. As-yet-undefined microtubule-associated (P) proteins (MAPx/y) remain in their equilibrium phosphorylated state as long as PP2A levels are controlled by this targeted degradation process. B: In OS, mutated MID1 is aggregated in the cytosol and can no longer juxtapose the ubiquitination controlled machinery and PP2Ac. Microtubule-associated PP2Ac accumulates and the phosphorylation-dephosphorylation equilibrium of the MAPs is shifted toward hypophosphorylation (taken from (Schweiger and Schneider, 2003)).

PP2A is able to associate with Shc through the Shc phosphotyrosine-binding domain (PTB domain). Insulin, IGF-1, or EGF treatment or small t expression causes dissociation of this complex with enhanced Shc phosphorylation. Thus, growth factor-stimulated Shc phosphorylation and downstream signaling increases in small-t-expressing cells. This suggests, that PP2A can negatively regulate growth factor signaling by binding to Shc and preventing its phosphorylation. In addition, these results indicate an additional level of control for Shc phosphorylation and a new mechanism for PP2A downregulation of the Ras/MAP kinase pathway (Ugi *et al.*, 2002).

| | Comments | Ref. |
|---|---|---|
| **Kinases inactivated by PP2A** | | |
| cAMP-dependent kinase | Major PKA phosphatase activity in cells is PP2A-like | (Liauw and Steinberg, 1996) |
| cGMP-dependent kinase b | | (Zhou et al., 1996) |
| PKB | PKB is activated in vivo by OA | (Andjelkovic et al., 1996a) |
| PKC | Dephosphorylated by membrane-associated PP2A | (Ricciarelli and Azzi, 1998) |
| PKCμ | | (Van Lint et al., 1998) |
| p70 S6 kinase | p70 S6 kinase forms a stable complex with PP2A | (Ballou et al., 1988) |
| CaM kinase I | | (DeRemer et al., 1992) |
| CaM kinase II | Downregulation of CaM-KII is prevented by OA in vivo | (Barnes et al., 1995) |
| CaM kinase IV | CaMKIV forms a stable complex with PP2A in vivo | (Park and Soderling, 1995) |
| AMP-activated kinase | The physiological AMPK phosphatase is probably PP2C | (Kudo et al., 1996) |
| MEK | Activated by OA and by expression of small t antigen | (Gomez and Cohen, 1991) |
| Ste7 | | |
| ERK MAP kinase | Activated by OA and by expression of small t antigen | (Anderson et al., 1990) |
| Fus3p | | (Errede et al., 1993) |
| SEK1 | | |
| p38/RK | | (Doza et al., 1995) |
| JNK1/SAPK | | |
| MAPKAP kinase 2 | | (Stokoe et al., 1992) |
| p90 RSK1, p90 RSK3 | | (Stokoe et al., 1992) |
| CDC2 (CDK1) | CyclinB-Cdc2 is activated in vivo by OA | (Lee et al., 1991a) |
| CDK2 | | (Poon and Hunter, 1995) |
| Polo-like kinase (Plk) | | (Mundt et al., 1997) |
| IkB kinase (IKK) | IKK-PP2A-Tax complex: PP2A inhibits, Tax activates | (DiDonato et al., 1997) |
| 31-kDa S6 kinase | | (Hei et al., 1994) |
| p21-activated kinase (PAK1) | PAK1 forms a stable complex with PP2A | (Westphal et al., 1999) |
| Mck1 | | (Hei et al., 1994) |
| Casein kinase II | | (Lebrin et al., 1999) |
| pp60 SRC | | (Ogris et al., 1999) |
| Pim kinase | | (Losman et al., 2003) |
| KSR1 | kinase suppressor of Ras binds to C and A subunit | (Ory et al., 2003) |
| **Kinases activated by PP2A** | | |
| Casein kinase I | | (Cegielska et al., 1998) |
| RAF-1 | RAF-1 forms a stable complex with PP2A | (Dent et al., 1995) |
| GSK-3 | | (Sutherland and Cohen, 1994) |
| MST1 | | (Creasy and Chernoff, 1995) |
| WEE1 | Phosphorylated on inhibitory sites | (Mueller et al., 1995) |

**Table 2 :** In vitro protein kinase substrates of PP2A (updated from (Millward et al., 1999))

| | Comments | Refs |
|---|---|---|
| **Protein kinases** | | |
| p70 S6 kinase | p70 S6 kinase is a PP2A substrate | (Westphal et al., 1999) |
| CaM kinase IV | Binds to ABC complex; substrate for C | (Westphal et al., 1998) |
| Casein kinase IIa | Binds to AC dimer in quiescent cells; stimulates activity of C | (Heriche et al., 1997) |
| RAF-1 b | RAF-1 can be dephosphorylated by PP2A | (Dent et al., 1995) |
| p21-activated kinase (PAK1) | PAK1 is a PP2A substrate | (Westphal et al., 1999) |
| JAK2 | Association upon IL11 stim. of adiposides | (Fuhrer and Yang, 1996) |
| TGF-β1 receptor | Binds to B subunit; inhibits p70S6K signaling | (Petritsch et al., 2000) |
| atypical PKC | Regulation of TJ formation | (Nunbhakdi-Craig et al., 2002) |
| PKCe | PKCe-PP2A-PDK1 complex regulated by integrins | (Ivaska et al., 2003) |
| KSR1 | Kinase suppressor of Ras binds to C and A subunit | (Ory et al., 2003) |
| IKK | IKK-PP2A-Tax complex: PP2A inhibits, Tax activates | (Agazie and Hayman, 2003) |
| **Other cellular proteins** | | |
| I-1PP2A (PHAPI, mapmodulin) | Endogenous, heat-stable inhibitor of PP2A | (Li et al., 1996a) |
| I-2PP2A (SET) | Endogenous, heat-stable inhibitor of PP2A | (Li et al., 1996b) |
| Tap42/4 | Binds to C; interaction dependent upon TOR1 | (Murata et al., 1997) |
| Cyclin G1 | Binds to B subunits of the B' (PR61) family | (Okamoto et al., 1996) |
| p107 (pRb-related) | Binds PR59-containing ABC complex | (Voorhoeve et al., 1999) |
| HOX11 | Binds to C subunit; inhibits phosphatase activity | (Kawabe et al., 1997) |
| HRX | Binds PP2A through I2PP2A; mutated in acute leukaemias | (Adler et al., 1997) |
| Caspase-3 | Activates PP2A during apoptosis by proteolysis of A | (Santoro et al., 1998) |
| PTPA (PPP2R4) | Binds dimer through A subunit; confers tyr-phos activity | (Cayla et al., 1994) |
| TAU | Dephosphorylated by ABC; promotes microtubule binding | (Sontag et al., 1996) |
| Neurofilament proteins | AC, binds and dephos. NF proteins, promotes assembly | (Saito et al., 1995) |
| eRF1 | Binds dimer through C subunit; targets dimer to ribosomes | (Andjelkovic et al., 1996b) |
| Paxillin | Binds PR61/B'γ, truncated PR61/B'γ causes malignancy | (Ito et al., 2000) |
| Cdc6 | Binds PR48/B'', modulates DNA replication in human cells | (Yan et al., 2000) |
| Bcl2 | Binds to C subunit | (Deng et al., 1998) |
| Vimentin | Binds to B subunit, no association without B | (Turowski et al., 1999) |
| HePTP | Complex has pERK phosphatase activity | (Wang et al., 2003) |
| Importin beta family | Native complex, regulating nuclear import | (Lubert and Sarge, 2003) |
| MID1 | Complex MID-PP2A-a 4, OptizBBB/G syndrome | (Schweiger and Schneider, 2003) |
| ERa | No complex with C upon OA, then ER-MAPK complex | (Lu et al., 2003) |
| tight junction complex | Complex with ABaC-TJ proteins at apical membrane | (Nunbhakdi-Craig et al., 2002) |
| bestrophin | Binds to Cb subunit | (Marmorstein et al., 2002) |
| Shc | No complex upon insulin/ EGF stim. or small t antigen inhibit. | (Ugi et al., 2002) |
| RelA/NFkB | Binds to A subunit | (Yang et al., 2001) |
| NMDA-R subunit NR3A | Dynamic complex regulation by NR3A and PP2A interaction | (Chan and Sucher, 2001) |
| E4orf4 | E4orf4-PP2A interacts with the APC/C | (Marmorstein et al., 2002) |
| GADD45A | Component of APC complex | (Hildesheim et al., 2003) |
| hUPF1 | Complex involved in nonsense-mediated mRNA degradation | (Ohnishi et al., 2003) |
| axin | Binds to PR61/B' in APC complex (Wnt signaling) | (Gao et al., 2002) |
| **Viral proteins** | | |
| SV40 small t | Binds to and inhibits AC dimer; displaces B subunits | (Scheidtmann et al., 1991) |
| Polyomavirus small t | Confers tyrosine phosphatase activity | (Cayla et al., 1993) |
| Polyomavirus middle T | Confers tyrosine phosphatase activity | (Cayla et al., 1993) |
| Adenovirus E4orf4 | Binds to ABC complex; causes downregulation of AP-1 | (Bondesson et al., 1996) |
| HIV Ncp7:Vpr | Binds to AB'C complex; activates C subunit | (Tung et al., 1997) |

**Table 3:** Proteins that form stable complexes with PP2A (updated from (Millward et al., 1999))

**PP2A and Transcription Regulated by Viral Proteins**

Reversible phosphorylation of transcription factors regulates their trans-activating potential, DNA-binding properties and intracellular location. In addition to its physiological targets, such as cell-surface receptors, ion channels, protein kinases, key regulatory enzymes and proteins involved in metabolism, PP2A is also involved in the direct and/or indirect regulation of transcription factors (Alberts et al., 1993; Cohen, 1989; Mumby and Walter, 1993; Wera and Hemmings, 1995). This regulation is partially due to the fact that viruses have developed distinct strategies to deregulate cellular signaling via PP2A. Some viruses simply incorporate PP2A holoenzymes or downstream components of the PP2A signaling pathway. Polyoma small t and middle T, as well as SV40 small t, form stable complexes with the PP2A core dimer by competing with the B regulatory subunits (Pallas *et al.*, 1990; Walter *et al.*, 1990). The A regulatory subunit of the PP2A core dimer is the only cellular protein known to bind to SV40 small t (Pallas *et al.*, 1990; Yang *et al.*, 1991). Experiments performed with SV40 small t and OA have clearly demonstrated that PP2A is a negative regulator of major transcription factors including AP1 (Frost *et al.*, 1994), NF-kB (Sontag *et al.*, 1997), Sp1 (Vlach *et al.*, 1995), and CREB (Wheat *et al.*, 1994) (Fig. 18). The proto-oncogene c-Jun, a major component of the activator protein-1 (AP-1) transcription-factor complex, is a substrate for PP2A. Evidence supporting this notion includes the observation that OA increases the concentration, phosphorylation and DNA binding of c-Jun as well as AP-1 (Black *et al.*, 1991; Lee *et al.*, 1994a). Compared to the other major mammalian protein serine/threonine phosphatases, purified PP2A preferentially dephosphorylates c-Jun isolated from OA-treated cells (Black *et al.*, 1991; Lee *et al.*, 1994a). In agreement with this, microinjection studies with purified PP2A (Alberts *et al.*, 1993) and $I_2^{PP2A}$ expression in HEK 293 cells (Al-Murrani *et al.*, 1999) both demonstrate that this phosphatase regulates c-Jun activity. Expression of SV40 small t antigen also increases the concentration and phosphorylation of c-Jun, as well as AP-1 transcriptional activity (Frost *et al.*, 1994; Howe *et al.*, 1998). SV40 small t promotes transformation and cell growth by activating PKCζ, resulting in MEK activation and NF-kB-dependent transactivation. Upon inhibition of PP2A, PKCζ and NF-kB

become constitutively activated (Sontag *et al.*, 1997). NF-kB also plays a critical role in the regulation of the expression of various viruses, including SV40 (Sassone-Corsi *et al.*, 1985), HIV-1 (Nabel and Baltimore, 1987), CMV (Cherrington and Mocarski, 1989), herpes simplex virus type 1 (HSV-1) (Cherrington and Mocarski, 1989) and hepatitis B (Siddiqui *et al.*, 1989). SV40 small t also inhibits dephosphorylation of PKA-phosphorylated CREB and, thereby, stimulates CREB-dependent transactivation (Wheat *et al.*, 1994). Finally, small t also induces transactivation of Sp1-responsive promoters through inhibition of PP2A activity (Garcia *et al.*, 2000). The association of adenovirus E4orf4 protein with a PR55/B subunit results in down regulation of JunB transcription (Bondesson *et al.*, 1996). This effect could play an important role during viral infection by regulating the apoptotic response of the infected cells in a p53-independent manner (Shtrichman and Kleinberger, 1998). In addition to further tumor viruses, the lentivirus HIV-1 encodes proteins that directly interact with PP2A. *Vpr*, the product of a regulatory gene of HIV-1, can induce G2 cell cycle arrest by inhibiting CDK1-cyclin B complex activation. This effect is reversed by OA, suggesting that *Vpr* activates PP2A (Re *et al.*, 1995). In line with this, HIV-1 *Vpr* interacts with PP2A *in vitro* and activates its catalytic activity (Tung *et al.*, 1997). PP2A has also been implicated in the regulation of STAT3. This transcription factor is activated through tyrosine phosphorylation by JAK kinases and serine phosphorylated by MAPK upon angiotensis II (Liang *et al.*, 1999). Inhibition of PP2A by specific phosphatase inhibitors induces (i) phosphorylation of STAT3 on serine and threonine residues, (ii) inhibition of STAT3 tyrosine phosphorylation and DNA binding activity, and (iii) relocation of STAT3 from the nucleus to the cytoplasm. This indicates, that PP2A plays a crucial role in the regulation of STAT3 phosphorylation and subcellular distribution in T cells (Woetmann *et al.*, 1999). As mentioned in “PP2A and Cell Cycle” the transcription factor HOX-11 was reported to interact with and inhibit PP2Ac and PP1c, abrogating a G2 checkpoint that could promote genomic instability and oncogenesis (see also Table 3) (Kawabe et al., 1997). The activity of a general coactivator, the four-and-a-half-LIM-only protein 2 (FHL2), is upregulated upon SV40 small t-antigen inhibition of PP2A. FHL2 functions as a coactivator for CREB-mediated transcription, and inactivation of PP2A further increases

FHL2-induced CREB-directed transcription. This effect can be reversed by overexpression of PR61/B'α, B'β and B'γ (Johannessen et al., 2003).

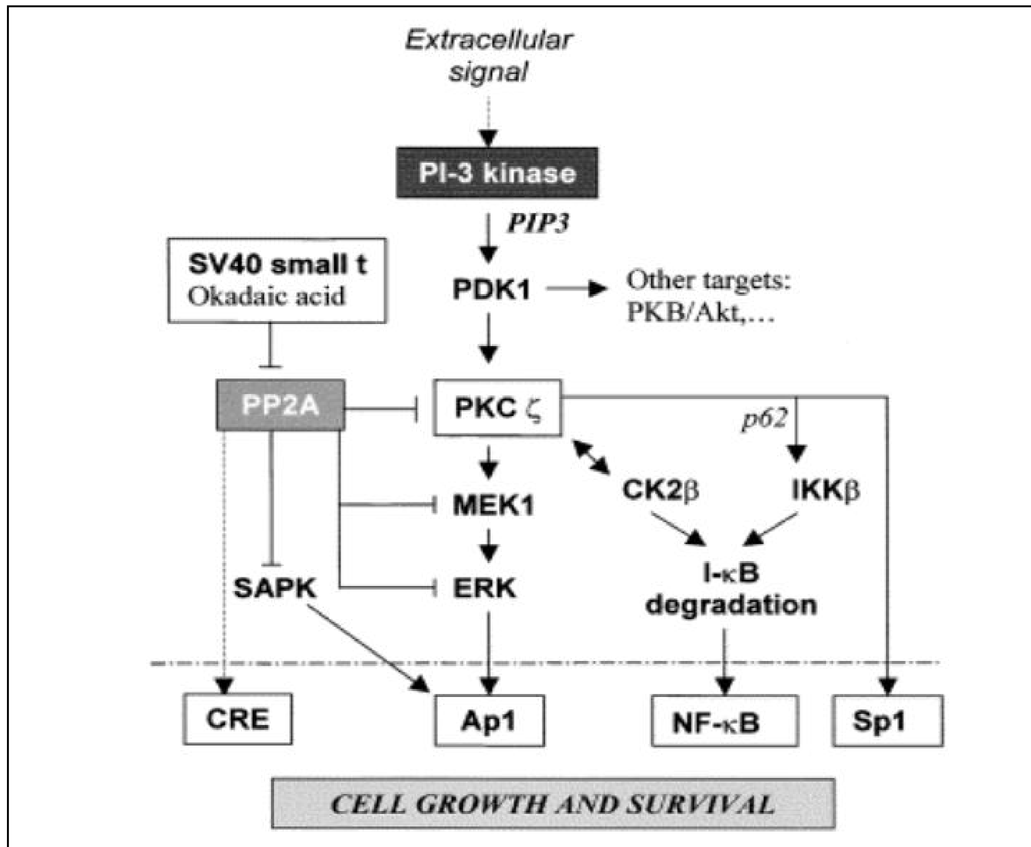


**Fig 18:** Simplified model for the regulation of gene transcriptional activation by interaction of SV40 small t with PP2A (taken from (Sontag, 2001)).

**PP2A and Disease**

PP2A appears to be critically involved in the regulation of a diverse set of cellular processes such as metabolism, transcription, translation, cell cycle, signal transduction, differentiation, and oncogenic transformation. Therefore, it is assumed that any dysfunction of PP2A will have severe consequences for cell physiology. Many observations support a role for PP2A in the pathology of human diseases, such as cancer, Alzheimer's, spinocerebellar ataxia, AIDS, malaria and BBB/G Opitz syndrome (Table 4).

| Class | | Name | Gene symbol | Synonym | GenBank accession | Position | Diseases (presumed*) |
|---|---|---|---|---|---|---|---|
| PSTP | | | | | | | |
| PPP | | PP1 | PPP1C | | M63960 | 11q13 | Alzheimer's disease |
| | | PP2A | PPP2C | | M66483 | 5q23 | Cancer, Alzheimer's disease |
| | | PP2B | PPP3C | Calcineurin A | M29550 | 4q21 | Alzheimer's disease, cardiac hypertrophy, cerebral ischemia |
| | | PP4 | PPP4C | PPX | AF097996 | 16p11 | |
| | | PP5 | PPP5C | | X89416 | 19q13 | |
| | | PP6 | PPP6C | | X92972 | Xq22 | |
| | | PP7 | PPP7C | | AF27977 | Xp21 | Retinitis pigmentosa |
| PPM | | PP2C | PPM1A | | AF070670 | 14 | |
| PTP | | | | | | | |
| RPTP | I | CD45 | PTPRC | LCA, GP180, | Y00062 | 1q31 | immunodeficiency disease SCID |
| | IIA | RPTPm | PTPRM | | X58288 | 18p11 | |
| | | RPTPk, l | PTPRK | | L77886 | 6q22 | |
| | IIB | LAR | PTPRF | | Y00815 | 1q34 | |
| | III | RPTPb | PTPRB | | X54131 | 12q15 | |
| | | SAP-1 | PTPRH | | D15049 | 19q13 | Colon & pancreas cancer |
| | IV | RPTPa | PTPRA | LPR | M34668 | 20p13 | |
| | | RPTPe | PTPRE | | X54134 | 10q26 | Mammary cancer |
| | V | RPTPz | PTPRZ1 | | M93426 | 7q31 | Lung & renal cancer |
| | | RPTPg | PTPRG | | L09247 | 3p14 | Lung & renal cancer |
| | VI | IA-2 | PTPRN | | L18983 | 2q35 | Diabetes type I, Celiac disease |
| | | IA-2B | PTPRN2 | ICAAR | U65065 | 7q36 | Diabetes type I |
| NPTP | | PTP1B | PTP1N | | M31724 | 20q13 | Diabetes type II |
| | | SHP1 | PTPN6 | SHPTP1, PTP1c | X62055 | 12p13 | Aortic valve disease |
| | | SHP2 | PTPN11 | SHPTP3 | X70766 | 12q24 | Noonan & LEOPARD syndrome |
| | | PTP-PEST | PTPN12 | | M93425 | 7q11 | |
| | | PTP-H1 | PTPN3 | | M64572 | 9q31 | |
| | | PTP-MEG | PTPN4 | | M68941 | | |
| DSP | | VHR | DUSP3 | | L05147 | 17q21 | |
| | | MKP-1 | DUSP1 | PTPN10, HVH1 | X68277 | 5q34 | Prostate & pancreas cancer |
| | | Cdc25A | CDC25C | | M34065 | 5q31 | Cancer & Alzheimer's disease |
| | | PTEN | PTEN | MMac-1 | U92436 | 10q23 | Bannayan-Zonana syndrome, Cowden's disease, Lhermitte-Duclos syndrome, Prostate cancer |
| | | EPM2A | EPM2A | LD, LDE, MELF | AF084535 | 6q24 | Myoclonic epilepsy, type lafora |

**Table 4:** List of human protein phosphatase catalytic subunits with classification, official gene symbol synonym, Genbank accession, chromosomal position, and predicted involvement in disease (updated from (Schmidt and Hemmings, 2002)).

**Cancer**

The exact role that protein phosphatases play in the pathology of cancer is much less clear than the involvement of protein kinases (reviewed in (Schonthal, 2001)). Only a few phosphatases have been found to be directly implicated in carcinogenesis, such as the dual-specificity protein phosphatases CDC25 and the dual-specificity protein phosphatase PTEN. CDC25, an important regulator of cell cycle progression, is able to transform cells in culture and, therefore, appears to harbor oncogenic potential (Galaktionov *et al.*, 1995). In contrast, PTEN exhibits many characteristics of a typical tumor suppressor, since it is frequently found mutated or deleted in various types of advanced cancers (Simpson and Parsons, 2001). Initial evidence for a negative role for PP2A in tumor development came from the observation that the tumor promoter OA (Suganuma *et al.*, 1988) is a potent inhibitor of PP2A (Bialojan and Takai, 1988). Additional evidences, such as complexing with DNA tumor viruses, indicate that PP2A is a negative regulator of cell growth and might even function as a tumor suppressor. In contrast, opposing results have emerged suggesting a positive function in tumorigenesis (Mordan *et al.*, 1990; Rivedal *et al.*, 1990). Recent investigations identified somatic alterations in the human PR65/Aβ sequence in 15% of primary lung and colon tumors-derived cell lines and one deletion mutation in PR65/Aβ was shown to restrict binding of PR65/Aβ to the catalytic subunit (Wang *et al.*, 1998). Although the frequency of these mutations is low, they clearly implicate PP2A as a participant in tumorigenesis. Based on the site of mutation, it was shown that binding of the A subunit not only to the C-subunit but also to the regulatory B subunits was defective (Ruediger *et al.*, 2001b). The eight PR65/Aβ mutants found in human lung and colon cancer were generated by site-directed mutagenesis and assayed for their ability to bind B and C subunits. Two mutants showed decreased binding of PR72/B", but normal C subunit binding; two mutants exhibited decreased binding of the C subunit and of B"/PR72; and one mutant showed increased binding of both the C subunit and B"/PR72. Of the three mutants that behaved like the wild-type PR65/Aβ subunit, one is a polymorphic variant and another is altered outside the binding region for B and C subunits. Interestingly, the wild-type PR65/Aα and PR65/Aβ isoforms, although 85% identical, are remarkably different in their ability to bind B and C subunits (Colella et al., 2001; Ruediger et al., 2001a; Ruediger et al., 2001b). In addition, alterations of PR65/Aα have

been found in human melanomas, breast and lung carcinomas. It is assumed, therefore, that PP2A suppresses tumor development through its involvement in cell cycle regulation and cellular growth control. This is also supported by the fact that overexpression of PR65/Aα in rat fibroblasts leads to multinucleated cells. A role for PP2A in melanoma tumor progression was suggested by overexpression of PR61γ in malignant melanomas (Francia *et al.*, 1999). Furthermore, a N-terminally truncated form of PR61γ1 was associated with a higher metastatic state of melanoma cells (Ito et al., 2000; Ito et al., 2003). In addition, PP2A inhibits nuclear telomerase activity in human breast cancer cells (Li *et al.*, 1997) and human leukemia cells (Liu et al., 2002b). While telomerase activity is not detected in normal somatic cells, it is elevated in many primary human malignancies. This suggests, that *de novo* synthesis of telomeres is crucial for unlimited cell division. PP2A can thus counteract uncontrolled cell growth by inhibiting this enhanced telomerase activity in cancer cells. Another aspect of PP2A function is its association with the APC tumor suppressor. The APC protein is mutated in over 80% of sporadic colon cancers and the PP2A-APC complex is considered to play a role in the turnover of the β-catenin protein. The stabilization of β-catenin plays an important role in the development of cancer (Polakis, 2000). Loss of protein phosphatase 2A expression correlates with phosphorylation of differentiation related transcription factor 1-polypeptide-1 (DP-1) and reversal of dysplasia through differentiation in a conditional mouse model of cancer progression (Tilli et al., 2003). Caveolin-1 (cav-1) is elevated in metastatic mouse and human prostate cancer (Yang et al., 1998) and it is involved in the inhibition of PP1 and PP2A through scaffolding domain binding site interactions leading to increased phosphorylation of specific PP1/PP2A substrates. Interestingly, cav-1-mediated increased PKB activities are suggested to be responsible for enhanced cell survival of prostate cancer cells (Li et al., 2003a).

**Alzheimer's disease**

Alzheimer's disease (AD) is a common neurodegenerative disorder characterized by the presence of two histopathological hallmarks called senile plaque formation and neurofibrillary tangles. Senile plaques are deposits of β-amyloid peptide (Aβ) produced by abnormal processing of amyloid precursor protein (APP) (Selkoe, 1991), whereas

neurofibrillary tangles consist of abnormally hyperphosphorylated tau protein assembled in paired helical filaments (PHF) (Grundke-Iqbal *et al.*, 1986). The amount of neurofibrillary tangles is reported to directly correlate to the degree of dementia in AD patients. The high levels of the PP2A catalytic subunit in the brain (Cheng *et al.*, 2000) and the brain-specific expression of some members of the PR55/B (Grundke-Iqbal and Iqbal, 1999; Khatoon et al., 1994; Lee et al., 1991b) and PR61/B'(Bennecib *et al.*, 2001) subunit families suggests that PP2A has unique functions in neuronal cells (reviewed in (Price and Mumby, 1999)). As major brain phosphatases, PP1, PP2A and PP2B regulate microtubule-associated proteins (MAP) such as tau and MAP2. Inhibition of PP2A leads to hyperphosphorylation of tau at multiple sites followed by its dissociation from microtubules (MT) and loss of MAP-mediated MT-stability. Of the serine/threonine phosphatases that were confirmed to be involved in dephosphorylating the 29 phosphorylation sites of the tau protein, PP2A is likely to be the major tau phosphatase. Inhibition of PP2A by OA in metabolically competent rat brain slices induced an increase in the phosphorylation/activation of ERK1/2, MEK1/2, and p70 S6 kinase as well as the phosphorylation of tau at several sites (Pei et al., 2003). Although tau hyperphosphorylation induced by OA-mediated protein phosphatase inhibition contributes to pathological aggregate formation, only hyperphosporylation of tau followed by proteasome inhibition leads to stable fibrillary deposits of tau similar to those observed in neurodegenerative diseases (Goldbaum et al., 2003). A pool of PP2A, especially a PR55/Bα containing trimer, associates with tau protein directly or interacts with microtubules through tau (Sontag *et al.*, 1996; Sontag *et al.*, 1999). *In vitro* studies have shown that hyperphosphorylated tau fails to promote microtubule assembly (Alonso et al., 1996; Bramblett et al., 1993; Gong et al., 1996; Iqbal et al., 1994; Wang et al., 1995) and, thus, it leads to microtubule destabilization, appearance of neurofibrillary tangles, and neurodegeneration in AD brain (Trojanowski and Lee, 1994). PP2A activity is reduced 30% in brain homogenates from AD patients (Gong *et al.*, 1995), and mRNA levels of Cα, PR55/Bγ and PR61/Bε are quantitatively decreased in the hippocampus of AD brains (Vogelsberg-Ragaglia *et al.*, 2001)..The role of PP2A in Alzheimers disease has been studied extensively in various transgenic mouse models, which are reviewed in (Gotz and Schild, 2003) and further described in the chapter "PP2A Mutant Mice".

**Spinocerebellar Ataxia 12**

Cerebellar dysfunction is a hallmark of different neurodegenerative disorders, like Spinocerebellar Ataxias (SCAs), but many also include abnormalities in other regions of the central and/or peripheral nervous system. Twenty distinct forms (SCAs 1–17, 19, 21 and 22) have been identified to date and most of them can be subclassified into three discrete groups based on pathogenesis: (i) the polyglutamine disorders, which result from proteins with toxic stretches of polyglutamine (SCAs 1, 2, 3, 7, and 17); (ii) the channelopathies, which result from disruption of calcium or potassium channel function (SCA6); (iii) the gene expression disorders, which result from repeat expansions outside of coding regions that may quantitatively alter gene expression (SCAs 8, 10, and 12) (for review see (Margolis, 2002). PR55/Bβ was predicted to be involved in SCA12. This form of autosomal dominant spinocerebellar ataxia is caused by CAG trinucleotide repeats in the 5' region of PR55/Bβ (Fig. 19). Onset of the disease is in the fourth decade of life and leads to loss of movement, head/extremity tremors and in a later stage to complete dementia. The CAG repeat associated with SCA12 lays 133 nucleotides upstream of the predicted transcription start side for PR55/Bβ. Up to 29 CAG repeats are considered normal, whereas more than 55 repeats are considered disease causative for SCA12. It is speculated, that the CAG repeat expansion affects PR55/Bβ expression and subsequently alters the function of PP2A in the brain (Holmes et al., 2001; Holmes et al., 1999).

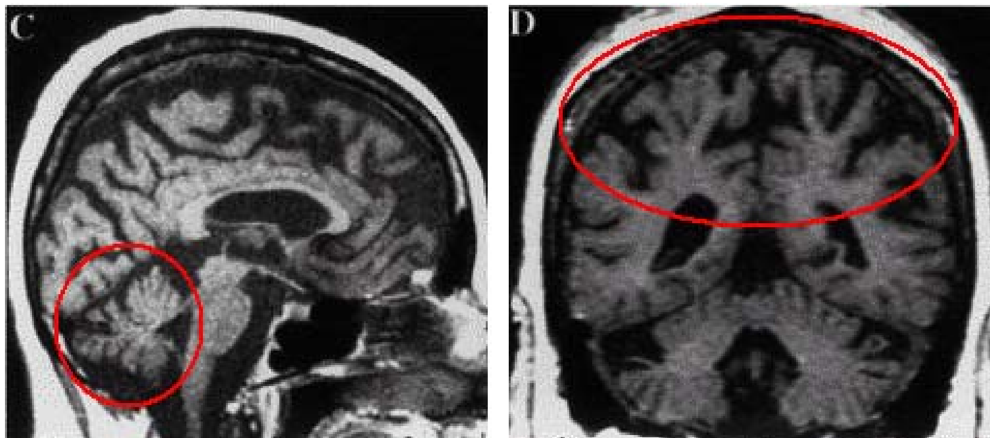


**Fig. 19:** Neuroradiologic images from a patient with spinocerebellar ataxia type 12. (C) (sagittal), (D) (coronal): T-1 weighted magnetic resonance images of a 59-year-old affected woman shows cerebellar and cortical atrophy (taken from (Holmes et al., 2001)).

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# V. Aim of Thesis

Protein Phosphatase 2A is on of the major multi-subunit serine/threonine protein phosphatases found in eukaryotic cells. It exerts pleiotropic effects on various cellular processes that may lead to the development of disease depending on the subunit assembly of the phosphatase.

The major aim of this thesis was to gain a more detailed insight into the structure and function of PP2A. Given the numerous cellular processes involving PP2A we sought to develop a method that allows overexpression of high levels of active PP2Ac for the investigation of active-site residues that were invariant for the catalytic function of PP2Ac.

Another goal was to investigate the biological function of PP2A and the involvement of its B regulatory subunits in the development of disease. Therefore, we determined the genomic organization, identified novel splice variants and analyzed expression levels and the subcellular localization of the PP2A B regulatory subunits in the murine brain. These studies will help to interpret phenotypes of transgenic or knockout mice that are altered or deficient in various PP2A subunits.

The protein serine-threonine phosphatase inhibitor okadaic acid was used in a GeneChip approach to model transcriptional effects of PP2A in HEK293 cells. In addition, we developed a software tool called StampCollector to predict potential transcription factor pairs that are involved in the regulation of genes based on their promoter sequence.

# VI. Results

**Part 1:** Active-site mutations impairing the catalytic function of the catalytic subunit of human protein phosphatase 2A permit baculovirus-mediated overexpression in insect cells.

T. Myles, **K. Schmidt**, D. R. H. Evans, P. Cron and B. A. Hemmings

Biochem. J. 2001, 357:225-232

**Part 2:** Diversity, developmental regulation and distribution of murine PR55/B subunits of protein phosphatase 2A.

**K. Schmidt**, S. Kins, A. Schild, R. Nitsch, B. A. Hemmings and J. Goetz

Eur. J. Neurosci. 2002;16(11):2039-2048

**Part 3:** Transcriptional effects of okadaic acid on promoter complexes using Affymetrix GeneChips.

**K. Schmidt**, E. J. Oakeley, Viktor Zhang, Herbert Angliker and B. A. Hemmings

# Active-site mutations impairing the catalytic function of the catalytic subunit of human protein phosphatase 2A permit baculovirus-mediated overexpression in insect cells

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Members of the phosphoprotein phosphatase (PPP) family of protein serine/threonine phosphatases, including protein phosphatase (PP)1, PP2A and PP2B, share invariant active-site residues that are critical for catalytic function [Zhuo, Clemens, Stone and Dixon (1994) J. Biol. Chem. **269**, 26234–26238]. Mutation of the active-site residues Asp[88] or His[118] within the human PP2A catalytic subunit (PP2Ac)α impaired catalytic activity *in vitro*; the D88N and H118N substitutions caused a 9- and 23-fold reduction in specific activity respectively, when compared with wild-type recombinant PP2Ac, indicating an important role for these residues in catalysis. Consistent with this, the D88N and H118N substituted forms failed to provide PP2A function *in vivo*, because, unlike wild-type human PP2Acα, neither substituted for the endogenous PP2Ac enzyme of budding yeast. Relative to wild-type PP2Ac, the active-site mutants were dramatically overexpressed in High Five® insect cells using the baculovirus system. Milligram quantities of PP2Ac were purified from $1\times10^9$ High Five cells and the kinetic constants for dephosphorylation of the peptide RRA(pT)VA (single-letter amino-acid notation) by PP2Ac ($K_m = 337.5\ \mu M$; $k_{cat} = 170\ s^{-1}$) and D88N ($K_m = 58.4\ \mu M$; $k_{cat} = 2\ s^{-1}$) were determined. The results show that the substitution impairs catalysis severely without a significant effect on substrate binding, consistent with the PPP catalytic mechanism. Combination of the baculovirus and yeast systems provides a strategy whereby the structure–function of PP2Ac may be fully explored, a goal which has previously proven difficult, owing to the stringent auto-regulatory control of PP2Ac protein levels *in vivo*.

Key words: kinetics, mutagenesis, PP2A, recombinant protein, yeast.

## INTRODUCTION

Protein phosphatase (PP)2A is a major protein serine/threonine phosphatase that plays an important role in diverse eukaryotic cellular processes regulated by reversible protein phosphorylation [1–4]. PP2A exists as a number of holoenzymes; the basic structure contains an invariant core dimer that is composed of a highly conserved 36 kDa catalytic subunit (PP2Ac) bound tightly to a 65 kDa regulatory subunit (PR65/A). PR65/A acts as a scaffold protein for the binding of PP2Ac and a large number of B-type regulatory subunits in the heterotrimeric holoenzyme [1,3]. The three major families of B-type subunits, PR55/B [5], PR61/B′ [6] and PR72/B″ [7] share no significant similarity in primary structure, and this diversity is believed to determine the enzymic activity and substrate specificity of PP2Ac, as well as its intracellular localization and the tissue specificity of distinct holoenzyme forms [8–11].

PP2Ac belongs to the phosphoprotein phosphatase (PPP) family of protein serine/threonine phosphatases and shares many invariant residues with the PP1 and PP2B (also known as calcineurin) catalytic subunits, and some active-site residues with bacteriophage λ phosphatase (λPPase), suggesting that these enzymes share a common catalytic mechanism [12–14]. Sequence alignment of the conserved phophoesterase domain (Figure 1) shows a consensus sequence important for metal and phosphate binding and catalysis [DXH-(∼25)-GDXXD-(∼25)-GNHD/E; where single-letter amino-acid notation has been used] [13]. Site-directed mutagenesis and kinetic analysis of PP1 [14,15] and λPPase [13] active-site residues, predicted to be involved in metal binding, substrate binding and catalysis, combined with the crystal structure of PP1 has defined a catalytic mechanism for members of the serine/threonine PPs [16]. The mechanism proposed for PP1 involves metal ion-mediated hydrolysis of the target substrate, where Ser(P)/Thr(P) is orientated for attack by a nucleophile in the active site by residues Arg[96] and Arg[122] (Arg[89] and Arg[115] in PP2Ac). An $H_2O$ molecule is activated to a hydroxide by two metal ions co-ordinated by several metal-binding residues. The hydroxide then makes a nucleophillic attack on Ser(P)/Thr(P). The PP1 residue His[125] (His[118] in PP2Ac) acts as a general acid, which protonates the leaving group oxygen, accelerating the dephosphorylation reaction. The acidic nature of His[125] is further enhanced by an ion-pair interaction with Asp[95] (Asp[88] in PP2Ac). The importance of the λPPase residues His[76] (His[125] in PP1) and Asp[52] (Asp[95] in PP1), and the PP1 residue Asp[95] in catalysis has been demonstrated by site-directed mutagenesis studies [13–15]. Recently, PP2Ac mutants mutated at the general acid residue His[118] or residues involved in metal-ion binding were expressed in NIH3T3 cells. Immunoprecipitated proteins had impaired phosphatase activity, revealing conservation of the PPP catalytic mechanism [17,18].

Abbreviations used: 5-FOA, 5-fluoroorotic acid; HA, haemagglutinin; moi, multiplicity of infection; PP, protein phosphatase; PP2Ac, PP2A catalytic subunit; PR65/A, PP2A regulatory subunit; PPP, phosphoprotein phosphatase; λPPase, bacteriophage λ phosphatase; *Sf*9, *Spodoptera frugiperda*; TBS, Tris-buffered saline.

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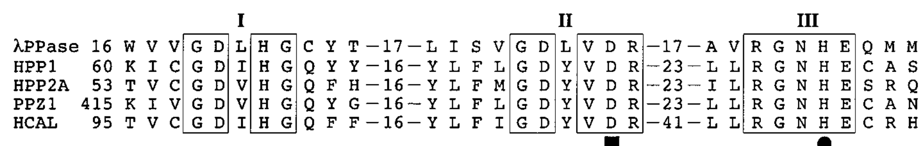


**Figure 1 Alignment of phosphoesterase domains of representative PPP family members**

λPPase, human PP1 (HPP1), human PP2Ac (HPP2A), *S. cerevisiae* PPZ1 and human calcineurin (HCAL) share the highly conserved phosphoesterase domains I, II and III (boxed). Numbers preceding the amino acid sequences denote the starting amino acids. Numbers between domains represent the number of amino acids between the conserved domains. The filled circle highlights the conserved histidine residue that acts as a general acid in catalysis. It's aspartate ion-pair partner is also shown (filled square).

Despite this, a detailed analysis of the structural and functional aspects of catalysis by PP2Ac is lacking, in part, due to the difficulty in overexpressing recombinant PP2Ac for purification. Thus in many cell lines, including NIH3T3 cells [19,20] and COS-7 cells [21], the expression of recombinant PP2Ac is maintained at a level comparable with that of endogenous PP2Ac by translational [19] or possibly transcriptional autoregulatory mechanisms [21]. In the present study we have analysed the effects of PP2Ac active-site mutations, involving the general acid residue $His^{118}$ and its ion-pair partner $Asp^{88}$.

## EXPERIMENTAL

### Materials

Purified rabbit skeletal muscle PP2Ac was a gift from Josef Goris (Katholieke Universiteit, Leuven, Belgium). The antibodies used in this study, AB45 and AB38, are rabbit anti-(human PP2Ac) and anti-(human PR65/A) respectively [22]. Horseradish-peroxidase conjugated goat anti-mouse and sheep anti-rabbit IgG antibodies, and Streptavidin Texas Red were purchased from Amersham Pharmacia Biotech. Secondary biotinylated goat anti-rabbit IgG antibodies were purchased from Bio-Rad, and okadaic acid was purchased from Alexis. The insect cell lines High Five® (*Trichoplusia ni* isolate BTI-TN-5b1-4) and *Spodoptera frugiperda* (*Sf*9) were obtained from Invitrogen.

### Construction of baculovirus transfer vectors and generation of recombinant baculovirus

The wild-type PP2Acα cDNA [23] was cloned into pBS $KS^-$ as a *Hin*dIII–*Bam*HI fragment and was used as a template for PCR amplification using *pfu* DNA polymerase to place a 5′ *Bam*HI haemagglutinin (HA) epitope in frame with the coding sequence downstream of the initiation codon and a 3′ *Eco*RI site immediately downstream of the termination codon. The fragment was cloned into pBS $KS^-$ (to generate pBSHACatα) and sequenced by an Applied Biosystems PRISM 377 sequencer. pBSHACatα was used for subcloning into the baculovirus transfer vector pBB4.5 (Invitrogen) to generate the transfer vector pBBHAPP2Ac. To generate the active-site substitutions H118N and D88N, pBSHACatα was used as template DNA for Quickchange PCR (Strategene) using appropriate mutagenic primers. Mutations were confirmed by DNA sequence analysis and subcloned into pBB4.5 to generate the transfer vectors pBBHAD88N and pBBHAH118N. Transfer vectors were co-transfected into *Sf*9 cells using the BAC-N-BLUE® linear transfection kit (Invitrogen) according to the manufacturer's instructions. Recombinant baculoviruses (vBBHAPP2Ac, vBBHAD88N and vBBHAH118N) were subjected to one round of plaque purification and identified by PCR amplification [24]. The recombinant baculovirus, PR65BV, was maintained on *Sf*9 cells as a high titre stock [25].

### Expression and Western-blot analysis of PP2Ac

Expression of HA-epitope-tagged wild-type and mutant PP2Ac was achieved by seeding 25 $cm^2$ flasks with High Five cells (Invitrogen) grown in Express Five® serum-free medium (Gibco BRL) at a density of $1 \times 10^5$ cells/$cm^2$, and infecting with recombinant baculoviruses (vBBHAPP2Ac, vBBHAD88N or vBBHAH118N) at a multiplicity of infection (moi) of 5. Cells were harvested and lysed in 4 ml of buffer A [50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 % (v/v) Nonidet P40, 0.1 mM PMSF and 1 mM benzamidine]. HA-tagged PP2Ac was detected by Western-blot analysis using the HA-epitope-specific 12CA5 monoclonal antibody or the polyclonal antibody AB45 (specific for PP2Ac). Detection was achieved using horseradish-peroxidase conjugated goat anti-mouse or sheep anti-rabbit IgG antibodies and ECL® (Amersham Pharmacia Biotech).

### Immunoprecipitations and Western-blot analysis

Flasks (185 $cm^2$; Nunc) were seeded with High Five cells grown in Express Five serum-free medium (Gibco BRL) at $1 \times 10^5$ cells/$cm^2$ and infected with recombinant virus at an moi of 5. For coexpression studies of HA–PP2Ac and PR65/A, cells were transfected with both the HA–PP2Ac recombinant virus and vPR65BV, at an moi of 5. Cells were harvested, lysed in buffer A and harvested by centrifugation at 190 ***g*** for 30 min. HA-tagged PP2Ac was immunoprecipitated from cleared lysate using 12CA5 saturated Protein A–Sepharose beads. Immunoprecipitates were washed three times in buffer A and two times in Tris-buffered saline [TBS; 50 mM Tris/HCl (pH 7.5)/150 mM NaCl]. Typically 50 $\mu$l of beads was boiled in 50 $\mu$l of Laemmli buffer [50 mM Tris/HCl (pH 6.8), 2 % (w/v) SDS, 0.004 % (w/v) Bromophenol Blue, 10 % (v/v) glycerol and 5 % (v/v) 2-mercaptoethanol] then analysed by Coomassie Brilliant Blue staining or Western blotting [25] using 12CA5 or AB45 as primary antibodies for the detection of HA–PP2Ac, and AB38 for the detection of PR65/A, followed by ECL® using the secondary antibodies described above. Quantification of immunoprecipitated HA–PP2Ac from cell lysates was determined by Western blotting, using okadaic-acid titrated rabbit skeletal muscle PP2Ac of known concentration as a standard. 12CA5–Protein A–Sepharose immunoprecipitated HA–PP2Ac (50 $\mu$l; diluted 1:1 in TBS) was added to 50 $\mu$l of Laemmli buffer and boiled for 5 min, then analysed by Western blotting as described

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previously [26] with samples run in duplicate. After transfer to Immobilin, the membranes were probed with AB45, washed extensively, then incubated with biotinylated goat anti-rabbit IgG antibodies and Streptavidin Texas Red. Blots were developed for red fluorescence using a Molecular Dymamics STORM 380 PhosphorImager. The signal for each band was quantified using ImageQuant software (Molecular Dymamics).

### Overexpression and purification of PP2Ac

Triple flasks (Nunc; 500 cm$^2$) with High Five cells in Express Five serum-free medium at a density of $1 \times 10^5$ cells/cm$^2$ were infected with recombinant virus at an moi of 5, and incubated at 27 °C for 72 h. At 72 h post-infection the majority of the infected cells had detached and the remaining cells were detached from the flasks with gentle tapping. The cells were collected by centrifugation at 800 rev./min (190 ***g***) for 20 min in a Sorval RC2B rotor, and pellets were washed with 1 × TBS, pooled and stored at −80 °C. Recombinant protein was purified from cell lysates by a modified ethanol precipitation procedure [27,28]. Briefly, cell pellets were thawed and diluted with 3 vol. of buffer B [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.5 mM EDTA and 0.2 % Triton X-100], containing Complete Protease Inhibitor Cocktail (Boehringer Mannheim), then lysed by several passes in a French press. Lysates were clarified by centrifugation at 10000 ***g*** at 4 °C, and 4 vol. of absolute ethanol (at 22 °C) was added to the clarified lysate to precipitate the proteins. The precipitate was collected by centrifugation at 10000 ***g***, then resolubilized in buffer B by trituration. The sample was dialysed extensively against 50 mM Tris/HCl (pH 7.4)/0.5 mM EDTA, and clarified by centrifugation at 10000 ***g*** at 4 °C. The resolubilized proteins were loaded on to a Protein A–Sepharose column crosslinked with 12CA5 monoclonal antibody (10 ml bead volume), washed extensively with 50 mM Tris/HCl (pH 7.4)/0.5 mM EDTA then eluted with HA peptide at 1 mg/ml in the same buffer. Fractions containing HA–PP2Ac were determined by Coomassie Brilliant Blue staining of SDS/PAGE gels then pooled, diluted 2-fold with glycerol and stored at −20 °C.

### Gel-filtration chromatography of PP2Ac

Gel-filtration chromatography was employed to ensure that monodisperse and correctly-folded PP2Ac was obtained after ethanol precipitation. Native and mutant HA–PP2Ac were dialysed from glycerol stocks against 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM dithiothreitol and 0.5 mM EDTA prior to concentration with Centricon YM-30 Centrifugal Filter Devices (Millipore). Approximately 0.2 mg of protein (PP2Ac) was loaded on to a Superdex 75HR 10/30 FPLC-column (Pharmacia). Fractions were collected based on FPLC profiles and the retention time for HA–PP2Ac was determined by Coomassie Brilliant Blue staining and Western-blot analysis of SDS/PAGE gels. The column was calibrated using the following proteins: BSA (66 kDa), carbonic anhydrase (29 kDa) and trypsin inhibitor (10 kDa).

### PP assays

A phosphatase assay using a $^{32}$P-labelled peptide (LRRASVA; kemptide) as the substrate has been described previously [10]. Briefly, phosphatase assays were performed in a 30 $\mu$l volume containing 50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM $MnCl_2$, 0.1 % 2-mercaptoethanol, 1 mg/ml BSA, 60 $\mu$M LRRAS-$^{32}PO_4$-VA and 1 nM purified HA–PP2Ac with or without 10 nM okadaic acid at 30 °C. For assays using immunoprecipitates, the appropriate dilution of each sample was used such that assays were performed within the linear range. Protein concentrations of immunoprecipitates determined by quantification of Western blots were then used to normalize PP2Ac concentrations in the phosphatase assays. For the kinetic analysis of purified proteins, phosphatase assays were performed according to the Promega non-radioactive phosphatase assay system (Kit V2460) using the peptide RRA(pT)VA as the substrate, and the same buffer used above. For the determination of $K_m$ and $k_{cat}$ for RRA(pT)VA of purified HA–PP2Ac and HA–D88N, the proteins were titrated with okadaic acid to determine the concentration of active molecules [29]. The initial rates of hydrolysis of RRA(pT)VA at 30 °C by native and mutant PP2Ac were determined for substrate concentrations ranging from 50 to 500 $\mu$M. The initial reaction velocities ($v$) were plotted against [S] and then fitted to the Michaelis–Menten equation by non-linear regression analysis to determine values of $K_m$ and $k_{cat}$. Reactions were performed in triplicate under conditions where substrate depletion was less than 20 %.

### Test for PP2Ac function *in vivo* by plasmid shuffling in yeast

Human *PP2Acα* wild-type and mutant cDNA clones were tagged with the HA epitope and expressed in yeast from the *PGK1* (phosphoglycerate kinase gene) promoter/*CYC1*(iso-1-cytochrome *c* gene) terminator of the *TRP1* (phosphoribosyl-anthranilate isomerase prototrophic selectable marker gene) vector pYPGE2 as previously described [30]. The *pph22-156* and *pph22-186* mutant alleles (where *PPH22* is the PP2A gene homologue 2, encoding yeast PP2Ac), encoding the D156N and H186N substitutions respectively, in yeast PP2Ac were generated by Quickchange PCR amplification (Promega) of the *PPH22* genomic clone in plasmid pDE22 [31] and were inserted (1.8 kb *Xba*I–*Eco*RI fragments) into the *TRP1* shuttle vector YCplac22 [32] for expression in yeast (plasmids YCpDE88 and YCpDE-186 respectively). For plasmid shuffling experiments, *TRP1* plasmids were introduced into cells of the haploid mutant strain DEY3 (*MAT***a** *pph21::LEU2 pph22Δ1::HIS3 pph3Δ1::LYS2 lys2-951 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 ssd1-d1* [YCpDE8: *URA3 PPH22*]) which is triply deleted for the chromosomal *PPH21*, *PPH22* and *PPH3* genes and contains the *URA3 PPH22* plasmid YCpDE8 providing essential yeast PP2Ac function [31]. Loss of YCpDE8 from these cells was tested by growing Trp$^+$ transformants on agar medium containing uracil and 5-fluoroorotic acid (5-FOA) [33]; 5-FOA is toxic to cells containing the *URA3* gene and permits the growth of cells that have lost YCpDE8 and are kept alive by PP2Ac function provided by the incoming *TRP1* plasmid.

## RESULTS

### High-level expression of recombinant PP2Ac in insect cells

To obtain recombinant PP2Ac for structure–function analysis, the baculovirus system was tested for expression of the human enzyme PP2Acα in the insect cell line, High Five. Western-blot analysis of lysates, prepared from cells infected with the recombinant virus vBBHAPP2Ac, revealed the presence of HA-tagged human PP2Ac using the 12CA5 monoclonal antibody (Figure 2). The PP2Ac-specific antibody, AB45, raised against a peptide based on the N-terminal 20 amino acids of PP2Ac [22], detected endogenous PP2Ac in non-infected High Five cells as expected, since the amino acid sequence similarity between *Drosophilia* and human PP2Ac is greater than 93 % [34]. However, the expression level of recombinant PP2Ac was approximately 5-fold greater than endogenous enzyme (Figure 2). Comparison of HA–PP2Ac

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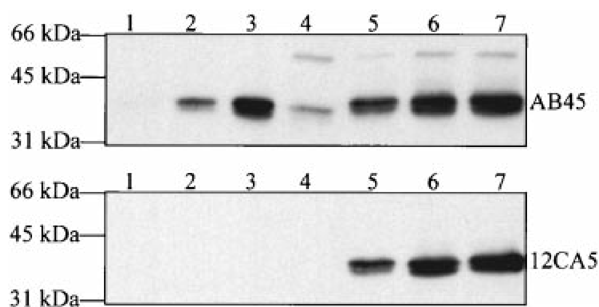


**Figure 2 Expression of human PP2Ac in High Five insect cells**

Lysates (10 $\mu$g) prepared from baculovirus-infected High Five insect cells 48 h post-infection were resolved by SDS/PAGE and analysed by Western blotting using the 12CA5 monoclonal antibody (bottom panel) or the PP2Ac peptide antibody AB45 (top panel). Gels were run in tandem and the first three lanes contained 1, 10 and 50 ng of purified rabbit PP2Ac as controls. Lane 4 contained uninfected High Five cells (control), and lanes 5, 6 and 7 contained lysates from High Five cells infected with baculoviruses for recombinant wild-type HA–PP2Ac, and the mutant HA–D88N and HA–H118N forms respectively.

present in High Five cells with known concentrations of purified rabbit skeletal muscle PP2Ac (Figure 2) indicated that the level of expression of recombinant protein was of the order of 1–2 mg/$10^9$ cells. By comparison, the insect cell line *Sf*9 expressed a 10-fold lower level of recombinant HA–PP2Ac (results not shown). Thus baculovirus infection of High Five cells is an efficient system for expressing recombinant HA–PP2Ac.

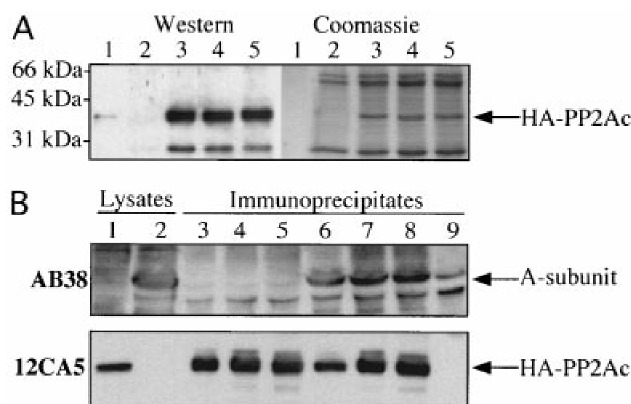


**Figure 3 Co-immunoprecipitation of PR65/A with wild-type and mutant PP2Ac**

(**A**) Immunoprecipitation of HA-tagged wild-type, D88N and H118N PP2Ac. Lysates were prepared from High Five cells and immune complexes were recovered with the 12CA5 monoclonal antibody. Two gels were run in tandem and subjected to Western blotting with 12CA5 or staining with Coomassie Brilliant Blue. Lane 1 contained 10–20 pg of rabbit PP2Ac as a control and lane 2 is a High Five cell lysate control. Lanes 3, 4 and 5 contained the HA-tagged wild-type PP2Ac, D88N PP2Ac and H118N PP2Ac proteins respectively. Coomassie Brilliant Blue staining revealed high and low molecular mass bands corresponding to heavy and light chain antibodies. (**B**) Recombinant human PP2Ac binds recombinant human PR65/A. Lysates were prepared from High Five cells expressing human HA–PP2Ac, human PR65/A or both (doubly infected). HA-tagged proteins were immunoprecipitated with the 12CA5 monoclonal antibody, resolved by SDS/PAGE, and analysed by Western blotting with 12CA5 to detect HA–PP2Ac or AB38 to detect the presence of co-precipitated PR65/A. Lanes 1 and 2 contained total lysate (10 $\mu$g) prepared from High Five cells infected with vBBHAPP2Ac and PR65BV respectively. Lanes 3–5 contained immune complexes prepared from High Five cells infected with vBBHAPP2Ac, vBBHAD88N and vBBHAH118N respectively. Lanes 6–8 contained immune complexes prepared from High Five cells doubly infected with vBBHAPP2Ac/PR65BV, vBBHAD88N/PR65BV and vBBHAH118N/PR65BV respectively. Lane 9 contained immune complexes prepared from High Five cells infected with the virus PR65BV.

**Table 1 Specific activities for immunoprecipitated wild-type and mutant HA-tagged PP2Ac**

Recombinant HA-tagged wild-type and mutant PP2Ac subunits were immunoprecipitated from High Five insect cell lysates using 12CA5–Protein A–Sepharose. The concentration of immunoprecipitated PP2Ac subunits was determined by quantitative Western-blot analysis (see the Experimental section) and used to standardize phosphatase assays using $^{32}$P-labelled kemptide as substrate. Assays were performed in triplicate with and without okadaic acid and were compared with the specific activity of purified rabbit skeletal muscle PP2Ac.

| | Specific activity (nmol [$^{32}$P]$P_i$ released/min per mg) | |
|---|---|---|
| PP2Ac | + Okadaic acid (10 nM) | − Okadaic acid |
| Rabbit PP2Ac | 10.0 ± 1.1 | 180.1 ± 30.4 |
| HA–PP2Ac | 4.4 ± 0.3 | 218.3 ± 17.4 |
| HA–D88N | 0.8 ± 0.1 | 23.5 ± 3.9 |
| HA–H118N | 2.2 ± 0.1 | 9.7 ± 1.3 |

### Overexpression of PP2Ac active-site mutant proteins in insect cells

To test the impact of PP2Ac active-site residues on catalytic function, and obtain maximum yields of recombinant PP2Ac$\alpha$ for structure–function analyses we generated mutant forms of HA–PP2Ac containing the H118N or D88N substitutions. Based on sequence alignment of the PPP family members (Figure 1) and kinetic studies of $\lambda$PPase and PP1 [13–16] the PP2Ac residue His$^{118}$ is believed to serve as a general acid, donating protons to the serine/threonine leaving group during the phosphatase reaction, while Asp$^{88}$ increases its acidic character. Changing either amino acid to a neutral asparagine residue is predicted to impair PP2Ac catalytic function. Mutant forms of HA–PP2Ac were expressed in High Five insect cells following infection with the recombinant viruses vBBHAD88N or vBBHAH118N. Remarkably, the expression level of HA–PP2Ac mutant proteins was approximately 5-fold greater than that of the native recombinant HA–PP2Ac (Figure 2). This observation, together with data shown below, suggests that, in High Five insect cells, overexpression of PP2Ac is facilitated by active-site mutations which impair catalytic function.

### Active-site mutations impair PP2Ac catalytic activity *in vitro*

To examine catalytic activity *in vitro*, native and mutant HA–PP2Ac proteins were immunoprecipitated from lysates prepared from High Five cells (Figure 3A). The amount of HA-tagged native and mutant (D88N or H118N) PP2Ac recovered in immune complexes was similar, as judged by Western-blot analysis (results not shown). However, phosphatase activity assays on the immune complexes revealed that, whereas the specific activity of the native HA–PP2Ac (218 nmol [$^{32}$P]$P_i$ released/min per mg) was similar to that of the purified rabbit skeletal muscle enzyme (180 nmol [$^{32}$P]$P_i$ released/min per mg), there was a 9-fold and 22-fold reduction in specific activity for the mutants D88N (24 nmol [$^{32}$P]$P_i$ released/min per mg) and H118N (9.7 nmol [$^{32}$P]$P_i$ released/min per mg) respectively, using a phospho-kemptide substrate (Table 1). These results are in agreement with those of Ogris et al. [17,18] who detected a dramatic decrease in PP2Ac activity towards phosphorylated

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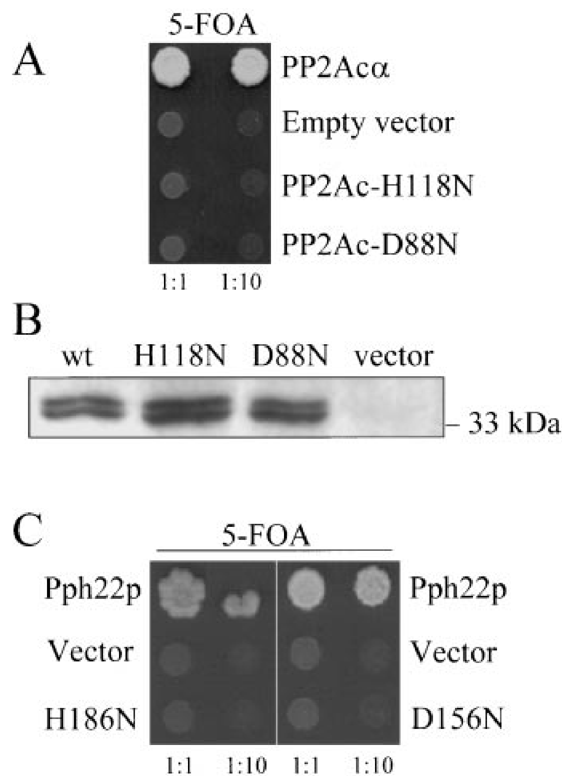


**Figure 4 Analysis of active-site mutant forms of PP2Ac in yeast**

(**A**) Functional replacement of yeast PP2Ac by active-site mutant forms of human PP2Ac by plasmid shuffling. *TRP1* plasmids encoding wild-type human PP2Ac (PP2Acα), no insert DNA (Empty vector) or a mutant form of human PP2Ac (containing the substitution indicated) were tested for PP2Ac function by plasmid shuffling in strain DEY3. $Trp^+$ transformant cells were grown to saturation in liquid SD medium [0.17% (w/v) yeast nitrogen base (Difco), 0.5% (w/v) ammonium sulphate and 2% (w/v) dextrose] with uracil. Cell suspensions were then diluted (1:1 or 1:10), spotted on to agar medium containing 5-FOA and incubated at 30 °C for 3 days. (**B**) Western-blot analysis of HA-tagged forms of human PP2Ac expressed in strain DEY3. Yeast cell extracts (30 μg) containing wild-type human PP2Ac (wt), a mutant form of PP2Ac (with the substitution indicated) or the empty expression vector were resolved by SDS/PAGE and probed with the 12CA5 monoclonal antibody. A molecular-mass marker is shown on the right-hand side. Human PP2Ac migrates as a doublet. (**C**) Functional replacement of wild-type PP2Ac with active-site mutant forms of yeast PP2Ac. *TRP1* plasmids encoding wild-type yeast PP2Ac (Pph22p), no insert DNA (Vector) or the active-site mutant form Pph22-H186N (left) or Pph22-D156N (right) were tested for essential function *in vivo* as in (**A**).

histone H1 (2% of wild-type) and phosphorylase (8% of wild-type) caused by a H118Q substitution [17].

PP2Ac complexes with PR65/A *in vivo* to form a core dimer [1,3]. To examine whether the HA–PP2Ac active-site mutations disrupted protein folding we tested whether the recombinant native and mutant HA–PP2Ac proteins bound recombinant PR65/A during coexpression in insect cells. Immunoprecipitation of HA-tagged PP2Ac proteins using the 12CA5 antibody caused co-precipitation of PR65/A with the native, D88N and H118N PP2Ac forms, indicating the formation of a stable protein–protein interaction in each case (Figure 3B). This suggests that the D88N and H118N mutations impair the catalytic function of PP2Ac without severely disrupting protein folding.

### Active-site mutations abolish essential PP2Ac function *in vivo*

Because the D88N and H118N substitutions inhibited PP2Ac catalytic activity *in vitro*, we tested whether they similarly impaired PP2Ac function *in vivo*. To do this, we employed a

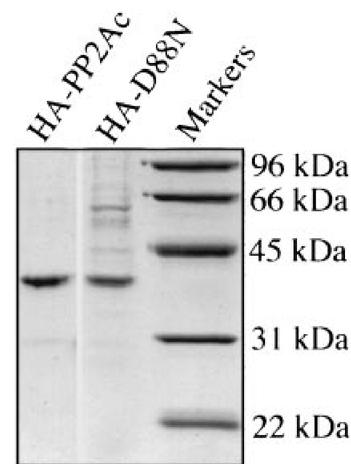


**Figure 5 Purification of recombinant PP2Ac**

Lysates were prepared from High Five cells using a French press. Proteins were purified by ethanol precipitation and 12CA5 monoclonal antibody affinity chromatography as described in the Experimental section. Approximately 1–3 μg of purified HA–PP2Ac (lane 1) and HA–D88N (lane 2) was resolved by SDS/PAGE and stained with Coomassie Brilliant Blue.

plasmid shuffling procedure (see the Experimental section) that tests the ability of human PP2Ac proteins to functionally replace the endogenous enzyme of the budding yeast *Saccharomyces cerevisiae* [30]. Functional analysis in yeast revealed that, unlike wild-type human PP2Acα, the PP2Ac-H118N and PP2Ac-D88N mutant proteins each failed to substitute for the endogenous yeast PP2Ac *in vivo* (Figure 4A), although each protein was expressed at a similar level (Figure 4B). To confirm this for the endogenous yeast PP2Ac we generated active-site mutations in *S. cerevisiae* Pph22p, which shares 71% amino acid sequence identity with human PP2Acα over the region of homology [30]. Like the equivalent human proteins, the active-site mutant forms of yeast PP2Ac, containing the H186N or D156N substitutions (analogous to H118N and D88N in human PP2Acα respectively) failed to provide PP2Ac function *in vivo* (Figure 4C). Together, these results demonstrate that active-site mutations impairing catalysis inhibit essential PP2Ac function *in vivo*.

### Purification and characterization of native and mutant PP2Ac

For the large-scale expression and purification of PP2Ac we employed an HA tag, placed in frame with the N-terminus of human PP2Acα, for immunoaffinity purification of recombinant protein. Constructs with N- and C-terminal His tags were also evaluated; however, binding of the tagged PP2Ac to $Ni^{2+}$-nitrilotriacetate agarose (Qiagen) was ineffective for large-scale purification, since significant amounts of endogenous insect PP2Ac were purified from lysates prepared from High Five cells. This would suggest that PP2Ac may bind to $Ni^{2+}$-nitrilotriacetate agarose matrix via the active site and as such would be a major source of contamination in the purification of His-tagged recombinant PP2Ac (results not shown). Large-scale expression of PP2Ac was achieved by seeding 20 triple flasks with $1 \times 10^9$ High Five cells. Recombinant protein was prepared from infected cells utilizing a two-step purification protocol based on ethanol precipitation [27,28] and monoclonal antibody affinity chromatography. Ethanol precipitation was used as the initial purification step in order to increase the yield of pure PP2Ac and significantly reduce non-specific binding to the 12CA5–Protein A–Sepharose

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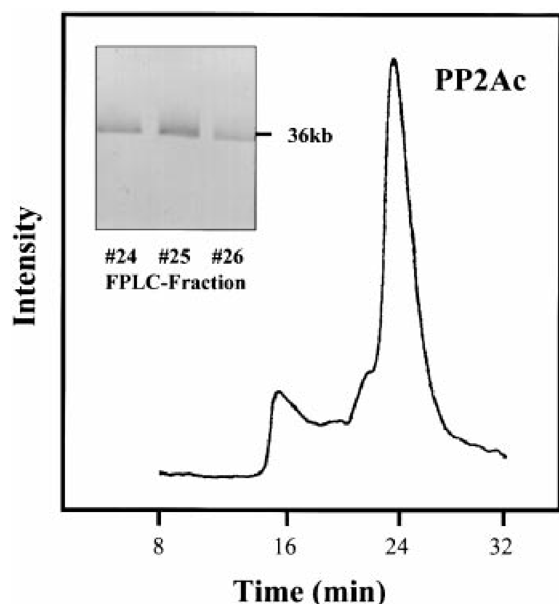


**Figure 6 Gel filtration of recombinant PP2Ac**

Approximately 0.2 mg of purified recombinant wild-type or mutant HA-tagged PP2Ac was loaded on to a Superdex 75HR 10/30 FPLC gel-filtration column. Fractions were collected over the course of the chromatography and the presence of PP2A was determined by Coomassie Brilliant Blue staining and Western-blot analysis. The chromatogram shows the elution profile of wild-type PP2Ac and is a representative trace for all PP2Ac forms tested. The inset shows a Coomassie Brilliant Blue-stained gel of the purified three main fractions of HA–PP2Ac in lanes 1–3 respectively. Each PP2Ac protein eluted off the column as a single peak with a retention time of 24 min. PP2Ac was not detected in any of the other fractions.

immunoaffinity column and to ensure that purified PP2Ac was not complexed to the insect cell A-subunit. This procedure permitted essentially quantitative recovery of 1 mg of pure wild-type HA–PP2Ac, while only 10 % of the HA–D88N PP2Ac (1 mg/$1\times10^9$ cells) was recovered with a purity greater than 90 % (Figure 5). The mutant HA–H118N PP2Ac did not survive ethanol precipitation well with 1 % recovery (results not shown). To ensure the purified PP2Ac was monodisperse and correctly folded using the ethanol precipitation procedure, we performed gel filtration on HA-tagged wild-type and mutant PP2Ac. The wild-type and mutant forms of PP2Ac eluted as a single peak with a retention time of 24 min suggesting that the proteins were not aggregated and were correctly folded (Figure 6), with the correct apparent molecular mass, as judged by standards.

Purified recombinant HA–PP2Ac and HA–D88N were titrated with okadaic acid revealing a concentration of active molecules similar to that determined by the Bradford assay using BSA as a standard (results not shown). The kinetic constants $K_m$ and $k_{cat}$ for the dephosphorylation of the peptide RRA(pT)VA were determined for titrated HA–PP2Ac and HA–D88N PP2Ac (Table 2). The $K_m$ for HA–D88N (58.4 $\mu$M) was reduced by approximately 6-fold compared with HA–PP2Ac (337.5 $\mu$M), whereas $k_{cat}$ was decreased 85-fold for HA–D88N (2 $s^{-1}$) compared with HA–PP2Ac (170 $s^{-1}$). For HA–PP2Ac, the value of $K_m$ for the substrate RRA(pT)VA was similar to that measured for the native bovine PR65/A–PP2Ac complex using $^{32}$P-labelled kemptide as a substrate ($K_m$ = 361 $\mu$M) [35], and for purified rabbit skeletal muscle PP2Ac using RRA(pT)VA as substrate where the $K_m$ was 310 $\mu$M (Promega).

**Table 2 Kinetic constants for purified wild-type HA–PP2Ac and HA–D88N PP2Ac**

The values for $K_m$ and $k_{cat}$ for the dephosphorylation of the peptide RRA(pT)VA were determined for purified wild-type HA–PP2Ac and HA–D88N PP2Ac. Substrate concentrations ranging from 50 to 500 $\mu$M were used in a 50 $\mu$l assay volume containing either 2 nM wild-type HA–PP2Ac or 10 nM HA–D88N and incubated at 30 °C for 10 and 30 min respectively. The reactions were stopped and free phosphate was measured as described by the manufacturers using a Molecular Devices Softmax plate reader. The initial reaction velocities ($v$) were plotted against [S] and then fitted to the Michaelis–Menten equation by non-linear regression analysis to determine values of $K_m$ and $k_{cat}$. Reactions were performed in triplicate under conditions such that substrate depletion was less than 20%.

| PP2Ac | $K_m$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}\cdot s^{-1}$) |
|---|---|---|---|
| HA–PP2Ac | 337.5 ± 37.4 | 170.0 ± 12.8 | $5.03\times10^5$ |
| HA–D88N | 58.4 ± 14.6 | 2.0 ± 0.1 | $0.34\times10^5$ |

## DISCUSSION

### Baculovirus system for expression of PP2Ac

The identification and analysis of PP2Ac structural determinants important for catalysis and its interaction with PR65/A and B-type subunits has been restricted by lack of a convenient eukaryotic expression system capable of generating high levels of recombinant PP2Ac significantly in excess over the endogenous enzyme. For example, expression of PP2Ac from the strong U3 promoter of the Moloney murine leukaemia virus led to an increase in the total (endogenous plus recombinant) PP2Ac in murine NIH/3T3 fibroblasts of only 30–50 % [20], while expression of recombinant PP2Ac from the cytomegalovirus promoter was achieved at a level only 3-fold higher than that of the endogenous protein in HEK-293 human kidney cells [36]. Accordingly, PP2Ac appears to control its intracellular levels stringently. Studies by Baharians and Schönthal [19] showed that the strict control of endogenous and recombinant PP2Ac levels in NIH/3T3 cells is due to auto-regulation at the level of translation, whereas Chung and Brautigan [21] show PP2Ac activity is strictly regulated at the level of transcription in COS-7 cells. These mechanisms are likely to operate in most cell types to maintain a constant intracellular pool size of PP2Ac. Our results indicate that the baculovirus system using High Five cells is appropriate for the overexpression of recombinant PP2Ac because it partially lacks these stringent mechanisms of PP2Ac auto-regulation. The baculovirus system has been used previously to investigate the regulation of PP2A enzyme specificity by regulatory B subunits in *Sf*9 cells, which were triply infected with baculoviruses for PP2Ac, PR65/A, and PR55/B, PR61/B′ or simian-virus-40 small tumour antigen [35]. Purification of PP2A heterotrimers yielded approximately 150 $\mu$g/$10^9$ *Sf*9 cells (approx. 50 $\mu$g of PP2Ac) similar to the yields from *Sf*9 cells infected with recombinant PP2Ac obtained in the present study (results not shown). Remarkably, we show that the level of recombinant HA–PP2Ac in High Five cells is in excess of 1 mg/$10^9$ cells (approximately 5-fold higher than endogenous PP2Ac).

### Active-site mutations inhibit catalysis and allow overexpression of PP2Ac

Mutating the active-site residues $Asp^{88}$ and $His^{118}$ to the neutral amino acid asparagine yielded substantially higher levels (5–10 mg/$10^9$ High Five cells) of mutant HA–PP2Ac compared with native recombinant HA–PP2Ac. The $Asp^{88}$ and $His^{118}$ residues were chosen based on analogous residues in the struc-

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turally related PP1 and λPPase (Figure 1), which, when similarly mutated, cause reduced values for $V_{max}$ in phosphatase reactions [13,14]. HA–PP2Ac activity assays revealed a 9- and 23-fold reduction in the relative activity of the HA–D88N and HA–H118N forms of PP2Ac respectively, while binding of the native and mutant PP2Ac proteins to PR65/A was similar, suggesting similar folding in each protein. The results for the HA–H118N substitution are similar to those observed for the H118Q substitution of PP2Ac, which had decreased activity towards the substrates phosphorylase *a* and histone H1 [17,18]. Moreover, functional analysis in yeast revealed that the D88N and H118N substitutions in human PP2Ac, as well as the analogous active-site mutations (D156N and H186N) in yeast PP2Ac, abolished essential PP2A function *in vivo*. These observations demonstrate that the human $Asp^{88}$ and $His^{118}$ residues perform a key, phylogenetically conserved, role in the catalytic mechanism of PP2Ac. Furthermore, our results indicate that the regulation of PP2Ac expression is apparently controlled by its PP activity, and could be due to the mechanisms outlined by Baharians and Schönthal [19] or Chung and Brautigan [21].

### Expression, purification and characterization of recombinant PP2Ac

Scaled-up expression and purification of recombinant PP2Ac from $1 \times 10^9$ High Five cells using a two-step purification procedure yielded 1 mg of pure HA–PP2Ac and 1 mg of HA–D88N, whereas the mutant HA–H118N did not survive the ethanol precipitation step particularly well. Only 1 and 10% of the total HA–H118 and HA–D88N was recovered respectively, and reflects the effect of the mutations on the stability of PP2Ac after ethanol precipitation. However, gel-filtration chromatography shows the mutant proteins to be monodisperse with a similar fold to wild-type PP2Ac. Expression of HA-tagged PP2Ac in conjunction with the two-step purification procedure has yielded milligram quantities of pure PP2Ac which has been complexed with recombinant PR65 from *Escherichia coli* (results not shown) to allow the structure determination of PP2Ac in complex with PR65/A using X-ray crystallography.

The kinetic parameters for the dephosphorylation of RRA(pT)VA by purified recombinant HA–PP2Ac ($K_m$ = 337.5 μM; $k_{cat} = 170\ s^{-1}$) were similar to values described previously for rabbit skeletal muscle PP2Ac (Promega) and the values obtained for the dephosphorylation of $^{32}$P-labelled kemptide by the PR65/A–PP2Ac complex where $K_m$ was 361 μM [18]. Substitution of $Asp^{88}$ with the isosteric asparagine shows a small decrease in $K_m$ (6-fold), but a significant reduction in $k_{cat}$ (85-fold), suggesting that the association of the Ser(P)/Thr(P) substrate with the PP2Ac active site is not impaired for complex formation; however, the mutation has an effect on catalysis. Mutation of λPPase residue $Asp^{52} \rightarrow Asn$ (D88N in PP2Ac) caused a small decrease in $K_m$ for the substrate *p*-nitrophenyl phosphate (3-fold) but a 35-fold decrease in $k_{cat}$, which is similar to the effects of HA–D88N PP2Ac towards RRA(pT)VA [13]. The effect of the PP2Ac D88N substitution on catalysis is consistent with the catalytic mechanism proposed for the PPP family member, PP1, based on its crystal structure, involving an ion-pair interaction between $Asp^{95}$ ($Asp^{88}$ in PP2Ac) and $His^{125}$ ($His^{118}$ in PP2Ac). The analogous PP1 residue $Asp^{95}$ promotes catalysis, both by enhancing the acidic character of $His^{118}$ via electron withdrawal and by holding the imidazole ring of PP1 $His^{125}$ in a rigid conformation permitting the protonation of the target substrate [16]. The PP2Ac residue $Asp^{88}$ is highly conserved between members of the PPP family and the effect of the D88N substitution on catalysis by PP2Ac provides support to the notion that the active-site topology and catalytic mechanism of this family of PPs is also conserved.

We show that the baculovirus system using High Five insect cells allows, for the first time, the high-level expression of biologically active PP2Ac for structural and functional studies. Site-directed mutagenesis of PP2Ac and purification of mutant proteins from High Five insect cells combined with functional analysis in yeast provides a powerful system for structure–function analysis of PP2Ac. We are currently employing these systems to further investigate the mechanism of PP2Ac catalysis and the role of post-translational modifications in PP2Ac function, activity and subunit interactions.

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## **Part 2:** Diversity, developmental regulation and distribution of murine PR55/B subunits of protein phosphatase 2A.

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**Diversity, developmental regulation and distribution of murine PR55/B subunits of protein phosphatase 2A**

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**Abstract**

Protein phosphatase 2A is a hetero-trimeric holoenzyme that consists of a core dimer composed of a catalytic subunit that is tightly complexed with the scaffolding subunit PR65/A. This core dimer associates with variable regulatory subunits of the PR55/B, PR61/B', PR72/B" and PR93/PR110/B"' families. As PP2A holoenzymes containing PR55/B have been shown to be involved in the pathogenesis of Alzheimer’s disease, we characterized the PR55/B family with particular emphasis on its distribution and expression in the brain. We determined the genomic organization of all members of the PR55/B family and cloned their murine cDNAs. Thereby, two novel splice variants of PR55/Bβ were identified. In addition, Northern blot analysis revealed multiple transcripts for the different PR55 subunits, suggesting a higher variability within the PR55 family. *In situ* hybridization analysis revealed that all PR55/B subunits were widely expressed in the brain. PR55/Bα and Bβ protein expression varies significantly in areas of the brain affected by neurodegenerative diseases such as the hippocampus or cerebellum. At the cellular level, PR55/Bβ protein expression was confined to neurons, whereas PR55/Bα was also expressed in activated astrocytes indicating that the PR55 isoforms confer a different function to the holoenzyme complex. As PP2A dysfunction has been demonstrated to contribute to various human diseases, dissecting the PP2A holoenzyme and its particular function in different cell types will assist in the development of novel therapeutic strategies.

## INTRODUCTION

Protein phosphatase 2A (PP2A, *PPP2*) is one of the major serine/threonine-specific phosphatases which, together with PP1 (protein phosphatase 1), PP2B (calcineurin), and PP2C, is involved in many diverse cellular processes (Millward *et al.*, 1999).

Several holoenzyme complexes of PP2A have been isolated from a variety of tissues and have been extensively characterized. The core enzyme is a dimer, consisting of a highly conserved catalytic subunit (C) and a structural scaffolding subunit (PR65/A) that forms complexes with variable regulatory B subunits. Four families of B subunits have been identified so far, termed PR55/B, PR61/B′, PR72/B′′ and PR93/PR110/B′′′ (Janssens & Goris, 2001). A striking feature of the regulatory subunits is the lack of sequence similarity between these families, despite the recognition of similar sequence motifs within the A subunit. Due to the expression in mammals of at least two A, two C, four B, at least eight B′, four B′′, and two B′′′ isoforms, a total of about 75 PP2A holoenzymes can be generated. Taking the different splice variants into account, even more holoenzymes can be formed (Janssens et al., 2001). This complexity, in addition to posttranslational modifications including phosphorylation and methylation of the C subunits, provides a vast variety of possibilities for the regulation of PP2A activity. Many substrates of PP2A have been described, including the microtubule-associated protein tau (Gong *et al.*, 1994). Hyperphosphorylated forms of tau form insoluble intracellular deposits in several human neurodegenerative diseases including Alzheimer's disease (AD) (Gotz, 2001). Previous studies have shown that in brain homogenates of AD patients, PP2A activity was 30% lower than in those of control subjects (Gong *et al.*, 1995) and, of five subunits analyzed, PP2A Cα, PR55/Bγ and PR61ε mRNA expression was quantitatively decreased in the CA3 region of the hippocampus (Vogelsberg-Ragaglia *et al.*, 2001). A role for PP2A in tau dephosphorylation is also supported by the finding that PP2A is localized on microtubules and that it binds directly to tau (Sontag *et al.*, 1999). In particular, PP2A holoenzymes containing regulatory subunits of the PR55/B family were shown to bind and dephosphorylate tau very efficiently (Goedert *et al.*, 1992; Sontag et al., 1999). Recent findings in subjects with spinocerebellar ataxia, in whom an expanded CAG repeat has been identified immediately upstream of the

PR55/Bβ gene, indicate that dysregulation of PR55 may have functional consequences in neurodegenerative disorders (Holmes *et al.*, 1999).

Genetic approaches in mice will play an integral role in understanding PP2A function *in vivo* (Janssens et al., 2001). To provide a framework for the interpretation of phenotypes of mice that either lack PP2A subunits (Gotz *et al.*, 1998; Gotz *et al.*, 2000) or display an altered PP2A composition or activity (Kins *et al.*, 2001; Planel *et al.*, 2001), we determined the genomic organization, identified novel splice variants and analyzed the mRNA and protein expression of the PR55/B regulatory subunits of PP2A in different tissues, in particular the brain.

## MATERIALS AND METHODS

### PR55 cDNA cloning

Different mouse brain cDNA libraries were screened to isolate cDNA clones of the three PR55/B isoforms that have been previously described in humans (α, β, and γ) (Mayer *et al.*, 1991), and a fourth isoform (δ) that has been identified in the rat (Strack *et al.*, 1999). In addition, the human sequences of the PR55/B isoforms were used to search murine expressed sequence tags (ESTs) in the NCBI nucleotide database. From EST libraries (Incyte Genomics, St. Louis, Missouri, USA and Invitrogen, Paisley, UK), a full-length IMAGE cDNA clone for PR55/Bα (thymus, accession # aa111189) and two splice variants for PR55/Bβ termed PR55/Bβ.1 (testis, EST accession # aa108896, GeneBank accession # AS512670) and PR55/Bβ.2 (lung, EST accession # bi150825, GeneBank accession # AF536771) were obtained. PR55/Bγ was obtained from a λ ZAP II mouse brain library (Stratagene, La Jolla, California, USA) by screening with a human cDNA probe at low stringency. PR55/Bβ and δ cDNAs were isolated by isoform-specific PCR amplification of the Marathon-Ready mouse brain cDNA library (Clontech, Palo Alto, California, USA). Nucleotide sequences were determined by automated sequencing (ABI PRISM 3700, Applied Biosystems, Foster City, California, USA).

### BAC genomic clones and chromosomal localization

The cDNAs encoding the PR55/B subunits were used to screen a mouse BAC library constructed from 129/SvJ genomic DNA (Genome Systems, Palo Alto, California, USA). A BAC sub-library was screened to establish the genomic organization of PR55/Bα. Positive clones were identified by filter hybridization using the corresponding $^{32}$P-labelled cDNA fragments. A 20kb PR55/Bα EcoRl fragment covering exons 4 to 10 was sub-cloned and sequenced. The complete genomic organization of PR55/Bα, β, and the partial organization of γ and δ was assembled by a Blast screening of the Ensembl murine database. The Ensembl database was also used to determine the chromosomal localization of the four PR55/B genes.

### Northern blot analysis

2 μg polyA$^+$ mRNA derived from different tissues of adult 3-month-old Balb/c mice and four embryonic stages of Balb/c mice (Clontech) were prehybridized at 50°C for 4 h in Express Hybridization buffer (Clontech) complemented with denatured and sonicated salmon sperm DNA (100 mg/ml), and sequentially hybridized with $^{32}$P-labeled PR55/B subunit-specific 45 bp-long antisense oligonucleotides
(α: 5'-tatctgcttctgctacgtcatcatctactgctcctttcacctgag-3',
β: 5'-gttgaattctaccgtagagataatgtcagctgtgaagggcttcat-3',
γ: 5'-agtggagatgacgtcagcttctgtcacatagctgtggtcccgcag-3',
δ: 5'-tattggaacccgtagtgccgtaattctaaatgggtctcgaagtcg-3').
The β antisense probe hybridizes to all PR55/Bβ transcripts including β.1 and β.2. A random-primed 2 kb human β-actin cDNA probe was used for normalization. The blots were washed sequentially using increasingly stringent conditions. The final wash was performed at 60°C for 30 min in a buffer containing 0.1 x SSC and 0.1% SDS, conditions used previously to differentiate between highly homologous transcripts (Kins *et al.*, 2000; Ramming *et al.*, 2000). Finally, the membranes were exposed to Biomax films (Kodak) for 10-60 h at –70°C.

## *In situ* hybridization

Wild-type mice were transcardially perfused with PBS containing 4% paraformaldehyde, postfixed overnight at 4°C, and paraffin embedded. 7 µm sections were dried overnight at 42°C on coated glass slides, dewaxed and permeabilized by acid treatment (0.1 M HCl for 10 min), followed by a proteinase K treatment (10 µg/ml) for 10 min at 37°C. After acetylation with 0.1 M triethanolamine and 0.4% acetic anhydride (20 min at room temperature), sections were incubated for 1 h at room temperature in hybridization buffer (25% deionized formamide, 4 x SSC, 5 x Denhardt's reagent, 0.25 mg/ml yeast tRNA, 10% dextransulfate, 50 mM DTT, 1 mM EDTA, 0.5 mg/ml salmon sperm DNA in 50 mM phosphate buffer pH 7). The sections were hybridized overnight at room temperature in hybridization buffer with DIG-labeled 45 bp antisense oligonucleotide probes and the complementary sense probes. Sections were washed at 37°C in solutions of decreasing salt concentrations (2 x, 1 x, 0.2 x SSC), blocked with 2% (v/v) normal sheep serum, and incubated with an anti-DIG antibody conjugated to alkaline phosphatase (Roche, Basel, Switzerland) in 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 1% normal sheep serum. Alkaline phosphatase activity was visualized with staining solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 1 mM levamisole, 450 ng/µl NBT, and 175 ng/µl BCIP) for 2-14 h. After washing, slides were mounted in Mowiol (Hoechst, Frankfurt, Germany).

## Western blot analysis

To determine the specificity of the PR55/B-specific antibodies, COS cells were transfected with constructs encoding HA-tagged PR55/Bα, PR55/Bβ, and PR55/Bγ, and lysates analyzed by western blotting. To ascertain the tissue distribution of PR55/Bα and PR55/Bβ, murine tissues were homogenized in TBS containing protease inhibitors (Complete® containing EDTA, Roche). Triton X-100 was added to a final concentration of 1%, and the homogenate was mixed in an overhead incubator for 1 h at 4°C. After centrifugation at 5000 g for 5 min at 4°C, the supernatant was used for western blot analysis. As a control, recombinant PR55/Bα was loaded. For sub-fractionation, brains

were homogenized in 10 mM Tris pH 7.5, 1 mM EGTA, 1 mM EDTA, and 1 mM DTT in the presence of protease inhibitors. The homogenate was centrifuged at 100,000 g for 60 min, and the supernatant (S1, cytosolic proteins) was removed. The pellet was rehomogenized in 10 volumes of homogenization buffer plus 1% Triton X-100 and centrifuged as before. The supernatant (S2, membrane fraction) was removed, and the pellet was resuspended in 10 volumes of homogenization buffer (P2, cytoskeletal proteins) (McNeill & Colbran, 1995; Strack *et al.*, 1998). Protein concentrations were determined with the DC-protein assay (BioRad, Hercules, California, USA) following the instructions of the manufacturer. 40 µg of protein were separated on 10-20% tricine gradient gels (Novex), and transferred to Hybond ECL membranes. Ponceau stainings of the membranes were included to confirm loading of comparable amounts of protein. Blots were developed with the following rabbit antisera: anti-β-actin (Abcam, Cambridge, United Kingdom, 1:5000), anti-GAPDH (BioDesign, Saco, Maine, United States, 1:500), anti-APP (Amyloid precursor protein, C-terminal, Sigma, 1:400), anti-phospho-APP (Thr668 phosphorylated, Cell Signaling, Beverly, MA, USA, 1:1000), anti-GFAP (Innogenex, San Ramon, California, USA 1:300), anti-PP2A/Bα 14-57 (Calbiochem, 1:200), anti-PP2A/Bβ 2-14 (Calbiochem, 1:200), anti PP2A/Bγ 53-66 (Calbiochem, 1:200), and anti-PP2A/Cα #45 (Gotz & Kues, 1999), and HRP-conjugated secondary antibodies as described (Kins et al., 2001).

**Immunohistochemistry**

Brains from three-month-old wild-type mice were used for immunohistochemical analysis. Animals were perfused transcardially with 4% paraformaldehyde in sodium phosphate buffer. 40 µm sections were cut on a vibratome and permeabilized with 0.1% NP-40 (Calbiochem), using standard published procedures (Gotz et al., 1998; Kins et al., 2001). Some of the sections were pretreated either with 5 µg/ml proteinase K or 0.1% Triton X-100 in TBS or PBS at 37°C for 2.5 mins for signal enhancement. The PR55/Bα and PR55/Bβ antisera (Calbiochem) were used at 1:200 dilutions, anti-GFAP mouse monoclonal (Innogenex, San Ramon, California, USA) at 1:1000, and the mouse monoclonal anti-MAP-2 (Chemicon, Temecula, California, USA) at 1:200. For

peroxidase/DAB stainings, secondary antibodies were obtained from Vector Laboratories (Burlingame, California, USA, Vectastain ABC kits PK-6101 and PK-6102). For immunofluorescence, secondary antibodies were obtained from Molecular Probes (Eugene, Oregon, USA, ALEXA-FLUOR™ series). Sections were dehydrated in an ascending series of ethanol and flat-embedded between glass slides and coverslips in Eukitt (Kindler, Germany).

## RESULTS

### Cloning of all murine PR55/B subunits and two novel splice variants

Four murine cDNAs encoding the PR55/Bα, β, γ, and δ subunits were identified, as well as two novel splice variants, $\tilde{\beta}1$ and $\tilde{\beta}2$ (Fig. 1). PR55/Bα was obtained as an IMAGE mEST clone (accession # 111189, from thymus). In addition, a PR55/Bα pseudogene was identified in a genomic database with exons 3 to 10 fused. The nucleotide sequence contained many point mutations, indicating that the pseudogene is not functional. PR55/Bβ was isolated by isoform-specific PCR amplification of a mouse brain cDNA library (Clontech). In addition, two PR55/Bβ splice variants were identified, encoding amino-terminally spliced variants of the β subunit that do not result in a shift of the open reading frame (ORF). PR55/Bγ was isolated from a λ ZAP II mouse brain library (Stratagene), and PR55/Bδ was isolated by tissue-specific PCR from a mouse brain cDNA library (Clontech). All mRNA and corresponding protein sequences were compared to those obtained in other species. Alignment of the murine protein sequences revealed, as for other species, a high degree of sequence conservation of the four subunits, with the exception of a highly diverse amino-terminus (Fig. 2). The overall sequence identity is in the range of 90%, the inter-species conservation of the individual subunits is between 94% (α) and 97% (γ), when compared to the human isoforms.

### The genomic organization of PR55/B subunits is highly conserved

Specific cDNA probes were used to screen a mouse BAC library constructed from 129/SvJ genomic DNA. We isolated three PR55/Bα clones, and one each for PR55/Bβ and PR55/Bγ. The genomic organization was established by restriction enzyme analysis and Southern blotting. A 20kb EcoRI fragment of the α isoform containing exons 4-10 was sub-cloned and sequenced. The genomic maps of the remaining isoforms were partially established *in silico* (Fig. 1). The size of the introns was determined based on publicly-available sequence data. Some of the introns are quite large which is indirectly

supported by the finding that the respective genes are not present in their full length on single BAC clones.

PR55/Bα is localized on chromosome 14D2, the likely PR55/Bα pseudogene on chromosome 12C3, PR55/Bβ on chromosome 18B3, PR55/Bγ on chromosome 5B2, and PR55/Bδ on chromosome 7F5.

Alignment of the cDNA and genomic sequences of the PR55/B isoforms revealed that the exon/intron boundaries of the final eight exons are conserved for each isoform, the PR55/Bβ splice variants, and when compared with other species. The PR55/Bα gene consists of ten coding exons, whereas all other isoforms have nine coding exons. The transcription start site for the splice variant PR55/Bβ.2 is on exon 1, followed by the start codon for PR55/Bβ.1 on exon 2, and the start codon for PR55/Bβ on exon 3. Alignment of the murine sequences revealed that all isoforms, including the novel PR55/Bβ.1 and PR55/Bβ.2 isoforms, contain a structural WD-40 repeat motif of five to seven imperfect repeats, depending on the stringency of the parameters, as in humans (Fig. 2).

**High diversity of PR55/B mRNAs**

We performed a northern blot analysis using isoform-specific probes, to determine the mRNA expression of PR55/B (Fig. 3A). The β antisense probe hybridizes to all PR55/Bβ transcripts including β.1 and β.2. No transcripts were detected for α, γ, or δ in mice at embryonic days E7, E11, E15, or E17. In contrast, a 2.5 kb β transcript was detected as early as embryonic day E11, with increasing levels until E17. Hybridization of multiple tissue northern blots revealed a PR55/Bα transcript of 2.5 kb in all tissues examined, similar to the expression pattern in humans (Mayer et al., 1991). Likewise, PR55/Bδ was expressed in all tissues. In testis, two mRNA species of 2.1 and 2.3 kb were found, whereas in all other tissues 2.0 and 2.2 kb transcripts were present. However, in brain, an additional transcript of 2.5 kb was detected. A major PR55/Bβ transcript of 2.5 kb and a minor transcript of 2.0 kb were found in brain, whereas in testis a 1.8 kb transcript was detected. Upon longer exposure, transcripts were found in additional tissues including lung and spleen. In contrast to PR55/Bβ, the 4.4 kb transcript of PR55/Bγ was solely restricted to brain. Control hybridizations with a probe specific for β-actin revealed that

equal amounts of mRNA were loaded (not shown). These results, together with the identification of two novel splice variants of the Bβ subunit, indicate both a high complexity of the PR55/B family and a very fine-tuned regulation of distinct functions of the heterotrimeric PP2A holoenzyme.

***In situ* hybridization analysis of brain sections**

To visualize the distribution of PR55/B subunit mRNAs in mouse brain, DIG-labeled antisense oligonucleotide probes specific for all PR55/B subunits were hybridized to parasagittal mouse brain sections. The distribution of the PR55/Bα subunit mRNA is shown in cortex, hippocampus, and cerebellum (Fig. 3B). No significant staining was obtained using the corresponding sense probes on adjacent sections (insets Fig. 3B). All PR55/B subunits were widely distributed in the brain, with a predominant expression by neurons. In general, hybridization was strongest in cell-dense areas such as the cerebellum, hippocampus, and olfactory bulb (Table 1). PR55/Bα and PR55/Bδ showed a similar distribution, and hybridization was more intense in brain stem than in cortex. In the dentate gyrus, the PR55/Bβ probe hybridized strongly to singulate cells, whereas the three other antisense probes hybridized more uniformly. With the exception of PR55/Bγ that was absent in the brain stem, the four PR55/B subunits were transcribed in all brain areas analyzed (Table 1).

**Immunoblot analysis of PR55/B subunits**

For detection of PR55/B, commercial antibodies were used that have been shown previously to be specific for the PR55/Bα and Bβ subunits on western blots and by immunohistochemistry (Strack et al., 1998). The commercially-available Bγ antiserum has been shown to be cross-reactive (Strack et al., 1998) and was therefore excluded from our analysis of the tissue distribution of PR55 subunits. We confirmed the specificity of the above antibodies by transfecting COS cells with constructs encoding HA-tagged PR55/Bα, Bβ, and Bγ, and analyzed lysates using western blot analysis. Whereas the HA-antibody detected all PR55 isoforms, the PR55/Bα- and PR55/Bβ-specific antibodies

detected only the respective isoforms (Fig. 4A and (Strack et al., 1998)). To determine the expression profile of PP2A Cα and of the PR55/B subunits, protein extracts were obtained from different tissues (Fig. 4B). A β-actin specific antibody (data not shown) and a GAPDH specific antibody (Fig. 4B) were included as controls. Cα was present in all tissues examined with levels highest in the brain. Next, we determined the tissue distribution of the PR55 subunits. PR55/Bα was ubiquitously present; levels were very low in kidney, liver, and heart, intermediate in testis, muscle, and spleen, and highest in lung and brain, as shown with the commercially-available antiserum: similar results were obtained with a second PR55/Bα-specific antiserum. PR55/Bβ was found in brain and testis, consistent with the northern blot data. Upon longer exposure, Bβ was also detected in additional tissues including lung and spleen (Fig. 4B). The commercially-available anti-PR55/Bγ antiserum was not reactive, and anti-PR55/Bδ antisera were not available.

To determine the subcellular localization of the PR55/B subunits, we generated brain homogenate fractions enriched for cytosolic, membranous and cytoskeletal proteins (Fig. 4C). This sub-fractionation protocol enriches for cytosolic, membraneous and cytoskeletal proteins, but does not completely separate them; the membrane fraction contains trans-membrane as well as membrane-associated proteins, and the cytoskeletal fraction contains all proteins that are insoluble in 1% Triton X-100, including RAFTs. Enrichment was demonstrated by Western blot analysis of the three fractions and probing the blot using a GAPDH-specific antibody as cytoplasmic marker, two APP (amyloid precursor protein) -specific antibodies as markers for the membrane fraction, and a GFAP (glial fibrillar acid protein) -specific antibody as marker for the cytoskeletal fraction. The APP-specific antibody revealed a doublet representing the immature and mature form of APP, whereas the phospho-APP-specific antibody detected only the mature and phosphorylated form of APP. Our data are consistent with previous studies in the rat using identical experimental conditions (Strack et al., 1998). Cα was present in all fractions, with slightly lower levels in the cytoskeletal fraction. PR55/Bα and β were present in all fractions, with highest levels in the cytosolic fraction.

## PR55 subunits are differentially expressed in brain and display a diverse subcellular localization

To determine the distribution of PR55/Bα and β proteins in the brain, we analyzed parasagittal sections using immunohistochemistry (Fig. 5). Both isoforms were widely expressed in the brain; stainings were generally weak in the hippocampus, more intense throughout the cortex and brain stem, and even stronger in the cerebellum. In the cortex, Bα was present in somata and apical dendrites of pyramidal neurons, whereas Bβ was mainly confined to the soma (Fig. 5a,b). In the cerebellum, the anti-Bβ antiserum intensely stained somata and dendrites of Purkinje cells, whereas Bα was present in additional cell types and weakly-stained Purkinje cells (Fig. 5c,d). In the brain stem, both subunits were expressed in different cell types, including motor neurons (Fig. 5e,f). PR55/Bβ was stronger in neurons of the hilus of the dentate gyrus, whereas Bα was not detected (Fig. 5g,h); in the CA1 region of the hippocampus, PR55/Bβ was more strongly expressed than PR55/Bα: however, the PR55/Bα-specific antiserum strongly stained cells resembling activated astrocytes (Fig. 5i,k).

Our RNA *in situ* hybridization data indicated a preferential expression of PR55/Bα and β by neurons. To determine neuronal versus glial expression of PR55/Bα and β proteins, we analyzed parasagittal brain sections using double-immunofluorescence analysis using PR55/Bα- and β-specific antisera together with a MAP-2-specific antibody as a dendritic marker for neurons, and a GFAP-specific antibody as a marker for activated astrocytes, respectively (Fig. 6). PR55/Bα was localized to cell bodies and dendrites of neurons (Fig. 6a-c), whereas PR55/Bβ was mainly confined to the cell body (Fig. 6d-f). In areas of the brain with activated astrocytes, PR55/Bα was expressed by these cells at much higher levels than by neurons, as judged by the fluorescence settings (Fig. 6g-i). In contrast, PR55/Bβ was only expressed at background levels, indicating a distinct role of PR55/Bα during astrocytosis (Fig. 6k-m). Together, as neurons outnumber astrocytes in healthy brain, the number of Bα and Bβ-expressing neurons by far exceeded that of Bα-expressing astrocytes, confirming the findings of our RNA *in situ* hybridization analysis (Fig. 3).

## DISCUSSION

In the present study, we performed an analysis of the distribution and developmental regulation of the murine PR55/B subunit family of PP2A, with particular emphasis placed on its role in the brain.

We identified two novel splice variants of the β subunit, PR55/Bβ.1 and Bβ.2, encoding amino-terminally spliced forms of the β isoform that preserve the ORF and result in a protein that has the first 23 amino acids replaced by 5 and 26 novel amino acids, respectively (Fig. 1). Whereas Bβ and Bβ.2 had a size of roughly 55 kDa, Bβ.1 had a size of 53 kDa. Different PR55/Bβ transcripts were revealed after northern blot analysis, a major transcript of 2.5 kb and a minor transcript of 2.0 kb in the brain, and a 1.8 kb transcript in the testis. Similarly, for PR55/Bδ, multiple transcripts between 2.0 and 2.5 kb were detected in all tissues examined. However, as no δ-specific antiserum was available, there was no possibility of testing for the presence of this isoform. Together, our data indicate that different PR55/B transcripts are tightly regulated at the transcriptional level in different cell types. For comparison, ribonuclease assays were performed in the rat for all four subunits, precluding the possibility of detecting splice variants (Strack et al., 1998; Strack et al., 1999). Thus, it appears likely that in mammals the variability of the PR55/B family is even higher than previously thought. For other families of PP2A regulatory subunits, multiple splice variants have also been described (McCright *et al.*, 1996; Janssens et al., 2001).

No transcripts were detected for the α, γ, and δ subunits in mouse embryos until embryonic day E17, in contrast to a 2.5 kb β transcript that was detected as early as E11, with increasing levels until E17. These data indicate that the β subunits exert a distinct function during development. Previous data in rat have also shown a differential expression of the four subunits during peri- and postnatal development using ribonuclease protection assays. RNA levels were quantified in E18, P1, P7, P14, P21, and adult rat brain. α levels stayed constant throughout all stages of development, β levels were approximately threefold reduced, and γ levels threefold increased when adult brain was compared with E18 brain (Strack et al., 1998). RNA levels were determined in eight

subdissected brain regions. Differences in RNA levels were generally moderate, with the exception of the β subunit mRNA that was sevenfold higher in the olfactory bulb compared with the cerebellum (Strack et al., 1998).

Our northern blot analysis of adult tissues including testis, kidney, muscle, liver, lung, spleen, brain, and heart revealed that the α and δ subunit were ubiquitously expressed, indicating that these subunits are involved in general cellular processes of adult, but not immature tissues. In contrast to the α and δ subunits, the γ subunit was transcribed exclusively in the brain, and the β subunit was restricted to the brain and testis. This indicates that these subunits confer the PP2A holoenzyme with brain-specific functions. Also, expression of all four subunits in adult tissues indicates a diversification of the function of the four subunits. A tight transcriptional control of the individual PP2A regulatory subunits may be critical for proper cell function, as indicated by the recent finding of a CAG expansion in the 5' region of PR55/Bβ associated with spinocerebellar ataxia 12, an autosomal dominant neurodegenerative disease (Holmes et al., 1999).

The high levels of expression of both PP2AC isoforms in brain (Khew-Goodall & Hemmings, 1988) and the brain-specific expression of some members of the PR55/B (Mayer et al., 1991; Zolnierowicz *et al.*, 1994; Strack et al., 1998) and B′/PR61 (McCright & Virshup, 1995; Csortos *et al.*, 1996) subunit families indicate that PP2A has unique functions in neuronal cells (Price & Mumby, 1999). These include the dephosphorylation of neurofilaments (Saito *et al.*, 1995; Strack *et al.*, 1997) and neuronal-specific microtubule-associated proteins including tau and MAP-2 (Mandelkow *et al.*, 1995). Interestingly, the aggregation of hyperphosphorylated tau in neurofibrillary tangles is a pathological hallmark of AD, and hyperphosphorylation of tau has been proposed as a mechanism leading to neuronal degeneration (Lee, 1995; Billingsley & Kincaid, 1997). As mentioned above, a pool of PP2A mainly containing PR55/B subunits associates with microtubules (Sontag *et al.*, 1995). In a more recent report, it was shown that only trimeric PP2A forms containing PR55/Bα or PR55/Bβ, but not PR55/Bγ or B′/PR61, associate with neuronal microtubules, and that this interaction depends on an as yet unidentified anchoring factor (Price *et al.*, 1999).

Previous studies with rat brain have shown that the α, β, and γ isoforms are transcribed in all brain areas examined (Strack et al., 1998). In agreement with these findings, our *in*

*situ* hybridization and immunohistochemical analysis of the murine brains showed that all PR55/B subunits, including δ, were widely distributed in brain with regional differences in expression levels. At the protein level, PR55/Bα and β were differently distributed in the cerebellum. PR55/Bβ was mainly localized to somata and dendrites of Purkinje cells, whereas α was present in additional cell types and present at lower levels in Purkinje cells. As the cerebellum is affected in spinocerebellar ataxia, a disease for which an association with an expanded CAG repeat immediately upstream of the PR55/Bβ gene has been shown, a detailed analysis of the PP2A holoenzyme composition may contribute to the understanding of the pathogenesis (Holmes et al., 1999). In the cortex and hippocampus, areas prominently affected in AD brains, the distribution of the two PR55 subunits also varies. Whereas PR55/Bα is present in apical dendrites, β is mainly confined to cell bodies suggesting distinct subcellular substrates. The presence of PR55/Bα in activated astrocytes at staining intensities far exceeding those seen for neurons indicates a pivotal role of PR55/Bα in activated astrocytes. Remarkably, astrocyte activation is an early step in the pathogenesis of AD and related disorders (Kurosinski & Gotz, 2002). Collectively, our data indicate that the individual PR55/B subunits exert specialized functions in different subcellular compartments of neurons and in the course of astrocytic activation, but their widespread distribution in the brain indicates that they are also involved in central functions of both neurons and glial cells. Finally, sequence analysis of all murine PR55/B subunits revealed the presence of five to seven degenerate WD-40 repeats depending on the setting of the search parameters (Fig. 2). WD-40 repeats are minimally conserved sequences of approximately 40 amino acids that typically end in tryptophan–aspartate (WD) and are thought to mediate protein–protein interactions (Neer *et al.*, 1994). Insight into the distinct role of PR55/B subunits and their specific interaction partners may emerge from knock-out studies in mice. In *Drosophila*, PR55/B mutant phenotypes have been described; however, in contrast to mice, *Drosophila* only expresses one PR55/B isoform (Gomes *et al.*, 1993; Mayer-Jaekel *et al.*, 1993; Uemura *et al.*, 1993; Mayer-Jaekel *et al.*, 1994; Silverstein *et al.*, 2002). Considering the putative role of PP2A in the pathogenesis of human diseases, all these studies may eventually lead to the discovery of therapeutic agents that can specifically counteract PP2A dysfunction.

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## Abbreviations

AD, Alzheimer's disease; ORF, open reading frame; PBS, phosphate-buffered saline; PP2A, protein phosphatase 2A; TBS, Tris-buffered saline

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## TABLES

### Table 1

Distribution of PR55 mRNAs in brain

| | PR55/Bα | PR55/Bβ | PR55/Bγ | PR55/Bδ |
|---|---|---|---|---|
| hippocampus | +++ | +++ | +++ | ++++ |
| olfactory bulb | ++++ | ++++ | ++++ | ++++ |
| cortex | ++ | + | + | ++ |
| cerebellum | +++++ | +++++ | +++ | +++++ |
| striatum | ++ | ++ | +++ | ++ |
| thalamus | ++ | + | + | + |
| brain stem | +++ | ++ | - | +++ |
| pons | ++ | + | + | + |

(-, no; +, low; ++, low to intermediate; +++, intermediate; ++++, high; and +++++, very high signal intensities)

## FIGURE LEGENDS

**Figure 1**

Genomic structure and chromosomal localization of the four murine PR55/B isoforms including the novel PR55/Bβ.1 and PR55/Bβ.2 splice variants. Exon/intron boundaries of the final eight exons are conserved in each isoform and the PR55/Bβ splice variants. Boxed numbers represent the exons, and the size of the introns is indicated. The first coding exon of each isoform is indicated by an arrow.

**Figure 2**

Alignment of the amino acid sequences of the murine PR55/B isoforms. The highly diversive amino-terminus is highlighted in black. The overall sequence similarity is in the range of 90%, and amino acid differences are highlighted in black using Bα as reference. The WD-40 repeat motifs are shown in dark grey whereas the overlap of the PR55/Bα N-terminus with the first WD-40 repeat is highlighted in light grey. The exon boundaries are indicated with vertical lines for PR55/Bα.

**Figure 3**

A: Embryonic and tissue-specific distribution of murine regulatory PR55/B subunits mRNAs. Blots with mRNA of different embryonic stages or multiple tissues were hybridized with probes directed against PR55/B α, β, γ, and δ subunit transcripts, respectively. E7, embryonic day 7; te, testis; ki, kidney; li, liver; lu, lung; sp, spleen; br, brain; he, heart.
B: The distribution of the PR55/Bα subunit mRNA is shown by *in situ* hybridization analysis of cortex (a), hippocampus (b), cerebellum (c), and olfactory bulb (d). No significant staining was obtained using the corresponding sense probes on adjacent sections (insets).

**Figure 4**

A: For detection of PR55/B, commercial antibodies were used that have been shown previously to be specific for the PR55/Bα and Bβ subunits on western blots and by immunohistochemistry (Strack et al., 1998). The specificity of the above antibodies was determined by transfecting COS cells with constructs encoding HA-tagged PR55/Bα, Bβ, and Bγ, and analyzing lysates using western blot analysis. Whereas the HA-antibody detected all PR55 isoforms, the PR55/Bα- and PR55/Bβ-specific antibodies detected only the respective isoforms. The commercially-available Bγ antiserum was not specific as shown previously (Strack et al., 1998) and was therefore excluded from the subsequent analysis.

B: The expression profile of PR55/Bα, PR55/Bβ and PP2A Cα determined by western blot analysis. Protein was extracted from: te, testis; ki, kidney; li, liver; lu, lung; sp, spleen; br, brain; and he, heart.

C: To determine the subcellular localization, brain homogenates were fractionated into fractions enriched for cytosolic (cy), membranous (me), and cytoskeletal (sk) proteins as confirmed by the staining with antibodies specific for GAPDH (cy, 43 kDa), APP (me, 115 kDa), phosphorylated APP (me, 115 kDa), and GFAP (sk, 50 kDa). Cα was present in all fractions, with slightly lower levels in the cytoskeletal fraction. PR55/Bα and β were present in all fractions, with highest levels in the cytosolic fraction.

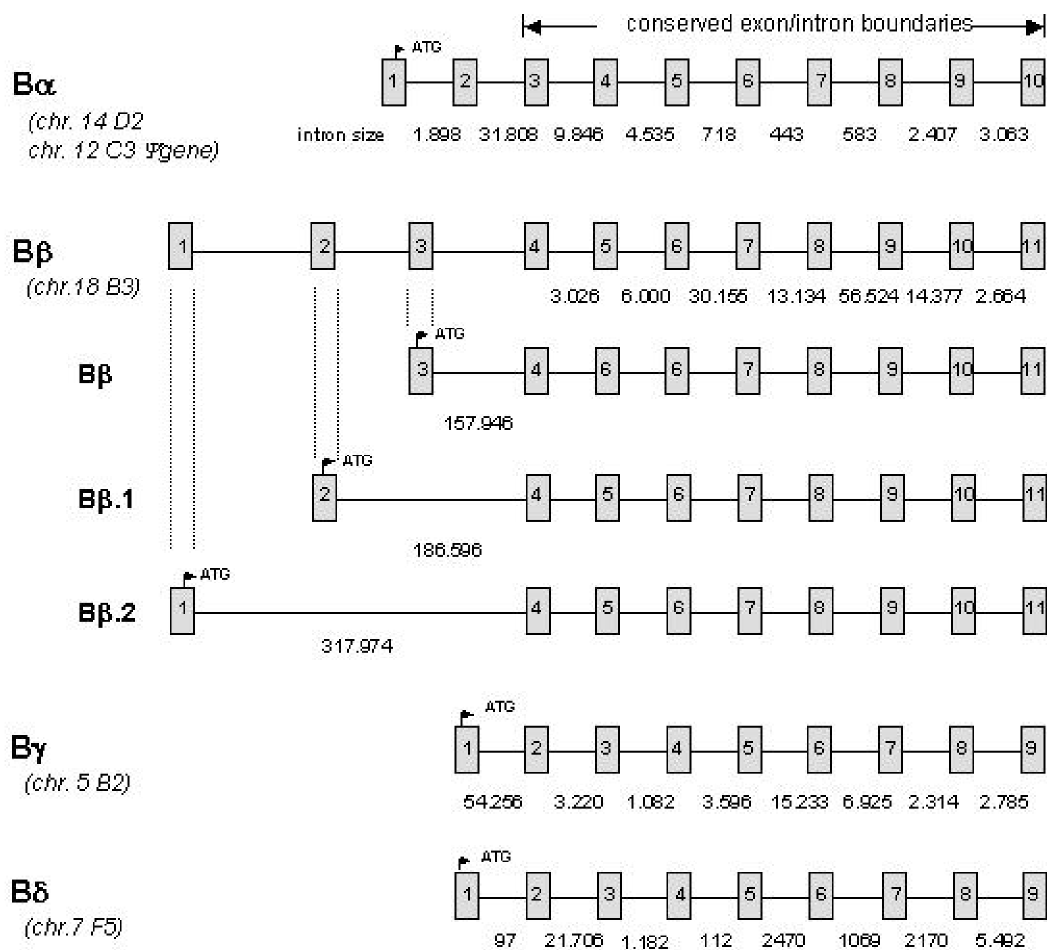
**Figure 5**

To determine the distribution of PR55/Bα and Bβ protein in the brain, we analyzed parasagittal sections by immunohistochemistry. Both isoforms are widely expressed. The distribution in cortex, cerebellum, brain stem, in the hilus of the hippocampus, and the CA1 region of the hippocampus is shown for Bα (a,c,e,g,i) and Bβ (b,d,f,h,k). In the cortex (a,b), Bα is present in somata and apical dendrites of pyramidal neurons, whereas Bβ is mainly confined to the soma. In the cerebellum, the anti-Bβ antiserum intensely stains somata and dendrites of Purkinje cells, whereas Bα is present in additional cell types and weakly stains Purkinje cells (c,d). In the brain stem, both subunits are

expressed in different cell types, including motor neurons (e,f). While Bβ is stronger in neurons of the hilus of the dentate gyrus, Bα is not detected (g,h); in the CA1 region of the hippocampus (indicated by an asterisk), Bβ is more strongly expressed than α; however, the Bα-specific antiserum strongly stains cells resembling activated astrocytes (see inset in (i) and Fig. 6).
Scale bar: a,b: 20 µm; c,d,i,k: 30 µm; e-h: 40 µm.

**Figure 6**

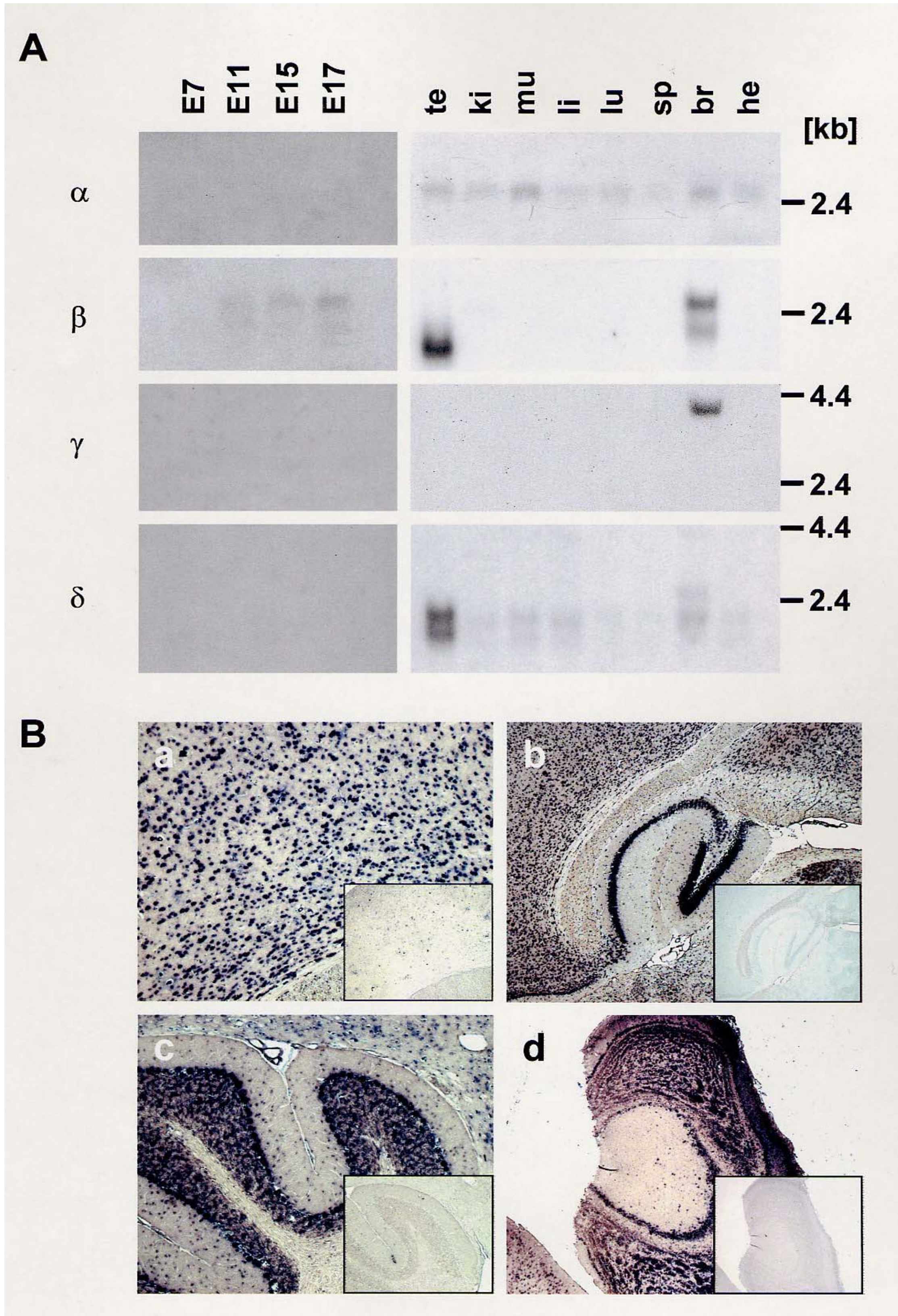
PR55/Bα is, in addition to neurons, strongly expressed by activated astrocytes. To determine neuronal versus glial expression of PR55/Bα (a-c, g-i) and Bβ (d-f, k-m) in the cortex, we analyzed parasagittal sections by double-immunofluorescence analysis using PR55-specific antisera together with a MAP-2-specific antibody as dendritic marker for neurons (a-f), and a GFAP-specific antibody as marker for activated astrocytes (g-m), respectively. PR55/Bα is localized to cell bodies and dendrites (a-c; a:Bα/Cy2, b:MAP-2/Cy3, c:merge), whereas PR55/Bβ is mainly confined to the cell body of neurons (d-f; d:Bβ/Cy2, e:MAP-2/Cy3, f:merge). PR55/Bα is expressed by activated astrocytes (g-i; g:Bα/Cy2, h:GFAP/Cy3, i:merge) at much higher levels than by neurons, whereas PR55/Bβ is only expressed at background levels (k-m; k:Bβ/Cy2, l:GFAP/Cy3, m:merge). Neuronal expression of PR55/Bα and PR55/Bβ in (g) and (k) is visible upon longer exposure. Scale bar: 20 µm



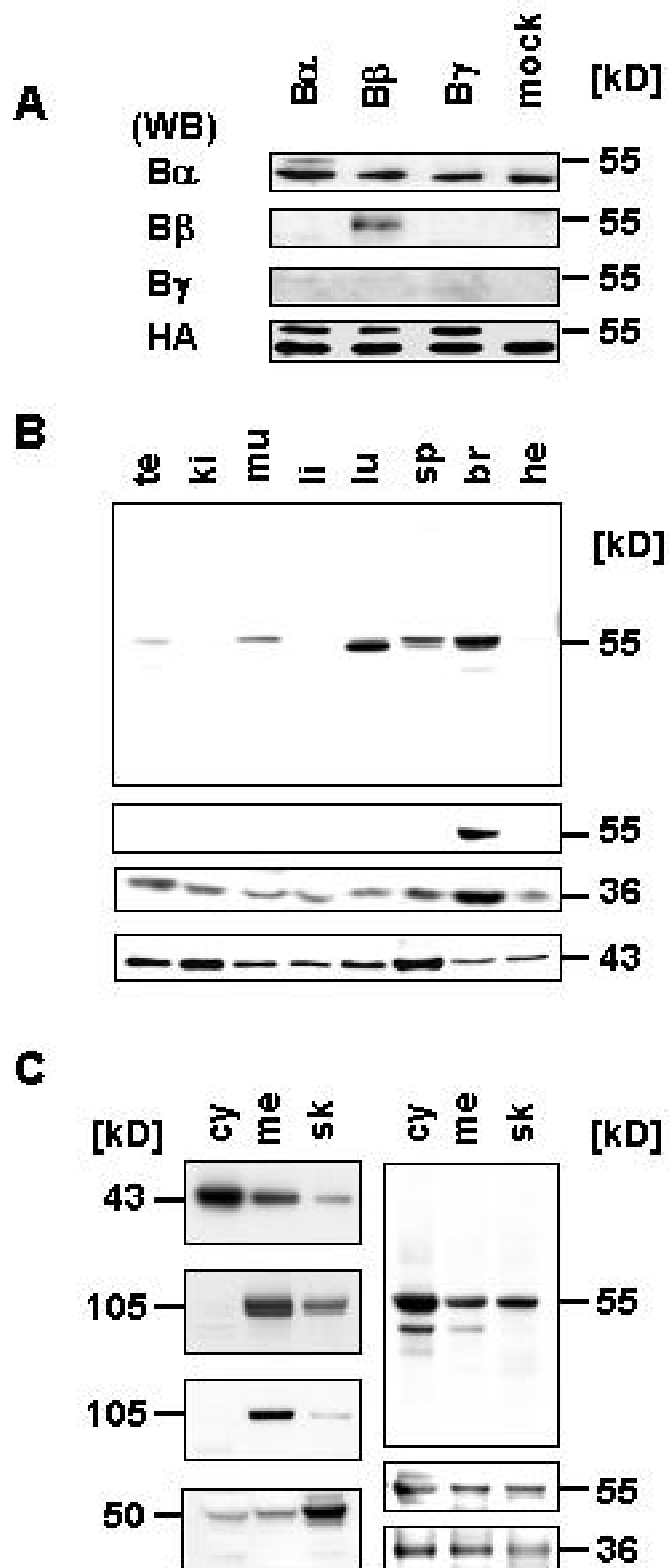

Schmidt *et. al.*, Figure 1

```
        1                                                                                                      100
alpha   ~~~~~~MAGA GGGNDIQWCF SQVKGAVDDD VAEADIISTA EFNHSGELLA TGDKSGRVVI FQQEQENKIQ SHSRGEYNVY STFQSHEPEF DYLKSLEIEE
beta    ~~~~~~~~~~~MEEDIDTRKI NNSFLRDHSY ATEADIISTV EFNHTGELLA TGDKGGRVVI FQREQESKNQ VHRRGEYNVY STFQSHEPEF DYLKSLEIEE
beta.1  ~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~MK PFTADIISTV EFNHTGELLA TGDKGGRVVI FQREQESKNQ VHRRGEYNVY STFQSHEPEF DYLKSLEIEE
beta.2  ~~~~~~~MKC FSRYLPYIFR PPNTILSSSC HTEADIISTV EFNHTGELLA TGDKGGRVVI FQREQESKNQ VHRRGEYNVY STFQSHEPEF DYLKSLEIEE
gamma   ~~~~~~~~~~ MGEDTDTRKI NHSFLRDHSY VTEADVISTV EFNHTGELLA TGDKGGRVVI FQREPESKNA PHSQGEYDVY STFQSHEPEF DYLKSLEIEE
delta   MAGAGGGGCP AGGNDFQWCF SQVKGAVDED VAEADIISTV EFNYSGDLLA TGDKGGRVVI FQREQENKGR AHSRGEYNVY STFQSHEPEF DYLKSLEIEE

        101                                                                                                    200
alpha   KINKIRWLPQ KNAAQFLLST NDKTIKLWKI SERDKRPEGY NLKEEDGRYR DPTTVTTLRV PVFRPMDLMV EASPRRIFAN AHTYHINSIS INSDYETYLS
beta    KINKIRWLPQ QNAAYFLLST NDKTVKLWKV SERDKRPEGY NLKDEEGRLR DPATITTLRV PVLRPMDLMV EATPRRVFAN AHTYHINSIS VNSDYETYMS
beta.1  KINKIRWLPQ QNAAYFLLST NDKTVKLWKV SERDKRPEGY NLKDEEGRLR DPATITTLRV PVLRPMDLMV EATPRRVFAN AHTYHINSIS VNSDYETYMS
beta.2  KINKIRWLPQ QNAAYFLLST NDKTVKLWKV SERDKRPEGY NLKDEEGRLR DPATITTLRV PVLRPMDLMV EATPRRVFAN AHTYHINSIS VNSDYETYMS
gamma   KINKIKWLPQ QNAAHSLLST NDKTIKLWKI TERDKRPEGY NLKDEEGKLK DLSTVTSLQV PVLKPMDLMV EVSPRRTFAN GHTYHINSIS VNSDCETYMS
delta   KINKIRWLPQ QNAAHFLLST NDKTIKLWKI SERDKRAEGY NLKDEDGRLR DPFRITALRV PILKPMDLMV EASPRRIFAN AHTYHINSIS VNSDHETYFS

        201                                                                                                    300
alpha   ADDLRINLWH LEITDRSFNI VDIKPANMEE LTEVITAAEF HPNSCNTFVY SSSKGTIRLC DMRASALCDR HSKLFEEPED PSNRSFFSEI ISSISDVKFS
beta    ADDLRINLWN FEITNQSFNI VDIKPANMEE LTEVITAAEF HPHHCNTFVY SSSKGTIRLC DMRASALCDR HTKFFEEPED PSNRSFFSEI ISSISDVKFS
beta.1  ADDLRINLWN FEITNQSFNI VDIKPANMEE LTEVITAAEF HPHHCNTFVY SSSKGTIRLC DMRASALCDR HTKFFEEPED PSNRSFFSEI ISSISDVKFS
beta.2  ADDLRINLWN FEITNQSFNI VDIKPANMEE LTEVITAAEF HPHHCNTFVY SSSKGTIRLC DMRASALCDR HTKFFEEPED PSNRSFFSEI ISSISDVKFS
gamma   ADDLRINLWH LAITDRSFNI VDIKPANMED LTEVITASEF HPHHCNLFVY SSSKGSLRLC DMRAAALCDK HSKLFEEPED PSNRSFFSEI ISSVSDVKFS
delta   ADDLRINLWH LEITDRSFNI VDIKPANMEE LTEVITAAEF HPHQCNVFVY SSSKGTIRLC DMRSSALCDR HAKFFEEPED PSSRSFFSEI ISSISDVKFS

        301                                                                                                    400
alpha   HSGRYMMTRD YLSVKIWDLN MENRPVETYQ VHEYLRSKLC SLYENDCIFD KFECCWNGSD SVVMTGSYNN FFRMFDRNTK RDITLEASRE NNKPRTVLKP
beta    HSGRYIMTRD YLTVKVWDLN MENRPIETYQ VHDYLRSKLC SLYENDCIFD KFECVWNGSD SVIMTGSYNN FFRMFDRNTK RDVTLEASRE NSKPRAILKP
beta.1  HSGRYIMTRD YLTVKVWDLN MENRPIETYQ VHDYLRSKLC SLYENDCIFD KFECVWNGSD SVIMTGSYNN FFRMFDRNTK RDVTLEASRE NSKPRAILKP
beta.2  HSGRYIMTRD YLTVKVWDLN MENRPIETYQ VHDYLRSKLC SLYENDCIFD KFECVWNGSD SVIMTGSYNN FFRMFDRNTK RDVTLEASRE NSKPRAILKP
gamma   HSGRYMLTRD YLTVKVWDLN MEARPIETYQ VHDYLRSKLC SLYESDCIFD KFECAWNGSD SVIMTGAYNN FFRMFDRNTK RDVTLEASRE NSKPRAVLKP
delta   HSGRYMMTRD YLSVKVWDLN MEGRPVETHQ VHEYLRSKLC SLYENDCIFD KFECCWNGSD SAIMTGSYNN FFRMFDRNTR RDVTLEASRE NSKPRASLKP

        401                                              450
alpha   RKVCASGKRK KDEISVDSLD FNKKILHTAW HPKENIIAVA TTNNLYIFQD KVN*
beta    RKVCVGGKRR KDEISVDSLD FSKKILHTAW HPSENIIAVA ATNNLYIFQD KVN*
beta.1  RKVCVGGKRR KDEISVDSLD FSKKILHTAW HPSENIIAVA ATNNLYIFQD KVN*
beta.1  RKVCVGGKRR KDEISVDSLD FSKKILHTAW HPSENIIAVA ATNNLYIFQD KVN*
gamma   RRVCVGGKRR RDDISVDSLD FTKKILHTAW HPAENIIAIA ATNNLYIFQD KVNSDMH*
delta   RKVCTGGKRK KDEISVDSLD FNKKILHTAW HP ESIIAVA ATNNLYIFQD KIN*
```

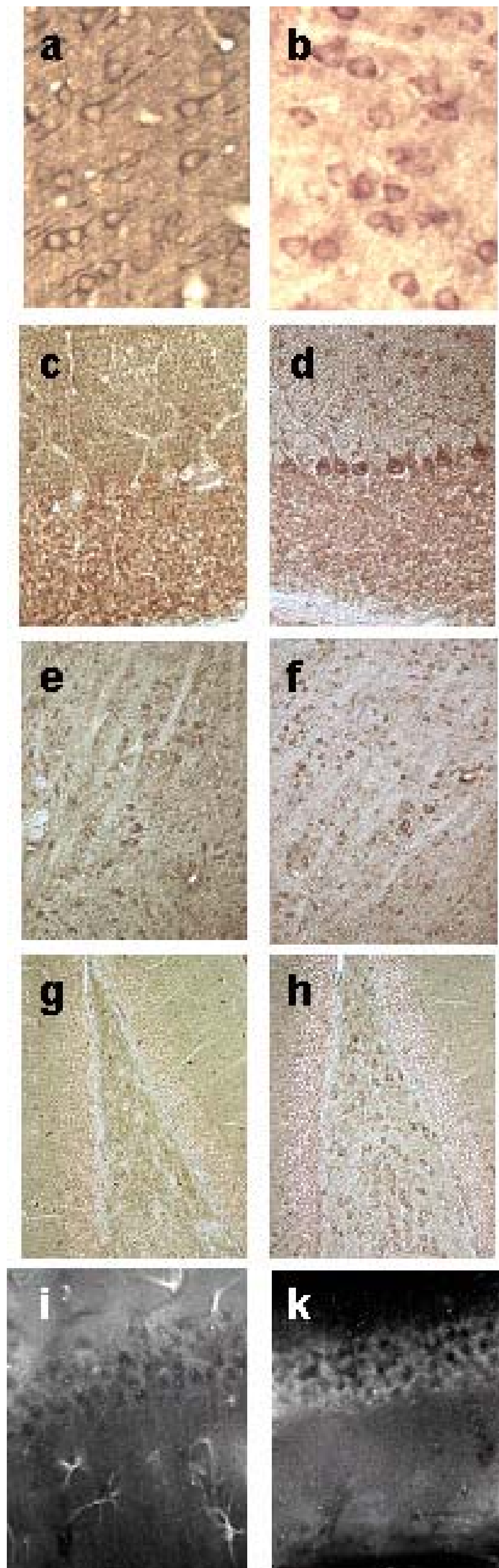
Schmidt *et. al.*, Figure 2



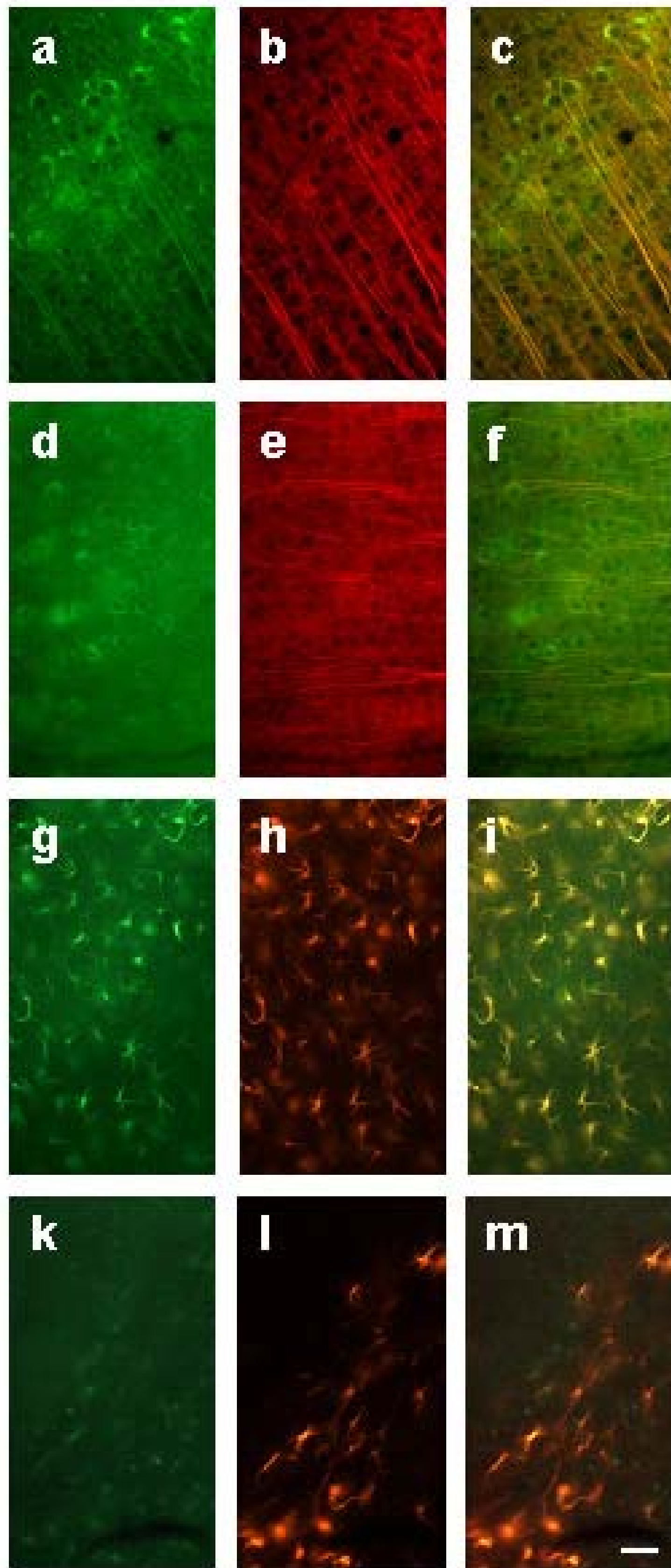

Schmidt *et. al.*, Figure 3




Schmidt *et. al.*, Figure 4




Schmidt *et. al.*, Figure 5




Schmidt *et. al.*, Figure 6

## **Part 3:** Transcriptional effects of the protein phosphatase 2A inhibitor okadaic acid on promoter complexes using Affymetrix GeneChips

**K. Schmidt**, E. J. Oakeley, Victor Zhang, Herbert Angliker and B. A. Hemmings

# Transcriptional Effects of the Protein Phosphatase 2A Inhibitor Okadaic Acid on Promoter Complexes using Affymetrix GeneChips

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**Abstract**

GeneChip technology has become a powerful tool for investigating the regulation of global gene expression. We have used Affymetrix U133A micro arrays to define the effects of okadaic acid (OA) on gene expression in HEK293 cells with time. We developed and applied a variety of bioinformatics tools, since the primary goal was to find genes that are co-regulated under the same experimental conditions. As co-regulated genes should have similar regulatory mechanisms at the transcriptional level, we explored their promoter regions (2 kb upstream of the start of transcription. Source: Genomatix GMBH). We identified common motifs that may act as binding sites for transcriptional regulators (TransFac Pro 7.2) and correlated them with transcription factors that are themselves altered in their expression profiles. In this way, we identified a key group of transcription factors that may be central to the primary transcriptional effects of OA.

## Introduction

Regulation of signal transduction pathways plays a crucial role in a variety of cellular processes and results in changes in both the phosphorylation status of the components and the expression of specific target genes. While much is known about the effect of kinases and phosphatases on protein levels; the involvement of these enzymes, especially protein phosphatases, on transcriptional events is less clear. The naturally occurring phosphatase inhibitor okadaic acid (OA) has become an essential tool for the investigation of protein phosphatases in signaling events *in vivo*. This potent tumor promoter is a $C_{38}$ polyether fatty acid produced by marine dinoflagellates and the causative agent of diarrhetic shellfish poisoning. OA induces various biological effects *in vivo*, including promotion of tumor growth, prolonged smooth muscle contraction and promotion of genomic instability (reviewed in (Fernandez et al., 2002)). Malignant transformation and cell growth is found to be both promoted and inhibited, depending on the system used. The variety of effects caused by OA is presumably a result of the down-regulation of OA-sensitive protein phosphatases. Following these studies on OA, several other phosphatase-inhibiting compounds have been identified, including calyculin A (Ishihara et al., 1989), microcystin-LR (Honkanen et al., 1990), tautomycin (MacKintosh and Klumpp, 1990), nodularin (Honkanen et al., 1991), and cantharidin (Li and Casida, 1992).

OA regulates gene expression at transcriptional, posttranscriptional and posttranslational levels on several signaling pathways, including the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3'-kinase (PI3K) pathways. Although both pathways are known to be regulated at multiple levels by protein phosphatase 2A (PP2A), the main criterion used to link PP2A involvement to the regulation of transcriptional activity is the application of OA (Schonthal, 1995). Several genes, including c-Jun , Early growth response 1 & 3 (Egr1&3), interleukin 6 (IL-6) (Guy et al., 1992), collagenase and stromelysin (Westermarck et al., 1995a) are transcriptionally induced in fibroblasts in the presence of OA, whereas elastin (Westermarck et al., 1995b) and different collagen subtype genes (Westermarck et al., 1995a) are repressed. Induction of *IL-8*, nerve growth factor (NGF) and c-fos (Miskolci et al., 2003; Rosenberger et al., 1999) *(Sonoda et al.,*

*1997)* by OA involves both transcriptional and posttranscriptional mechanisms in different cell types.

At the posttranslational level, OA causes increased phosphorylation of p53 but inhibits phosphorylation of the Rb gene product (Zhang et al., 1994). Inhibition of PP2A by OA in metabolically competent rat brain slices induced a increase in the phosphorylation/activation of ERK1/2, MEK1/2, and p70 S6 kinase as well as the phosphorylation of tau at several sites (Pei et al., 2003). Although tau hyperphosphorylation induced by OA-mediated protein phosphatase inhibition contributes to pathological aggregate formation, only hyperphosporylation of tau followed by proteasome inhibition leads to stable fibrillary deposits of tau similar to those observed in neurodegenerative diseases (Goldbaum et al., 2003). Treatment of SCC-25 carcinoma cells with OA enhanced the expression of both Fas receptor and Fas ligand mRNA and protein levels. OA treatment did not only lead to translocation of NFkB from the cytosol to the nucleus, its levels also increased, whereas the amount of IkB-α decreased. This suggests that NF-kB activated at early stages by OA stimulated the promoter activity of Fas receptor in the cells leading to apoptosis (Fujita et al., 2004). Another apoptotic effect of OA was shown in HL-60 cells, where OA induces bcl-2 mRNA destabilization which is associated with decreased binding of trans-acting factors such as nucleolin to the AU-rich element (ARE) of Bcl-2 (Sengupta et al., 2003). Direct involvement of PP2A has been shown in the inactivation of CREB activity (Wadzinski et al., 1993; Wheat et al., 1994). Also, indirect activity of PP2A in the regulation of transcription at the phosphorylation level is known. Treatment of cells with OA leads to concentration-dependent inhibition of serine/threonine protein phosphatases. While PP2A is inhibited most efficiently, PP1 is 100-fold less sensitive to OA *in vitro*. The effect on PP2B is even lower and PP2C is insensitive to the treatment. Since OA does not penetrate cell membranes rapidly to accumulate on the catalytic subunit of the phosphatases, it is very difficult to control the actual concentration of the compound *in vivo*. Nevertheless, conditions for the selective inhibition of PP2A in intact cells have been established (Favre et al., 1997) and 1 μM OA applied to the living cell is sufficient to block PP2A activity. As less abundant protein phosphatases like PP4, PP5 and PP6 are just as sensitive to OA as PP2A, the cellular effects of OA can no longer be entirely attributed to

PP2A inhibition. On the other hand, the physiological role of these novel phosphatases is still unclear and they represent only a minor fraction of total cellular phosphatase activity (Brewis et al., 1993)(Chen et al., 1994).

The aim of this study was to model the common transcriptional events induced in genes that are up-regulated in response to OA treatment using microarray and bioinformatics tools. Using DNA microarrays, we identified both novel and known target genes for OA/PP2A. Further, we developed a computational tool called StampCollector that allowed us to confirm established (Schonthal, 1995) and predict novel regulatory mechanisms for the control of gene expression at the transcriptional level due to the action of OA.

## Experimental Procedures

Cell Culture, protein and RNA extraction

Human embryonic kidney cells (HEK293) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS, Life Technologies) and 50 U/ml penicillin/streptomycin (Gibco) and incubated with 5% $CO_2$-in-air at 37°C. Cells were seeded at 50% confluency and treated with 1 μM OA in 0.1% *N*,*N*-dimethylformamide (Alexis Biochemicals, Switzerland) for 0, 30, 60 and 90 min after 24 h pre-incubation. Total RNA from two 10-cm dishes was extracted at each time point using the RNeasy mini kit (Qiagen). NP40 protein extracts were prepared from one 10-cm dish at each time point to monitor the state of inhibition by OA.

*Western blotting*

NP40 protein (30 μg) extracted from OA-treated HEK293 cells at each time point was used to test the effect of OA on protein levels. The extracts were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked in TBST containing 10% horse serum for 1 h and incubated for a further 1 h at room temperature with 1:500 Ndr kinase anti-Thr-444P purified antibody (Tamaskovic et al., 2003). The antibody was detected using horseradish peroxidase-conjugated donkey anti-rabbit Ig antibody (Amersham Biosciences) and ECL.

*Microarray analysis*

Microarray analysis was performed using HG_U133A GeneChips™ (Affymetrix, Santa Clara, USA). A 10-μg aliquot of total RNA (isolated from HEK293T cells) was reverse transcribed using the SuperScript Choice system for cDNA synthesis (Life Technologies) according to the protocol recommended by Affymetrix (GeneChip Expression Analysis: Technical Manual (2001) p. 2.1.14-2.1.16). The oligonucleotide used for priming was 5'-ggccagtgaattgtaatacgactcactatagggaggcgg-$(t)_{24}$-3' (GenSet Oligo, France), as recommended by Affymetrix. Double-stranded cDNA was cleaned by phenol:chloroform extraction and the aqueous phase removed by centrifugation through Phase-lock Gel (Eppendorf). *In vitro* transcription was performed on 1 μg of cDNA using the Enzo

BioArray High Yield RNA transcript labeling kit (Enzo Diagnostics, USA) following the manufacturer's protocol. The cRNA was cleaned using RNAeasy clean-up columns (Qiagen) and fragmented by heating in 1x fragmentation buffer (40 mM Tris-acetate pH 8.1, 100 mM KOAc, 30 mM MgOAc). A 10-µg aliquot of this fragmented cRNA was hybridized to an HG-U133A GeneChip (45°C, 16 h). Washing and staining were performed in a Fluidics Station 400 (Affymetrix) using the protocol EukGE-WS2v4 and scanned in an Affymetrix GeneChip 2500 scanner. Chip analysis was performed using the Affymetrix Microarray Suite v5 and GeneSpring 5.1 (Silicon Genetics). Changes in gene expression were assessed by looking for concordant changes between replicates using a signed Wilcoxon rank test (as recommended by Affymetrix $P$-value threshold $<0.003$). A gene that changed in the same way in at least 6 out of 9 replicate comparisons was considered to be changing reproducibly. Gene with detection $P$ values of $>0.05$ in all experimental conditions were discarded from the analysis as unreliable data. Final data quality assessment was by a Student T-test ($P$ value threshold $<0.05$). Genes failing the Student T-test were eliminated due to their highly variable expression profiles within the replicates. A Benjamini and Hochberg false discovery rate multiple testing correction was applied to the T-test data.

*Transfac database*

The Transfac 7.2 Pro database was installed locally together with ActivePerl 5.8.0 (Sophos, Inc. Mass., USA) and an Apache 1.3.28 web server (Apache Software Foundation, Minn., USA). The Transfac Pro database is a commercial product that is required for the StampCollector application.

*Genomatix database*

The Genomatix Chip Promoter Resource CD for the HG-U133A and MOE430A GeneChips (Genomatix Software GmbH, Munich, Germany) contains all of the annotated promoters from the commercial Genomatix database, indexed according to their Affymetrix accession numbers. The sequences provided on the CD were derived from the NCBI Human Genome build 33.

*StampCollector*

The StampCollector program imports the provided sequences in FASTA format. Transcription factor binding sites are predicted using the Match 1.1 component of Transfac 7.2 via an automated batch query. The identified transcription factors are compared to the expression data and those factors that are not expressed in the samples are removed from the analysis. The distances between each remaining factor and all the others are calculated and pairs generated for those factors 10-100 bp apart. This threshold was selected to minimize false pairs (multiple binding sites for the same factor located over a stretch of 1-10bp are common; Setting the minimum to 10-bp decreases the risk of generating false homodimers). The maximum of 100 represents an arbitrary measure beyond which paired-binding interactions are not expected to be as important as DNA-binding interactions. Pairs were considered to be the minimum simplification state as they should allow the modeling of higher order complexes while minimizing disruption caused by extra predicted sites in the sequence.

*DNA Block Aligner*

The DNA Block Aligner (DBA) is an online tool (http://www.ebi.ac.uk/Wise2/dbaform.html) that aligns two sequences under the assumption that the sequences share a number of co-linear blocks of conservation separated by potentially large and varied lengths of DNA in the two sequences. The conserved blocks may be regions important for regulation of the gene. The final model is a probabilistic finite state machine (or pair-HMM) that aligns the two sequences. Each block can choose one of four different parameter Sets, roughly with conservation at 65% (A), 75% (B), 85% (C) or 95% (D) identity. Linear gaps (gaps where the open gap is the same as the extension) were modeled in the blocks at a fixed probability 0.05 and each block is expected at around 1% of the DNA sequence. We used DBA to compare upstream regions of a gene from mouse and human.

*MEME and MOTIFSampler*

We used the online version 3.0 of the MEME motif discovery program (http://meme.sdsc.edu/meme/website/intro.html). MEME is a tool for discovering motifs in a group of related DNA or protein sequences. MEME represents motifs as position-dependent letter-probability matrices that describe the probability of each possible letter at each position in the pattern. Individual MEME motifs do not contain gaps. Patterns with variable-length gaps are split by MEME into two or more separate motifs. MEME uses statistical modeling techniques to automatically choose the best width, occurrence and description of each motif. We downloaded the command line version 0.1.2 of the MotifSampler (http://www.esat.kuleuven.ac.be/~thijs/Work/MotifSampler.html), which attempts to find over-represented motifs in the promoter region of a Set of co-regulated genes. This motif-finding algorithm uses Gibbs sampling with a probabilistic framework and higher-order background models to find a position probability matrix representing the optimum motif. The command line was automated so that each sequence could be resampled 20 times. The parameters were Set to search for 15 different motifs, each with a length of 8 bp.

## Results

*OA treatment leads to phosphorylation of NDR kinase*

The level of phosphorylation of the nuclear Dbf2-related (NDR) kinase in HEK293 cells was used to test for effective inhibition of PP2A by OA. The anti-Thr-444P rabbit polyclonal antisera (Tamaskovic et al., 2003) detected endogenous activated NDR kinase at the 60- and 90-min time points.

*Microarray analysis identifies OA-regulated genes*

In order to identify genes transcriptionally co-regulated by OA, we treated human embryonic kidney (HEK293) cells with 1 μM OA for 30, 60 and 90 min prior to total RNA extraction (Fig. 1). The RNA was converted into cRNA following the protocol recommended by Affymetrix and was hybridized to human HG-U133A oligonucleotide arrays for 16 h. Of the 22283 transcripts present on the human HG-U133A chip 11581 were identified as present in HEK293 cells based on the filter 'data quality flags' in the original data files. The chips were subjected to an Affymetrix change call filter that required that each condition had a change *P* value of $<0.003$ for increase and $>0.997$ for decrease genes. Concordance filtering was applied to the lists by requiring that a gene called as changing did so in at least six out of nine replicate comparisons. The remaining genes were then subjected to a Student T-test to eliminate those genes whose expression values were highly variable (*P* value cutoff 0.05).

Of those genes remaining, 115 were found to be up-regulated between 2-fold and 994-fold (Table 1), whereas only five genes were down-regulated more than twofold (35 genes down more than 1.5-fold) (Table 2). This suggests that OA mainly stimulates transcription activators or inhibits transcription repressors, probably by inhibition of PP2A. Of the 115 genes that were up-regulated at any time point, six were genes encoding hypothetical proteins without any reported function. Of these 115 genes, 33 were transcription factors and DNA-binding proteins. The group of genes up-regulated by OA was further analyzed on the basis of functional similarity using gene ontology grouping of the Genespring 6.0 program. This classification revealed a wide range of different biological functions for the regulated genes, such as cell communication, signal

transduction, cell cycle and apoptosis regulators, signal transducers, and genes involved in cell transformation. Notable examples include AP-1 family members like Fos and Jun transcription factors, other early intermediate genes like the Egr-1 and Egr-3, growth arrest and DNA damage-induced genes like Gadd45B, phosphatases of the MAPK pathway like dual specific phosphatases DUSP1 and DUSP5, and other activators (VEGF) and suppressors (SOCS1) of various signaling cascades. With the exception of the Fos and Jun family, most of the genes were not known to be regulated by OA prior to this study. Thus, the results provide a more comprehensive and deeper knowledge about OA regulation of gene expression in HEK293 cells.

*Genes regulated jointly by TPA*

About 20 genes that were up-regulated upon OA treatment coincide with genes up-regulated by treatment of HEK 293 cells with the phorbol ester 12-O-tetradecanoyl-phorbol-13 acetate (TPA) (Table 3). Although there is contradictory evidence about the co-regulation of gene expression by two different treatments, it has been suggested that some of the effects of OA and TPA are mediated via the same pathways. TPA-activated PKC initiates a phosphorylation cascade that can be reversed by OA-sensitive phosphatases. Inhibition of these phosphatases may lead to altered phosphorylation of substrates of the PKC pathway and, thereby, may affect gene expression in a manner similar to TPA. For example, Fos and Jun family members were up-regulated by both OA and TPA in our chip experiments. In addition, this is supported experimentally in the literature (Rahmsdorf and Herrlich, 1990). This well-characterized relationship can be used as an internal control for inhibitor efficiency at the transcriptional level.

*Clustering of OA up-regulated genes by K-means*

The 115 OA-regulated genes were clustered according to their different expression profiles at 10, 30, 60 and 90 min. Clustering was achieved in five Sets (SET1-5) by the *K-means* algorithm with a standard correlation for the similarity measure and the number of iterations Set to 100 (Fig. 2). No significant change in transcription was observed at 10 or 30 min for almost all genes. This may be because OA needs time to penetrate the cell wall, bind and inhibit the catalytic subunits of PP2A and PP1 and trigger the transcription

machinery of the target genes. The 23 genes in SET1 showed an increase in transcription between 30 and 60 min incubation with OA that persisted up to the 90-min time point, whereas the 32 genes in SET 2 followed a slower but steady increase in rate from 30 to 90 min. SET3 contains 14 genes that increased moderately up to 60 min and showed no further change in transcription rate from 60 to 90 min (Fig. 3 and 4). The change in transcription rate of the 29 genes in SET4 started between 60 and 90 min and was thus delayed for 30 min relative to SET1-3. The transcription of seven of 17 genes in SET 5 decreased slightly between 30 and 60 min and then increased between 60 and 90 min (Table 1).

*StampCollector identifies potential transcription factor pairs involved in regulation of OA treated cells*

As described in Experimental Procedures, we have developed a software tool called "StampCollector" that is able to predict potential transcription factor pairs (TF pairs) involved in the regulation of genes based on their promoter sequences. The rational behind predicting TF pairs is that most genes are activated by TF complexes rather than by a single activator protein. On this assumption, we were able to reduce the number of TF hits from the Transfac 7.2 Pro database significantly (Matys et al., 2003). We obtained putative promoters for all of the target genes from Genomatix (Genomatix, 2003), a promoter being defined as the sequence extending 2 kb upstream of every mapped transcriptional start site for a gene. The mapping information fell into three quality classes: gold (experimentally verified by oligo-capping); silver (predicted start sites derived from Genomatix's promoter inspector software) and bronze (no prediction or experimentally verified start available, so the start of translation was used). For the purposes of this experiment, we restricted ourselves to gold and silver promoters. The promoters were loaded into the StampCollector program, which generated three output files: 1) all potential transcription factor-binding sites and their positions according to Transfac 7.2 Pro, using all vertebrate matrices optimized to minimize both false positive and false negative hits, are identified; 2) after calculation of the distance between each potential binding site, every neighboring pair more than 10 and less than 100 bp away are recorded for each promoter. The minimum of 10 bp was chosen to prevent the same

binding site being selected as a pair with itself; the maximum of 100 bp was an arbitrary distanced based on 10 helical repeats of the DNA to minimize the number of distant pairs that should be considered; 3) after assigning all possible distance pairs for each promoter, those pairs common to at least 50% of the promoters are recorded (Fig. 3).

In order to generate a control Set of genes, we also examined the expression values of all 22283 transcripts on the HG-U133A chip actually expressed (detection $P$-value $< 0.05$) at all time points for those classed as robustly unchanging (change $P$ value = 0.50000) at all time points relative to time zero and whose fold change relative to time zero was 0.95-1.05.

Of the 54 genes that passed all selection criteria, we retrieved a total of 90 promoter sequences from the Genomatix database and identified common motifs that function as binding sites for transcriptional regulators. Several transcription factor pairs identified were simply over-represented in the promoter sequence of the clustered genes in SET1-5 compared with the 90 promoter sequences of the 54 control genes. Potential binding sites for a TF pair had to be present in a minimum of 50% of genes in at least one SET in order to be included in the analysis for the StampCollector approach. The reason for the StampCollector approach is that certain transcription factors may be involved in regulating a certain Set of genes if the frequency of its binding motif in the putative promoter region of OA-regulated genes is significantly over-represented compared with the 90 promoter sequences of the 54 control genes. Table 4 shows 11 different transcription factors involved in the formation of 18 transcription factor pairs that pass the 50% over-representation and 1-out-of-5 Set restriction (Table. 4): Gut-enriched Krueppel-like factor (GKLF), spermatogenic Zip (SPZ1), zink finger protein (ZF5), simian-virus-40-protein1 (SP1), paired domain, paired box/homeodomain (PAX-4), homeodomain TF (MSX), general initiator signal (GEN_INI), fetal Alzheimer antigen (FAC-1), caudal homolog homeobox protein (CDXA), CCAAT/enhancer binding protein (C/EBP) and vitamin D receptor (VDR).

Binding sites for about seven different TF pairs were present in over 50% of the 23 genes in SET1. Binding sites for four of these nine pairs were over-represented >2-fold compared with the 90 control sequences: GKLF+SPZ1 (47.8%, 4.7-fold), ZF5+ZF5 (56.5%, 3.6-fold), SP1+ZF5 (60.9%, 3.2-fold) and PAX-4+SPZ1 (56.5%, 2.4-fold). This

suggests that TF pairs containing GKLF, ZF5, SP1, PAX-4 or SPZ1 are involved in the transcriptional regulation of these genes (Fig. 4).

The genes clustered in SET2 showed a similar expression profile, but only six different TF pairs were present in over 50% of the 32 genes in SET2. As in SET1, binding sites for SP1+ZF5 (50%, 2.6-fold) and PAX-4+SPZ1 (56.3%, 2.4-fold) were over-represented >2-fold compared with the 90 control sequences. Interestingly, binding sites for GKLF+SPZ1 and ZF5+ZF5 were over-represented 4.2-fold and twofold, respectively, but did not match the 50% occurrence restriction (46.9% and 31.3%). We also found that the occurrence of the binding sites for MSX+GEN_INI is 9.4% and thus 3.1-fold less then in the 90 control sequences (Fig. 4).

Binding sites for nine different TF pairs were present in over 50% of the 14 genes in SET3. FAC-1+GEN_INI (57.1%, 3.4-fold), PAX-4+SPZ1 (57.1%, 2.5-fold), GEN_INI+GKLF (64.3%, 2.3-fold) and MSX-1+GEN_INI (57.1%, 2.0-fold) fulfilled these criteria. Conspicuously, binding sites for GEN_INI+PAX-4 were present in 93% of the promoter sequences in SET3, and 1.8-fold over-represented compared with all other SETs and the 90 control sequences. We observed a similar scenario for all other TF pairs containing bindings sites for GEN_INI and conclude from this that TF pairs containing GEN_INI are be involved in the transcriptional regulation of these genes in SET3. Surprisingly, there were no binding sites for ZF5+ZF5 in SET3 at all, compared with SET1, where binding sites for this pair were present in 56.5% and over-represented 3.6-fold. This emphasizes the importance of the presence/absence of specific TFBS for the regulation of transcription (Fig. 4).

None of the five genes that pass the 50% occurrence restriction in SET4 fulfilled the >2-fold over-representation requirement. Merely three TF pairs GKLF+SPZ1 (31.0%, 2.8-fold), ZF5+ZF5 (41.4%, 2.7-fold) and GKLF+ZF5 (41.4% and 2.5-fold) met the >twofold restriction. The 25 genes in SET4 contained a higher binding-site number of CDXA-containing TF pairs than the other SETs. In addition, the representation of these pairs in SET1-3 and SET5 was lower than in the 90 control sequences, whereas the representation of these pairs in SET4 was higher than in the control sequences (see PAX-4+CDXA, CDXA+CDXA and CDXA+C/EBP) (Fig. 8). Only ZF5+PAX-4 had a TFBS on more than 50% of the 17 genes in SET5, but the over-representation was <twofold

(1.7). Interestingly, the 4.2-fold over-representation of binding sites for GKLF+SPZ1 was comparable to all other SETs. As argued above (SET1), this strengthens the point that TF pairs containing GKLF, ZF5, SP1, PAX-4 or SPZ1 might be involved in the transcriptional regulation of these genes (Fig. 4).

*Human and Mouse Promoter Comparison*

Comparative analysis of genomic sequences is used more and more to investigate coding and regulatory regions in mammalian genomes, since only a very limited number of promoters have been experimentally evaluated to date. To further examine the results obtained from the StampCollector program, we compared sequence identity and TF binding-site similarity to mouse promoter sequences. For this we retrieved the mouse Affymetrix identification number corresponding to the human code obtained from the chip experiment and obtained 2 kb of putative promoter sequence for all of the mouse target genes from Genomatix. Both the human and the mouse sequences were loaded into the DNA Block Aligner (DBA) program that aligns two sequences under the assumption that the sequences share a number of co-linear blocks of conservation separated by potentially large and varied lengths of DNA. DBA models four different types of conserved blocks, with corresponding sequence identities: type A, 60-70%; type B, 70-80%; type C, 80%-90%; type D, 90-100%. Assuming that the human and mouse genomes share a sequence identity of 51% under non-selective pressure (Jukes and Cantor, 1969), the single blocks were considered to be conserved because of functional constraints and are, therefore, likely to play functional roles. Based on this, the sequences of the blocks were loaded into the StampCollector program, which then determined all TF binding sites present and all possible TF pairs within the conserved blocks of the human and mouse sequences. In an further step, the results were visualized by a graphic tool and could be easily evaluated. As shown in (Fig. 5), many predicted human TF-binding sites lined up well with the binding sites of the corresponding mouse homologues. Also, the number of TF hits was again reduced compared with the Transfac database. All of the TF-binding sites for c-Fos were identified by StampCollector in this analysis. Whether any of the other TFs are directly involved in the regulation of c-Fos remains to be tested experimentally.

*Supporting evidence*

Interestingly, when we examined the functional annotations of a training Set containing 23 "best hit" genes identified in the Affymetrix experiment, we observed that seven of them encoded the transcription factors c-Fos; FosB; EGR1; ATF3; c-Jun; GKLF and CITED. With the exception of c-Fos and ATF3, none of the transcription factors had binding sites on any of the 23 genes. Binding sites for GKLFwere present in every gene with the exception of DTR (22/23). C/EBP binding sites were identified in all of the promoters except those of the JUN, ID1 and SGK genes (20/23). Cyr61 had binding sites in all promoters except for ZF5 and SP1. In addition, no ZF5 binding sites were found on the VEGF gene (21/23 and 22/23). Interestingly, GKLF was not only identified by the StampCollector program as a candidate for the regulation of this Set of genes, it was also itself one of the 23 up-regulated genes. CITED, a co-activator of GKLF, was also in the list. GKLF and CITED are particularly interesting candidates because, in addition to having binding sites in most of the up-regulated gene promoters and being up-regulated themselves in response to OA treatment, they were not up-regulated by treatment with any of the other phosphatase inhibitors which we had tested in independent GeneChip experiments (preliminary results from treatment of HEK293 cells with Calyculin A, Tautomycin, Cyclosporin A, unpublished data, not discussed in this thesis). This leads us to suggest that the role of GKLF and CITED in the regulation of these genes is specific for OA treatment.

We used MEME and MotifSampler, a Gibbs sampling algorithm, to identify over-represented cis-regulatory motifs in the 2-kb promoter region of the 23 co-expressed genes. Both established computational methods confirmed our findings that the transcription factors GKLF, C/EBP, ZF5 and SP1, identified by StampCollector, are indeed cis-regulatory motifs over-represented upon OA treatment in all tested up-regulated genes (Table 5).

## Discussion

One of the most important breakthroughs in the investigation of how protein serine/threonine phosphatases function occurred with the discovery of OA more than 15 years ago (Haystead et al., 1989). Since then, many members of different signaling pathways have been identified with the help of OA-mediated inhibition of protein serine/threonine phosphatases. While many examples in the literature associate these events with PP2A or sometimes with PP1, the existence of other OA-sensitive protein serine/threonine phosphatases like PP4, PP5 and PP6 is widely ignored (Hastie and Cohen, 1998)(Chen et al., 1994).

In this study, we used Affymetrix GeneChips to identify genes in which expression levels are regulated by 1 µM OA, which is reported to be specific for inhibition of PP2A. However, even knowing that PP1 is two orders of magnitude less sensitive to OA than PP2A and that PP4, PP5 and PP6 are less abundant in the cell, it can not entirely be assumed that the cellular effects observed are only due to inhibition of PP2A. Treating HEK293 cells with OA had mainly positive effects on transcription, which suggests that OA mainly stimulates transcription activators or inhibits transcription repressors, probably by inhibition of PP2A.

Most of the 115 genes that were up-regulated upon OA treatment in HEK293 in our study were previously not known to be transcriptionaly regulated by OA. Amongst other genes, we were able to confirm the transcriptional up-regulation of AP-1 gene members like c-Fos, FosB, c-Jun and JunB (Park et al., 1992)(Holladay et al., 1992) and other early intermediate genes such as Egr-1 and Egr-3 following OA treatment (Kharbanda et al., 1993). The fact that all genes up-regulated have diverse functions in the cell shows that OA-inhibited serine/threonine phosphatases fulfill a wide variety of cellular functions (Janssens and Goris, 2001).

In addition, we found about 20 genes that were up-regulated upon OA treatment and also by the treatment of HEK 293 cells with TPA in an unrelated experiment. Although there is contradictory evidence about the co-regulation of gene expression due to these two different treatments (Guy et al., 1992), it has been suggested that some of the effects of OA and TPA are mediated via the same pathways (Rahmsdorf and Herrlich, 1990; Wakiya and Shibuya, 1999). In concordance with this, we found Fos and Jun family

members to be up-regulated by both OA and TPA in our chip experiments. OA significantly changes the expression levels of many cytokines and genes induced by DNA damage.

About 30% of the up-regulated genes encode transcription factors, which raises the question of whether these genes or even all genes up-regulated in response to OA follow similar activation pathways. It has been reported that OA-responsive sequences in up-regulated genes, such as c-Fos, map to AU-rich sequences in combination with an unidentified protein complex (Farhana et al., 2000). With our approach, we cannot necessarily confirm these findings, but we also identified transcription factor binding sites (TFBS) that may be involved in OA-mediated up-regulation and that included AU-rich sequences, e.g. PAX-4, CDXA and C/EBP. Our data reveal that activation of transcription of distinct genes occurs in a time-dependent manner and is in most cases irreversible due to the apoptotic effect of OA.

Accurate *in silico* detection of TF-binding sites is a challenge and needs a combination of different approaches. For this reason, we developed a software tool called "StampCollector" that is able to predict potential TF pairs involved in the regulation of genes, based on their promoter sequences. One reason for creating this computational tool was that identification of TF-binding sites using the Match search program of the Transfac database results in an overwhelmingly large number of false positive TF-site hits. Despite the fact that statistical analysis can be useful in some cases (Qiu et al., 2002), we were still concerned that the high number of false positives sometimes "dilutes" the frequency of key TF sites. The program identified 11 different TF-binding sites present and over-represented in up-regulated genes and involved in the formation of 18 transcription factor pairs (Table 4). At least four of these 11 TFs are regulated by phosphorylation. C/EBP is phosphorylated by PKA and translocates to the nucleus to induce p21-mediated apoptosis (Chinery et al., 1997). The activity of SP1 is regulated by PP2A and PP1 (Garcia et al., 2000). Interestingly, Sp1 activity at the nitric oxide synthase promoter has been proposed to be regulated by a CKll and PP2A complex, indicating that phosphorylation and dephosphorylation maybe linked (Cieslik et al., 1999). VDR is regulated by DNA-PK-mediated phosphorylation (Okazaki et al., 2003), and FAC1 is dephosphorylated by an OA sensitive phosphatase (Jordan-Sciutto et al., 1999). Given the

flexibility and variability of promoter regulatory mechanisms, we decided to combine the TF pair over-representation approach of StampCollector with a comparison of human and mouse promoter regions using the DNA Block Aligner tool. Only TFs located in these blocks in both human and mouse promoters were considered for further analysis. In this way, we were able to reduce the over-represented TF pairs to regions of the promoters conserved between human and mouse. We found differences in regulatory motifs of the promoter sequences clustered according to their expression patterns.

Of the 18 TF pairs, five (GKLF, ZF5, SP1, PAX-4 and SPZ1) passed our stringent filter criteria and are good candidates for a central role in the primary transcriptional effects of OA. The upstream regulatory region of the c-Fos promoter contains two growth factor-regulated promoter elements: the serum response element, which binds a ternary complex comprising serum response factor (SRF) and a ternary complex factor (TCF); and the sis-inducible element (SIE), which binds STAT transcription factors. In addition, AP-1/ATF- and CREB-binding sites have been reported (Hill and Treisman, 1995). The cellular retinoblastoma susceptibility gene protein Rb also binds to specific *cis* elements in the c-Fos gene promoter, leading to repression of c-Fos transcription (Robbins et al., 1990). StampCollector identified all of these TF-binding sites involved in the upregulation of c-Fos in this analysis. Whether any of the additional TFs identified with StampCollector are directly involved in the transcriptional regulation of c-Fos or other upregulated genes remains to be experimentally validated.

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| SET1 OA up | 0 | 10 | 30 | 60 | 90min | common name | name |
|---|---|---|---|---|---|---|---|
| 209189_at | 1.0 | 1.1 | 1.8 | 210.5 | 994.2 | FOS | v-fos FBJ murine osteosarcoma viral oncogene homolog |
| 222315_at | 1.0 | 0.7 | 1.0 | 48.9 | 279.7 | ESTs | ESTs no further annotation |
| 210090_at | 1.0 | 0.8 | 0.9 | 30.7 | 238.6 | ARC | activity-regulated cytoskeleton-associated protein |
| 202768_at | 1.0 | 0.9 | 1.0 | 33.6 | 175.1 | FOSB, GOS3 | FBJ murine osteosarcoma viral oncogene homolog B |
| 201694_s_at | 1.0 | 0.9 | 1.8 | 18.7 | 45.0 | EGR1, TIS8 | early growth response 1 |
| 207574_s_at | 1.0 | 0.8 | 1.0 | 12.3 | 30.8 | GADD45B | growth arrest and DNA-damage-inducible, beta |
| 214349_at | 1.0 | 1.1 | 1.0 | 7.3 | 24.8 | TPARL | Homo sapiens cDNA: FLJ23438 fis, clone HRC13275. |
| 202672_s_at | 1.0 | 0.9 | 1.0 | 4.1 | 14.2 | ATF3, ATF3 | activating transcription factor 3 |
| 202912_at | 1.0 | 1.3 | 1.2 | 5.2 | 14.1 | ADM, ADM | adrenomedullin |
| 201041_s_at | 1.0 | 0.9 | 1.0 | 4.3 | 11.3 | DUSP1, HVH1, MKP-1 | dual specificity phosphatase 1 |
| 201473_at | 1.0 | 1.3 | 1.1 | 3.6 | 8.0 | JUNB, JUNB | jun B proto-oncogene |
| 222162_s_at | 1.0 | 1.0 | 0.9 | 3.6 | 6.8 | ADAMTS1 | a disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1 |
| 206115_at | 1.0 | 1.1 | 1.3 | 4.1 | 6.6 | EGR3, EGR3 | early growth response 3 |
| 201289_at | 1.0 | 1.0 | 1.1 | 2.8 | 6.5 | CYR61, IGFBP10 | cysteine-rich, angiogenic inducer, 61 |
| 209357_at | 1.0 | 0.8 | 0.9 | 3.5 | 6.0 | CITED2 | Cbp/p300-interacting transactivator, Glu/Asp-rich C-terminal domain, 2 |
| 210592_s_at | 1.0 | 0.9 | 0.9 | 2.5 | 5.2 | SAT | spermidine/spermine N1-acetyltransferase |
| 201464_x_at | 1.0 | 1.0 | 1.0 | 3.3 | 5.2 | JUN | v-jun sarcoma virus 17 oncogene homolog |
| 213931_at | 1.0 | 1.2 | 1.1 | 3.2 | 4.9 | ID2 | inhibitor of DNA binding 2, dominant negative helix-loop-helix protein |
| 210512_s_at | 1.0 | 1.3 | 1.0 | 2.9 | 4.7 | VEGF | vascular endothelial growth factor |
| 201236_s_at | 1.0 | 1.2 | 1.1 | 3.1 | 4.3 | BTG2, TIS21 | BTG family, member 2 |
| 216248_s_at | 1.0 | 1.1 | 1.0 | 3.7 | 3.8 | NR4A2 | nuclear receptor subfamily 4, group A, member 2 |
| 201502_s_at | 1.0 | 1.0 | 0.8 | 2.7 | 3.5 | NFKBIA | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha |
| 213649_at | 1.0 | 1.0 | 0.9 | 3.5 | 3.2 | SFRS7 | splicing factor, arginine/serine-rich 7, 35kDa |
| 209293_x_at | 1.0 | 1.0 | 0.9 | 2.2 | 2.6 | ID4 | inhibitor of DNA binding 4, dominant negative helix-loop-helix protein |
| 202531_at | 1.0 | 1.2 | 1.2 | 2.3 | 2.5 | IRF1 | interferon regulatory factor 1 |

| SET2 OA up | 0 | 10 | 30 | 60 | 90min | common name | name |
|---|---|---|---|---|---|---|---|
| 203821_at | 1.0 | 1.8 | 1.0 | 3.9 | 22.0 | DTR | diphtheria toxin receptor |
| 209305_s_at | 1.0 | 0.9 | 1.1 | 4.4 | 21.7 | GADD45B | growth arrest and DNA-damage-inducible, beta |
| 202014_at | 1.0 | 2.1 | 1.9 | 5.0 | 19.4 | PPP1R15A, GADD34 | protein phosphatase 1, regulatory subunit 15A |
| 201939_at | 1.0 | 0.6 | 1.4 | 4.9 | 13.3 | SNK | serum-inducible kinase |
| 221841_s_at | 1.0 | 1.0 | 1.1 | 3.1 | 12.5 | KLF4 | Homo sapiens cDNA FLJ38575 fis, clone HCHON2007046. |
| 201693_s_at | 1.0 | 0.9 | 1.3 | 3.7 | 11.3 | EGR1, | early growth response 1 |
| 219480_at | 1.0 | 1.0 | 1.1 | 2.7 | 9.1 | SNAI1 | snail homolog 1 |
| 202081_at | 1.0 | 0.9 | 1.1 | 2.9 | 8.8 | ETR101 | immediate early protein |
| 209457_at | 1.0 | 0.8 | 0.9 | 2.1 | 8.3 | DUSP5 | dual specificity phosphatase 5 |
| 210764_s_at | 1.0 | 1.3 | 1.4 | 2.8 | 6.6 | CYR61, IGFBP10 | cysteine-rich, angiogenic inducer, 61 |
| 202388_at | 1.0 | 0.9 | 0.9 | 1.9 | 6.2 | RGS2 | regulator of G-protein signalling 2, 24kDa |
| 36711_at | 1.0 | 0.9 | 1.0 | 3.3 | 6.1 | MAFF, | v-maf musculoaponeurotic fibrosarcoma oncogene homolog F |
| 220319_s_at | 1.0 | 1.0 | 1.0 | 2.2 | 5.9 | MIR | myosin regulatory light chain interacting protein |
| 208937_s_at | 1.0 | 1.0 | 0.9 | 2.1 | 5.4 | ID1 | inhibitor of DNA binding 1, dominant negative helix-loop-helix protein |
| 207980_s_at | 1.0 | 1.1 | 1.2 | 2.4 | 5.0 | CITED2 | Cbp/p300-interacting transactivator, Glu/Asp-rich C-terminal domain, 2 |
| 203394_s_at | 1.0 | 0.9 | 0.8 | 2.0 | 4.5 | HES1, HRY | hairy and enhancer of split 1, |
| 203751_x_at | 1.0 | 1.1 | 1.3 | 1.9 | 4.4 | JUND | jun D proto-oncogene |
| 212099_at | 1.0 | 1.2 | 1.1 | 2.1 | 3.9 | ARHB | Human HepG2 3' region cDNA, clone hmd1f06. |
| 217028_at | 1.0 | 0.9 | 1.0 | 1.9 | 3.9 | CXCR4 | chemokine receptor 4 |
| 218611_at | 1.0 | 1.0 | 1.0 | 2.0 | 3.8 | IER5 | immediate early response 5 |
| 213281_at | 1.0 | 1.1 | 1.3 | 2.4 | 3.4 | JUN | v-jun sarcoma virus 17 oncogene homolog |
| 211965_at | 1.0 | 1.0 | 0.8 | 2.2 | 3.0 | ZFP36L2 | zinc finger protein 36, C3H type-like 1 |
| 209212_s_at | 1.0 | 1.1 | 1.1 | 1.8 | 3.0 | KLF5, CKLF | Kruppel-like factor 5 |
| 217168_s_at | 1.0 | 1.0 | 1.0 | 1.6 | 3.0 | HERPUD1 | homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain |
| 201235_s_at | 1.0 | 0.6 | 0.6 | 2.1 | 2.9 | BTG2 | BTG family, member 2 |
| 200920_s_at | 1.0 | 0.8 | 0.9 | 1.5 | 2.9 | BTG1 | B-cell translocation gene 1, anti-proliferative |
| 201367_s_at | 1.0 | 1.1 | 1.1 | 2.2 | 2.9 | ZFP36L2 | zinc finger protein 36, C3H type-like 2 |
| 214016_s_at | 1.0 | 1.3 | 0.8 | 1.7 | 2.7 | SFPQ | splicing factor proline/glutamine rich |
| 208707_at | 1.0 | 0.9 | 0.9 | 1.6 | 2.6 | EIF5 | eukaryotic translation initiation factor 5 |
| 204622_x_at | 1.0 | 1.1 | 0.8 | 1.9 | 2.5 | NR4A2 | nuclear receptor subfamily 4, group A, member 2 |
| 215224_at | 1.0 | 0.9 | 1.4 | 1.6 | 2.3 | | Homo sapiens cDNA: FLJ21547 fis, clone COL06206. |

**Table 1:** List of genes up-regulated by OA treatment in HEK293 cells, clustered in SET1-5 according to their expression profiles by K-Means (Table 1 continues on next page).

| SET3 OA up | 0 | 10 | 30 | 60 | 90min | common name | name |
|---|---|---|---|---|---|---|---|
| 210001_s_at | 1.0 | 1.8 | 3.9 | 14.9 | 26.7 | SOCS1, SSI-1 | suppressor of cytokine signaling 1 |
| 38037_at | 1.0 | 1.8 | 2.0 | 5.9 | 16.4 | DTR, DTS | diphtheria toxin receptor |
| 201531_at | 1.0 | 1.4 | 1.6 | 4.2 | 13.8 | ZFP36, TIS11 | zinc finger protein 36, C3H type, homolog |
| 210056_at | 1.0 | 2.0 | 0.7 | 6.8 | 10.3 | RHO6 | GTP-binding protein |
| 211527_x_at | 1.0 | 1.6 | 1.6 | 9.8 | 5.8 | VEGF | vascular endothelial growth factor |
| 214696_at | 1.0 | 2.2 | 1.8 | 8.8 | 5.0 | MGC14376 | hypothetical protein MGC14376 |
| 213805_at | 1.0 | 1.3 | 1.3 | 3.0 | 4.8 | CGI-58 | CGI-58 protein |
| 204602_at | 1.0 | 1.8 | 1.3 | 2.6 | 3.7 | DKK1 | dickkopf homolog 1 |
| 204748_at | 1.0 | 0.5 | 1.0 | 4.3 | 3.6 | PTGS2 | prostaglandin-endoperoxide synthase 2 |
| 217524_x_at | 1.0 | 0.9 | 1.7 | 5.1 | 3.0 | ESTs | ESTs, Moderately similar to hypothetical protein FLJ20489 |
| 215011_at | 1.0 | 1.5 | 1.3 | 3.0 | 2.8 | RNU17D, U17HG | RNA, U17D small nucleolar |
| 203780_at | 1.0 | 1.5 | 1.7 | 2.1 | 2.2 | EVA1 | epithelial V-like antigen 1 |
| 221919_at | 1.0 | 1.3 | 1.5 | 3.5 | 1.9 | HNRPA1 | heterogeneous nuclear ribonucleoprotein A1 |
| 204291_at | 1.0 | 1.0 | 1.2 | 2.8 | 1.5 | KIAA0335 | KIAA0335 gene product |
| 218750_at | 1.0 | 1.0 | 1.1 | 2.2 | 1.4 | MGC5306 | hypothetical protein MGC5306 |
| 209451_at | 1.0 | 1.0 | 1.0 | 2.1 | 1.3 | TANK, I-TRAF | TRAF family member-associated NFKB activator |

| SET4 OA up | 0 | 10 | 30 | 60 | 90min | common name | name |
|---|---|---|---|---|---|---|---|
| 201739_at | 1.0 | 1.0 | 1.0 | 3.5 | 33.6 | SGK | serum/glucocorticoid regulated kinase |
| 201631_s_at | 1.0 | 0.9 | 0.8 | 2.1 | 10.7 | IER3 | immediate early response 3 |
| 33767_at | 1.0 | 0.2 | 0.9 | 1.3 | 10.0 | NEFH | neurofilament, heavy polypeptide 200kDa |
| 220266_s_at | 1.0 | 0.8 | 0.9 | 1.9 | 7.5 | KLF4, GKLF | Kruppel-like factor 4 |
| 203725_at | 1.0 | 1.1 | 0.9 | 1.8 | 6.4 | GADD45A | growth arrest and DNA-damage-inducible, alpha |
| 218810_at | 1.0 | 1.2 | 0.9 | 1.5 | 6.2 | FLJ23231 | hypothetical protein FLJ23231 |
| 209325_s_at | 1.0 | 1.3 | 1.1 | 1.8 | 5.8 | RGS16 | regulator of G-protein signalling 16 |
| 213988_s_at | 1.0 | 0.8 | 1.1 | 1.7 | 5.7 | SAT | spermidine/spermine N1-acetyltransferase |
| 208078_s_at | 1.0 | 1.1 | 1.0 | 1.4 | 4.8 | TCF8 | transcription factor 8 |
| 221011_s_at | 1.0 | 1.5 | 1.2 | 1.5 | 4.4 | LBH | likely ortholog of mouse limb-bud and heart gene |
| 203304_at | 1.0 | 1.0 | 0.8 | 1.3 | 3.7 | NMA | putative transmembrane protein |
| 202814_s_at | 1.0 | 1.2 | 1.0 | 1.5 | 3.5 | HIS1 | HMBA-inducible |
| 203455_s_at | 1.0 | 0.8 | 0.8 | 1.2 | 3.5 | SAT | spermidine/spermine N1-acetyltransferase |
| 202887_s_at | 1.0 | 1.0 | 0.7 | 1.1 | 3.4 | RTP801 | HIF-1 responsive RTP801 |
| 203395_s_at | 1.0 | 1.0 | 1.0 | 1.1 | 3.4 | HES1 | hairy and enhancer of split 1, |
| 209006_s_at | 1.0 | 1.1 | 1.2 | 1.6 | 3.3 | DJ465N24.2.1, | hypothetical protein dJ465N24.2.1 |
| 206724_at | 1.0 | 1.2 | 1.0 | 1.3 | 3.2 | CBX4 | chromobox homolog 4 |
| 213348_at | 1.0 | 0.9 | 1.0 | 1.1 | 3.1 | CDKN1C | cyclin-dependent kinase inhibitor 1C |
| 203068_at | 1.0 | 1.2 | 1.3 | 1.5 | 3.0 | KIAA0469 | KIAA0469 gene product |
| 212171_x_at | 1.0 | 0.9 | 1.1 | 1.5 | 2.8 | VEGF | vascular endothelial growth factor |
| 208922_s_at | 1.0 | 1.0 | 1.1 | 1.3 | 2.7 | NXF1 | nuclear RNA export factor 1 |
| 201416_at | 1.0 | 0.9 | 0.9 | 1.1 | 2.7 | SOX4 | SRY -box 4 |
| 202636_at | 1.0 | 1.1 | 1.1 | 1.4 | 2.5 | ZFP103 | zinc finger protein 103 homolog |
| 212724_at | 1.0 | 0.9 | 1.1 | 1.5 | 2.5 | ARHE | ras homolog gene family, member E |

| SET5 OA up | 0 | 10 | 30 | 60 | 90min | common name | name |
|---|---|---|---|---|---|---|---|
| 206935_at | 1.0 | 1.6 | 4.2 | 1.8 | 20.8 | PCDH8 | protocadherin 8 |
| 203140_at | 1.0 | 2.4 | 1.4 | 1.7 | 5.6 | BCL6 | B-cell CLL/lymphoma 6 |
| 218559_s_at | 1.0 | 1.1 | 1.0 | 1.1 | 4.5 | MAFB | v-maf musculoaponeurotic fibrosarcoma oncogene homolog B |
| 209201_x_at | 1.0 | 1.1 | 1.1 | 0.7 | 3.3 | CXCR4 | chemokine receptor 4 |
| 209007_s_at | 1.0 | 0.6 | 0.8 | 0.9 | 3.3 | DJ465N24.2.1 | hypothetical protein dJ465N24.2.1 |
| 209803_s_at | 1.0 | 0.9 | 1.0 | 1.0 | 3.2 | TSSC3 | tumor suppressing subtransferable candidate 3 |
| 210425_x_at | 1.0 | 1.2 | 1.2 | 1.0 | 3.1 | GOLGIN-67 | golgin-67 |
| 212501_at | 1.0 | 1.0 | 0.9 | 1.1 | 3.1 | CEBPB | CCAAT/enhancer binding protein , beta |
| 221768_at | 1.0 | 1.2 | 1.5 | 1.1 | 3.0 | SFPQ | Homo sapiens cDNA FLJ38383 fis, clone FEBRA2003726. |
| 219142_at | 1.0 | 1.0 | 1.2 | 1.2 | 2.9 | MGC2827 | hypothetical protein MGC2827 |
| 202284_s_at | 1.0 | 1.2 | 0.9 | 1.2 | 2.9 | CDKN1A, P21, CIP1 | cyclin-dependent kinase inhibitor 1A |
| 202815_s_at | 1.0 | 1.0 | 0.9 | 1.1 | 2.8 | HIS1 | HMBA-inducible |
| 220306_at | 1.0 | 1.0 | 1.1 | 0.9 | 2.8 | FLJ20202 | hypothetical protein FLJ20202 |
| 204286_s_at | 1.0 | 1.1 | 0.8 | 1.1 | 2.7 | PMAIP1 | phorbol-12-myristate-13-acetate-induced protein 1 |
| 218012_at | 1.0 | 1.1 | 0.8 | 1.0 | 2.6 | SE20-4, CDA1, CTCL | cutaneous T-cell lymphoma-associated tumor antigen se20-4 |
| 204379_s_at | 1.0 | 1.1 | 1.1 | 0.9 | 2.4 | FGFR3 | fibroblast growth factor receptor 3 |

**Table 1 continued:** List of genes up-regulated by OA treatment in HEK293 cells, clustered in SET1-5 according to their expression profiles by K-Means.

| OA down | 0 | 30 | 60 | 90min | common name | name |
|---|---|---|---|---|---|---|
| 216855_s_at | 1.0 | -1.3 | -2.4 | -1.1 | HNRPU | Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A) |
| 213119_at | 1.0 | -1.1 | -2.2 | -1.1 | cDNA: FLJ21449 | wx23h08.x1 NCI_CGAP_Kid11 Homo sapiens cDNA IMAGE:2544543 3', |
| 204766_s_at | 1.0 | -1.2 | -1.2 | -2.0 | NUDT1 | Nudix (nucleoside diphosphate linked moiety X)-type motif 1 |
| 205967_at | 1.0 | -1.2 | -2.0 | -1.3 | H4FG | H4 histone family, member G |
| 202979_s_at | 1.0 | -1.0 | -1.6 | -2.1 | ZF | HCF-binding transcription factor Zhangfei |

**Table 2:** List of genes down-regulated by OA treatment in HEK293 cells

| | OA | TPA | common name | name |
|---|---|---|---|---|
| 209189_at | 994.2 | 44.1 | FOS | v-fos FBJ murine osteosarcoma viral oncogene homolog |
| 202768_at | 175.1 | 31.2 | FOSB, GOS3 | FBJ murine osteosarcoma viral oncogene homolog B |
| 201694_s_at | 45.0 | 20.9 | EGR1, TIS8 | early growth response 1 |
| 202672_s_at | 14.2 | 1.8 | ATF3 | activating transcription factor 3 |
| 201041_s_at | 11.3 | 2.6 | DUSP1, HVH1, MKP-1 | dual specificity phosphatase 1 |
| 201631_s_at | 10.7 | 2.2 | IER3 | immediate early response 3 |
| 202081_at | 8.8 | 2.7 | ETR101 | immediate early protein |
| 201473_at | 8.0 | 4.7 | JUNB | jun B proto-oncogene |
| 222162_s_at | 6.8 | 2.5 | ADAMTS1 | a disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1 |
| 206115_at | 6.6 | 22.4 | EGR3 | early growth response 3 |
| 210764_s_at | 6.5 | 3.4 | CYR61, IGFBP10 | cysteine-rich, angiogenic inducer, 61 |
| 36711_at | 6.1 | 5.3 | MAFF | v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian) |
| 209325_s_at | 5.8 | 2.2 | RGS16 | regulator of G-protein signalling 16 |
| 201466_s_at | 5.2 | 2.0 | JUN | v-jun sarcoma virus 17 oncogene homolog (avian) |
| 210592_s_at | 5.2 | 1.7 | SAT | spermidine/spermine N1-acetyltransferase |
| 201236_s_at | 4.3 | 1.7 | BTG2, TIS21 | BTG family, member 2 |
| 212099_at | 3.9 | 1.5 | ARHB | Human HepG2 3' region cDNA, clone hmd1f06. |
| 202887_s_at | 3.4 | 1.5 | RTP801 | HIF-1 responsive RTP801 |
| 209803_s_at | 3.2 | 2.2 | TSSC3, | tumor suppressing subtransferable candidate 3 |
| 212724_at | 2.5 | 1.5 | ARHE | ras homolog gene family, member E |

**Table 3:** Genes jointly up-regulated by OA and TPA. Maximum fold change at either 10, 30, 60 or 90 min is indicated

| | chip | up | TF class | function |
|---|---|---|---|---|
| **GKLF** | P | 2.7x | Zn | Growth arrest and cell cycle regulation |
| **C/EBP** | P | 3.3x | ZIP | Regulation of proliferation, differentiation and apoptosis Binds to AP-1 sites and heterodimerizes with Fos/Jun, interacts with SP1 |
| **PAX-4** | A | - | HTH-PD | Regulates pancreatic cell differentiation and development, represses glucagen expression |
| **SP1** | P | 2.5x | Zn | Ubiquitous factor, PP2A activates SP1. It's reversed in GeneChip!! OA induces SP1 driven gene expression |
| **ZF5** | P | 2.2x | Zn | Activator and repressor |
| **GEN_INI** | - | - | - | General initiator sequence, surrounding TSS, bound by general TFs |
| **SPZ1** | - | - | HLH-ZIP | Testis specific. Role in spermatogenesis by regulating proliferation and differentiation |
| **VDR** | A | - | Zn | VDR binding site includes AP-1 site |
| **FAC1** | P | - | Zn | Repressor, role in neural development/degeneration |
| **MSX-1** | P | 2.4x | HTH-HD | Activator of development and differentiation, also repressor, since interaction with proteins from core transcription complex (TBP, TFIIA) |
| **CDXA** | - | - | HTH-HD | - |

**Table 4:** Transcription factors involved in TF pair formation upon okadaic acid (OA) treatment of HEK 293 cells. Present (P) or absent (A) call from chip data is included as well as TF class and the known function of the TF.

| MotifSampler | MEME | TFBS |
|---|---|---|
| CCCGCGCs | - | AP2 |
| TGTGTrTn | - | - |
| GCsGCCGC | - | ZF5, SP1 |
| **GGGCGGGG** | **GGGGGCGGGG**<br>**T A A A** | **Sp1, VDR (100%), ZF5, SPZ1** |
| **TnATTwTT** | **TTATTATT**<br>**ATA T** | **PAX-4, CDXA, HNF** |
| - | TTTTTTTTTTCG<br>GG C | PAX-4, C/EBP, FAC1, HNF3 |
| - | AGAAAAAAAA<br>AG G | PAX-4, C/EBP, FAC1, GKLF, Oct-1 |
| - | TTTAAAAA<br>A T T | MEF-1, C/EBPg, CDC5 |

**Table 5:** Transcription factor binding sites identified using MEME and MotifSampler bioinformatic tools.

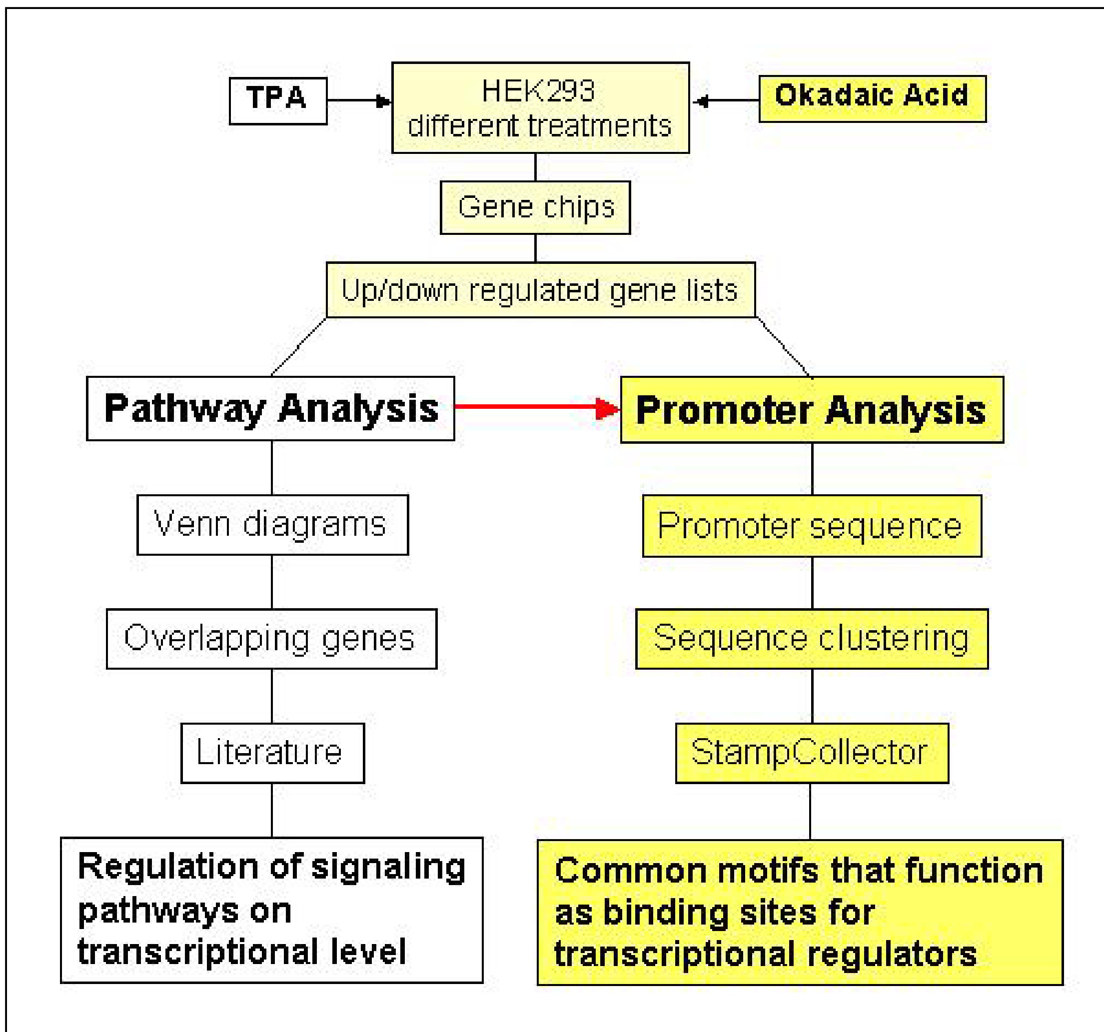



**Fig 1:** Flowchart of data analysis

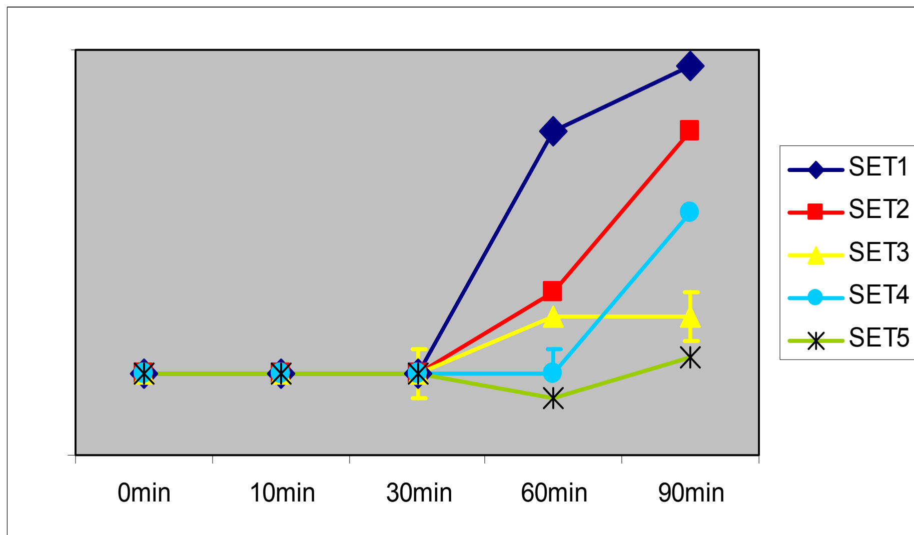



**Fig. 2**: Trend of expression profiles for K-Means clustered OA up-regulated genes

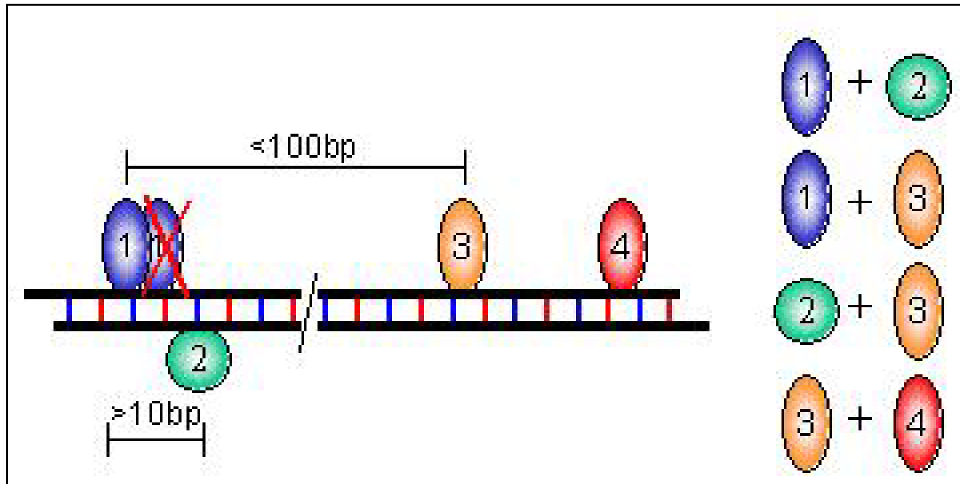



**Fig. 3:** Functional principle of the StampCollector program to predict potential transcription factor pairs involved in the regulation of genes based on their promoter sequences

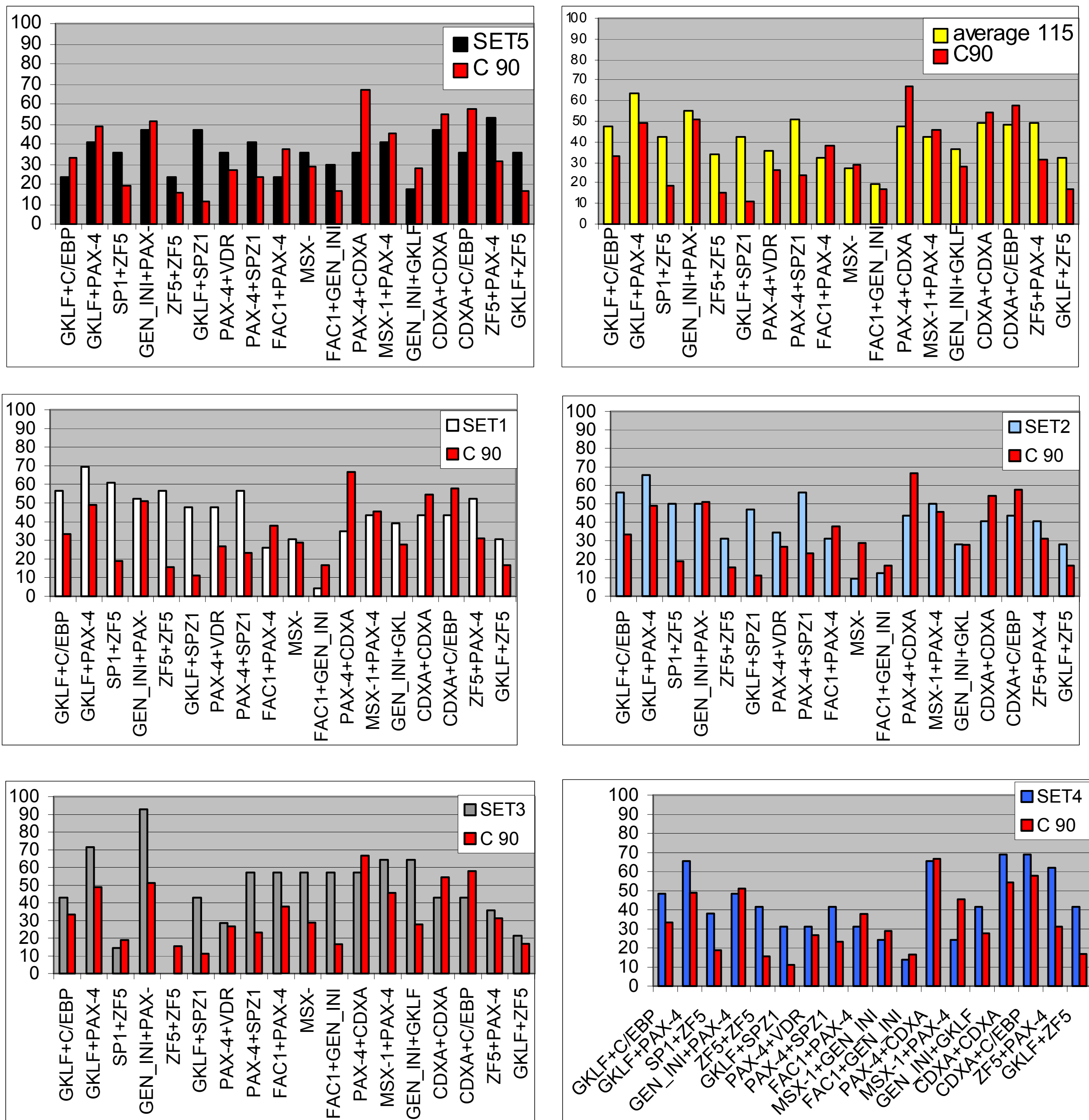
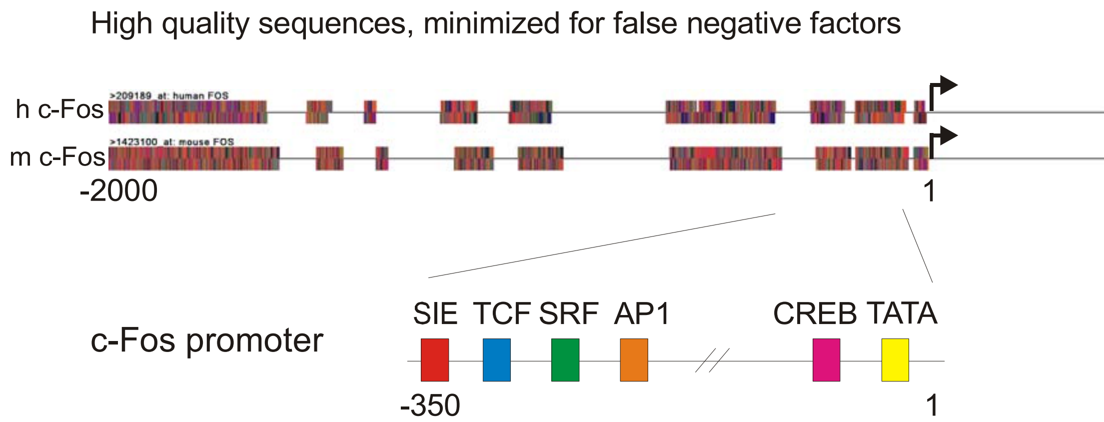



**Fig. 4:** Over-representation of TF pairs in SET1-5 compared with the control set.



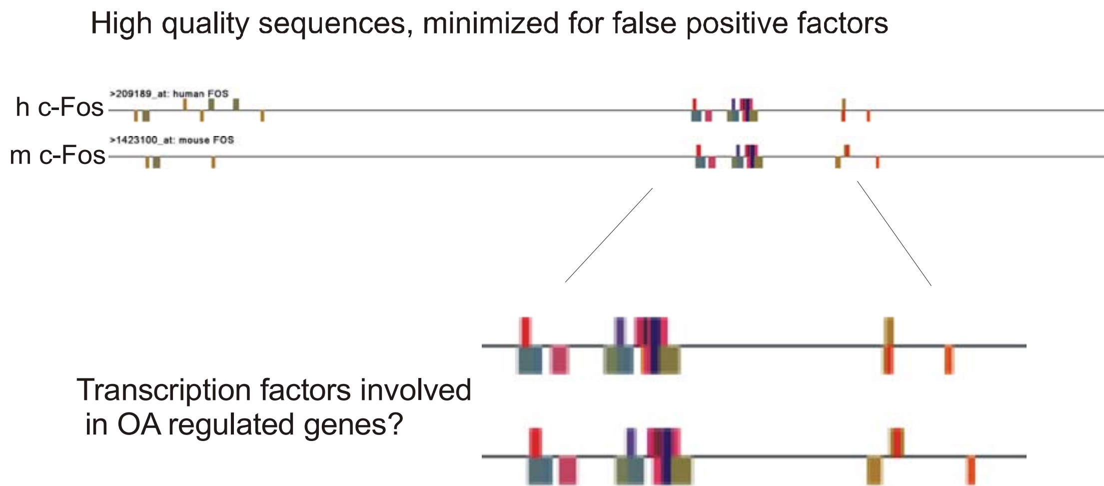




**Fig. 5:** Different sensitivities used for StampCollector program to predict transcription factos on c-Fos promoter that may play a regulatory role upon OA treatment of HEK293 cells.

## Abbreviations

| | |
|---|---|
| ARE | AU-rich element |
| CDXA | Caudal homolog homeoboxprotein |
| C/EBP | CCAAT/enhancer binding protein |
| CREB | c-AMP responsive element binding protein |
| DUSP | Dual specific phosphatase |
| Egr | Early growth response |
| ERK1/2 | Extracellular signal-related kinase1/2 |
| FAC-1 | Fetal Alzheimer antigen |
| GADD45B | Growth arrest and DNA damage-induced 45B |
| GEN_INI | General initiator sequence |
| GKLF | Krueppel-like factor |
| IkB-α | Inhibitor of NFkB |
| IL | Interleukin |
| MAPK | Mitogen-activated protein kinase |
| MEK1/2 | MAP/ERK kinase 1/2 |
| MSX | Homeodomain TF |
| NDR | Nuclear Dbf2-related |
| NFkB | Nuclear factor kappa B |
| NGF | Nerve growth factor |
| OA | Okadaic acid |
| PAX-4 | Paired domain, paired box/homeodomain |
| PI3K | Phosphatidylinositol 3'-kinase |
| pRb | retinoblastoma protein |
| SOCS1 | Suppressor of cytokine signaling 1 |
| SP1 | Simian-virus-40-protein1 |
| SPZ1 | Spermatogenic Zip |
| TF | Transcription factor |
| TFBS | Transcription factor binding site |
| VDR | Vitamin-D receptor |
| VEGF | Vascular and endothelial growth factor |
| ZF5 | Zink finger protein |

# VII. General Discussion

Protein phosphatase 2A (PP2A) is one of the major multi-subunit serine/threonine protein phosphatases found in eucaryotic cells. It exerts pleiotropic effects on various cellular processes and has been shown to be involved in the regulation of growth and metabolism, signal transduction, cell cycle progression, cell transformation, DNA replication and transcription, RNA splicing and translation and neuronal development (reviewed in (Janssens and Goris, 2001)). Defective or inappropriate function of PP2A may lead to deregulation of signal transduction and in the worst-case scenario to the development of diseases such as diabetes, cancer or immune dysfunction. Altered interaction or dysfunction of PP2A in the brain may also lead to neurodegenerative disorders such as Alzheimer's disease (AD) (Tian and Wang, 2002).

Given the numerous cellular processes involving PP2A, we developed a method that yields over-expression of high levels of active PP2Ac for the investigation of active-site residues invariant for the catalytic function of PP2Ac. PP2Ac shares many invariant residues with the PP1 and PP2B catalytic subunits and some active-site residues with bacteriophage λ phosphatase (λPPase), suggesting that these enzymes share a common catalytic mechanism (Barford et al., 1998; Huang et al., 1997; Zhuo et al., 1994). Mutation of two of these sites (D88N and H118N) resulted in a dramatic reduction in phosphatase activity *in vitro* and *in vivo*, which is in concordance with functional analysis of yeast, where the substitutions abolished *in vivo* function (Ogris et al., 1999a; Ogris et al., 1999b). One aim of this study was to establish a method producing over-expression of recombinant protein in insect cells at a level significantly higher than that in mammalian cell lines. Detailed analyses of the structural and functional aspects of catalysis by PP2A are rare, due to the fact that over-expression in mammalian cells is restricted to endogenous levels by a transcriptional (Chung and Brautigan, 1999) and/or translational autoregulatory mechanism (Baharians and Schonthal, 1998). Mutating the active-site residues $Asp^{88}$ and $His^{118}$ to the neutral amino acid asparagine yielded substantially higher levels of mutant HA–PP2Ac than native recombinant HA–PP2Ac. Taken together, these observations demonstrate that the human $Asp^{88}$ and $His^{118}$ residues perform a key, phylogenetically conserved role in the catalytic mechanism of PP2Ac. Furthermore, we

suggest that the regulation of PP2Ac expression is controlled by its phosphatase activity and may be due to mechanisms outlined (Baharians and Schonthal, 1998; Chung et al., 1999). This method will also be a useful tool for generating large amounts of biologically active PP2Ac protein, which can then be used for resolving the crystal structure and further functional analysis.

In its active *in vivo* complex, the catalytic subunit of PP2A is bound to the regulatory A subunit and to one of the variable B regulatory subunits. About 75 different holoenzymes can be theoretically generated, given that there are two C, two A, four B, at least eight B', four B'' and two B''' isoforms. The fact that we identified two novel splice variants for mouse PR55/Bβ (Schmidt et al., 2002) and that there are additional splice variants for other B' regulatory subunits clearly shows that the variability of the PR55/B family, and consequently for the PP2A trimer, is even greater than previously thought (McCright et al., 1996). We were also able to show that the brain- and testis-specific PR55/Bβ isoform exerts a distinct function during mouse brain development, since transcripts of other isoforms were not detected during embryogenesis. Interestingly, the main PP2A complex in the brain is ABαC and it probably performs general cellular functions, in contrast to PR55/Bβ and Bγ. PR55/Bα- and Bβ-containing trimers have been reported to be involved in the association with the tau protein, which in its hyperphosphorylated form is involved in the development of Alzheimer's disease (Billingsley and Kincaid, 1997; Goldbaum et al., 2003). Neither the PR55/Bγ nor the PR61/B' regulatory subunits are involved in the association with the tau protein or microtubules (Price et al., 1999). A distinct function of the brain-specific PR55/Bγ in promoting differentiation in neuronal cells has been suggested recently (Strack et al., 2002). Our *in situ* hybridization and immunohistochemical analysis of murine brains showed all PR55/B subunits to be widely distributed in brain, with regional differences in expression levels. In concordance with this, significant differences in distribution at the protein level have been found in the cerebellum, an area reported to be involved in a spinocerebellar ataxia (Costa Lima and Pimentel, 2004). This disease, called SCA12, is probably caused by a CAG repeat in the non-translated region of the PR55/Bβ gene (Holmes et al., 2001; Holmes et al., 1999). In addition, the distribution of the PR55/Bα and Bβ subunits also varies in the cortex and hippocampus, areas prominently affected in AD brains. Interestingly, we identified high

levels of PR55/Bα staining in activated astrocytes, which suggests a role for this isoform in AD, since astrocyte activation is an early step in the pathogenesis of AD and related disorders (Kurosinski and Gotz, 2002).

Taken together, the results presented in this part of the thesis provide insights into the regulation and distribution of the PR55/B regulatory subunits in mouse brain, which may be extremely valuable for understanding the biological role of this subunit family in the overall function of PP2A. These results will help in the interpretation of the phenotypes of transgenic or future knockout mice (Gotz and Schild, 2003) that are altered or deficient in various PP2A subunits.

A useful tool in studying cellular functions of PP2A is the tumor promoter OA. As already mentioned, the effects of OA can not be entirely attributed to the inhibition of PP2A; even though PP1 and PP2B are not affected at this concentration, the less abundant PP4, PP5 and PP6 are probably affected (Fernandez et al., 2002). For characterizing the effects of inhibitors on a distinct protein phosphatase, it would be extremely helpful to know the crystal structure of each protein phosphatase and so to identify unique binding interactions. To date, only PP1 (Egloff et al., 1995; Goldberg et al., 1995), PP2B (Kissinger et al., 1995) and PP2C (Das et al., 1996) have been characterized. Characterization of the other members will not only increase our understanding of the function of the different phosphatases and their inhibitors, but will also allow development of specific and selective phosphatase inhibitors and provide deeper insights into the regulation of phosphatase activity.

Given that all diseases involve deficiencies in cellular signaling, it is not surprising that protein kinases and protein phosphatases have become targets for the design of novel therapeutic tools (Dancey and Sausville, 2003). Understanding of the role of protein phosphatases in these systems, however, is behind that of protein kinases, but it is already apparent that the role of protein phosphatases is just as complex and elegant as that of protein kinases. Consequently, efforts have increased to elucidate the biological roles of protein phosphatases and, thus, their potential medical implications (Honkanen and Golden, 2002). The development of type-specific protein phosphatase inhibitors has become an interesting task, since they are believed to be extremely useful research tools with potential for development as novel therapeutic agents (McCluskey et al., 2002). In

the case of PP2A, several inhibitors have been described that are more or less specific (Sheppeck et al., 1997). The most prominent of these is the tumor-promoter okadaic acid (OA), whose ability to inhibit protein serine-threonine phosphatases in a dose-dependent manner has been proven to be extremely valuable in the investigation of major signaling events. One crucial disadvantage of OA is that it induces tumor formation in a mouse skin two-stage model (Suganuma et al., 1988). This almost immediately disqualifies it for therapeutical use in patients, since no physician wishes to apply a treatment that produces more harm than good. Another drawback of OA is that it equally inhibits minor abundant protein phosphatases like PP4, PP5 and PP6 (Cohen, 1997). This complicates the development of a specific phosphatase inhibitor for clinical use, because from a drug development point of view the target for development remains uncertain. It is also important to remember that a potential drug must distinguish between different isoforms of the phosphatase. Given that there are almost always multiple phosphatase complexes in a cell, this may complicate the issue dramatically. In some cases, for example that of AD, inhibition of PP2A may even promote development of the disease it restrains the level of tau phosphorylation. Thus, therapeutic use of such an inhibitor would be counterproductive (Gong et al., 1995). The use of a PP2A inhibitor as an anti-cancer agent also seems counterintuitive, since PP2A is considered to be a negative regulator of the cell cycle and would, therefore, stimulate rather than inhibit cell growth (Ghosh et al., 1996). Nevertheless, some inhibitors such as cyclosporine A (Kosch et al., 2003), fostriecin (de Jong et al., 1999) or cantharidin (Langley et al., 2003; Sakoff et al., 2002; Wang, 1989) have been used clinically to treat patients, since they are immunosuppressive or are toxic for a wide range of cancer cell types.

Even if OA is not suitable for the *in vivo* treatment of patients, it is still one of the most valuable tools for investigating cellular mechanisms mediated (in part) by PP2A in cell culture. We treated HEK293 cells with OA in a GeneChip approach using microarray and bioinformatics tools to define the common transcriptional events induced in genes that are up-regulated in response to OA. We identified both novel and known target genes for OA/PP2A (Schonthal, 1995). Whereas about 115 genes were up-regulated >2-fold, only two genes passed the >2-fold down-regulated restriction, which suggests that OA exerts activating effects on transcription activators and/or suppressing functions on transcription

suppressors by inhibiting PP2A. We found that about 30% of the up-regulated genes encode transcription factors such as the AP-1 members c-Fos and c-Jun. This finding is in concordance with earlier reports demonstrating that OA induces expression of c-Fos and c-Jun proto-oncogenes in human breast cancer cells (Kiguchi et al., 1992) and increases activity (Rosenberger et al., 1999). This effect is reported to be shared by other tumor promoters such as TPA (Rosenberger and Bowden, 1996) and was confirmed by our findings in an independent GeneChip experiment. Activation of the c-Fos gene is regulated through reversible phosphorylation of the ternary complex factor (TCF) by kinases of the MAPK family, and OA stimulates TCF via activation of the MAPK pathway (Schonthal, 1998). In addition, it was shown that tumor promotion by OA is due to increased AP-1 DNA transactivation mediated by c-Jun hyperphosphorylation (Peng et al., 1997). This and other reported evidence makes it very likely that the effect of OA on AP-1 family of transcription factors is due to the inhibition of PP2A.

As discussed above, OA exerts its function through modulating regulatory mechanisms like dephosphorylation of the TCF complex that is responsible for activating c-Fos. One aim of this project was to investigate such regulatory events arising from OA treatment of HEK293 cells. Therefore, we developed a software tool we named "StampCollector" that allowed us to test established mechanisms and, based on their promoter sequences, to predict potential transcription factor (TF) pairs involved in the regulation of genes. The major challenge in developing *in silico* tools is the filtering of false positive and false negative results to give reliable readout. This is certainly a drawback of the Transfac database, a commercially available tool that includes the highest number of transcription factors and their putative binding sites. Use of the Match search program of the Transfac database resulted in an overwhelmingly high number of false positive TF site hits, which we attempted to reduce by combining the TF pair over-representation approach of StampCollector with a comparison of human and mouse promoter regions using the DNA Block Aligner tool. We used c-Fos as a training gene for StampCollector since its promoter region is well characterized (Hill and Treisman, 1995). We were able to confirm all regulatory elements for c-Fos reported in the literature and, in addition, we identified 11 transcription factors forming 18 different TF combinations that seem to be important for OA-mediated up-regulation of different genes in HEK293 cells. Whether

any of these TF pairs play an important role in transcriptional regulation remains to be tested experimentally.

In conclusion, the work presented in this thesis is in many ways comprehensive for the understanding of "how PP2A does its job" or, in more scientific terms, the multiple functions that PP2A and in particular its B regulatory subunits exert in all major signal transduction pathways.

In the first part of this thesis, we set the course for structural and functional analysis of the catalytic subunit of PP2A with the study "Active-site mutations impairing the catalytic function of the catalytic subunit of human protein phosphatase 2A permit baculovirus-mediated over-expression in insect cells". In addition to the new insights gathered by this work, structural biologists will also profit from the devised method that allows over-expression of biologically active protein sufficient for crystallizing this extremely important molecule.

Investigating the "Diversity, developmental regulation and distribution of murine PR55/B subunits of protein phosphatase 2A" clearly indicates that PP2A is a global player at the cellular level and that the deregulation or altered function of the phosphatase on any level can lead to severe disorders, such as Alzheimer's disease or related neurodegenerative disorders. This shows how important it is to accomplish a detailed functional analysis of PP2A.

In the third part of this thesis, we tried to pursue this challenge by defining the "Transcriptional effects of protein phosphatase 2A inhibitor okadaic acid on promoter complexes using Affymetrix GeneChips". With this approach, we underline the importance of the PP2A inhibitor OA in the regulation of transcriptional effects prior to or based on the activation or suppression of signaling events.

Considering the putative role of PP2A in the pathogenesis of human disease, all these results will at least help to understand the molecular mechanisms involved and may eventually lead to the discovery of therapeutic agents that specifically target PP2A dysfunction.

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# VIII. Appendix

My aim was to dissect the function of the PR55/B family of subunits using a gene knock out strategy in mice. The project is still ongoing with our collaborator Juergen Goetz in Zurich, but was not presented in this thesis due to the lack of homologous recombination of our targeting constructs in ES cells for all PR55/B isoforms. The strategy will be described briefly for further attempts.

*Characterization of Murine PR55/B BAC Clones*

cDNAs for the murine PR55/Bα, Bβ, Bβ.1, Bβ.2, Bγ and Bδ were cloned or obtained from various libraries (described in Results: "Diversity, developmental regulation and distribution of murine PR55/B subunits of protein phosphatase 2A"). PR55/Bα, Bβ.1 and Bγ cDNAs were digested with the corresponding enzymes in order to release the full-length insert. The PR55/Bα insert (2.3kb) was released with EcoRI/NotI, PR55/Bβ.1 (1.8kb) with NotI/XhoI and PR55/Bγ (2.5kb) with HindIII/BamHI. The released and gel-purified full-length inserts of PR55/Bα, Bβ and Bγ were used to screen a mouse BAC library constructed from 129/SvJ genomic DNA (Incyte Genomics). We received three PR55/Bα BAC clones (GS control number 23175-23177), and one each for PR55/Bβ (23029) & Bγ (22922). The PR55/Bγ BAC clone was sent to J. Goetz (University of Zurich) for further characterization. The PR55/Bα and Bβ.1 BAC clones were digested with a set of restriction enzymes for Southern blotting and hybridisation with $P^{32}$-labelled cDNA probes. Hybridization was performed with the entire cDNA labeled as well as with exon specific cDNA fragments obtained from PCR amplification (e.g. E1-4, E3-5) for verification of the completeness of the BAC clones. A BAC sub-library was screened in order to establish the genomic structure of the PR55/B isoforms. Positive clones were identified by filter hybridization with the corresponding $P^{32}$-labelled cDNA fragments. A 14kb PR55/Bα sub-clone was sequenced to verify the exon/intron boundaries, as well as the genomic structure between exon 3 and exon 9. The full genomic structures for all PR55/B genes were assembled by screening the genomic databases (Ensembl and Celera).

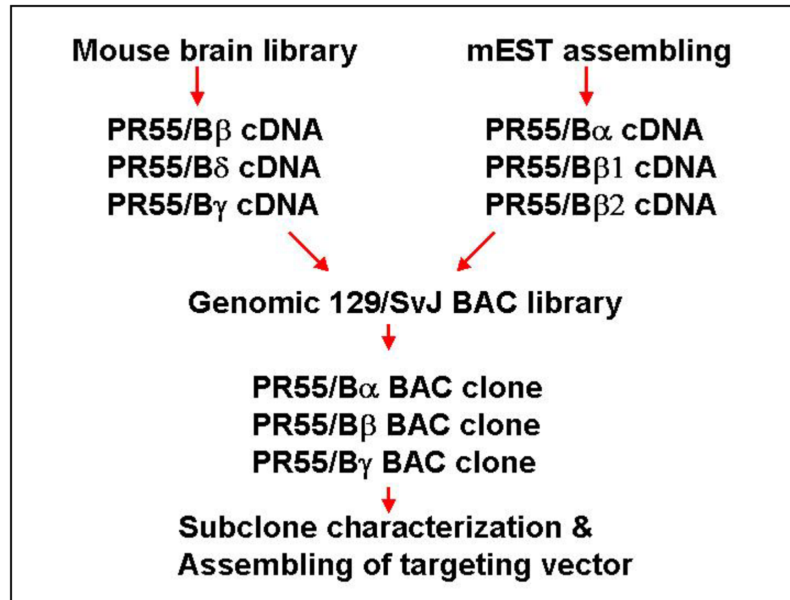


***Fig. A:*** *Cloning and screening process for assembling of PR55/B targeting vectors*

*Targeting constructs for PR55/B isoforms*

A knockout targeting vector was assembled from the 14kb PR55/Bα subclone encompassing 9kb of genomic PR55/Bα sequence with exons 4 to 6 replaced by a neomycin-resistance cassette. In addition, a PR55/Bα control vector was generated to establish PCR conditions for the ES-cell screen. The PR55/Bβ vector was cloned by direct amplification from the PR55/Bβ BAC clone and encompassed 11.4kb genomic sequence. The IRES/lacZ/NEO cassette replaced 5kb of genomic sequence including parts of exon 4 and exon 5. A knock-in approach was used for the complete disruption of the PR55/Bγ gene. A targeting vector was assembled encompassing exon 2 to exon 5 with a stop-codon introduced into exon 3 to prevent alternative splicing. In addition, intron 3 was replaced by a neomycin/thymidine kinase (TK)-resistance cassette.

| PR55/Bα | PR55/Bβ | PR55/Bγ |
|---|---|---|
| 500 ES cell screened by PCR<br>400 ES cell screened by PCR and Southern blot<br>→ no homologous recombination<br>→ new strategy, new vector | 500 ES cell screened by PCR and Southern blot<br>→ no homologous recombination<br>→ new strategy, new vector | 1400 ES cell screened by PCR<br>→ 2 positive clones<br>→ chimeras don't go germ line<br>→ new ES screens, new strategy |

**Table A:** Status of the screening process

.

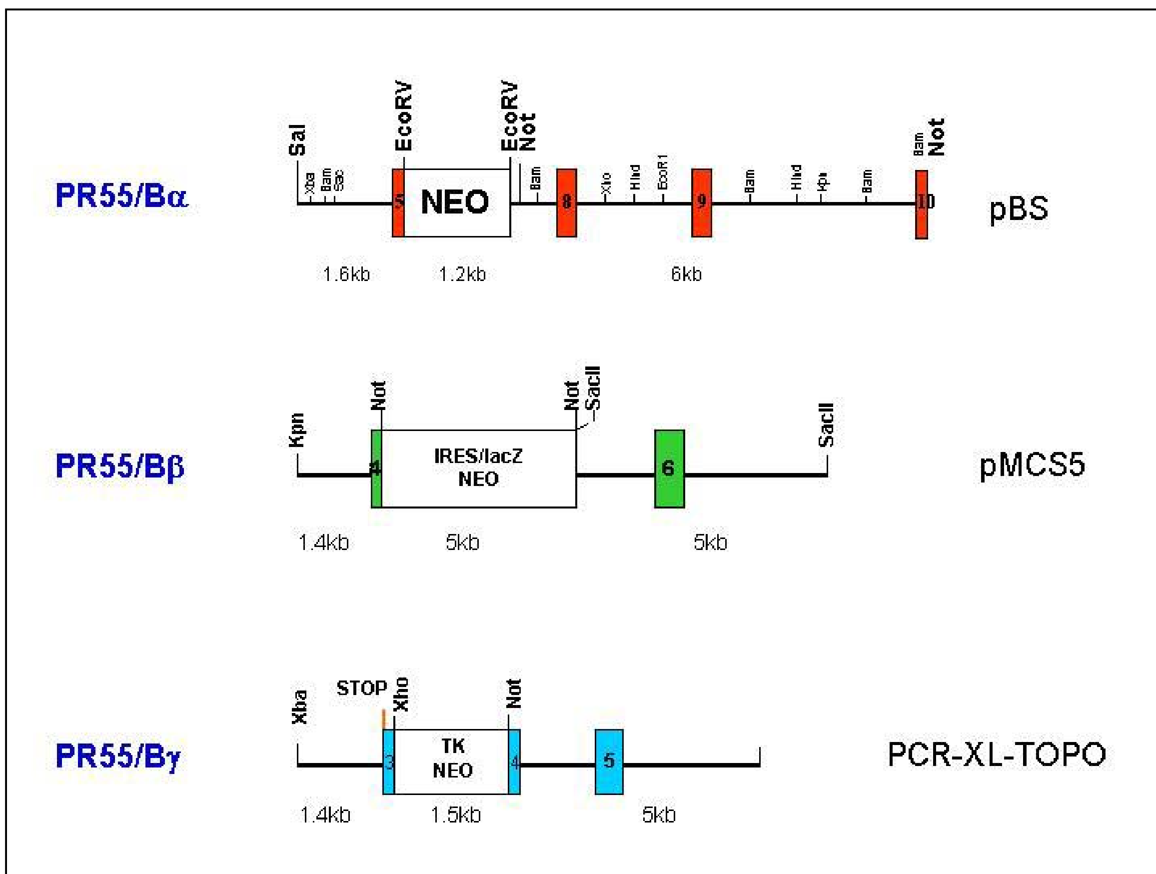


**Fig B:** Assembled PR55/B targeting vectors.

# IX. Curriculum Vitae

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Nationality: German

Marital Status: Single

Languages: German and English

## Education

1999- 2004 International Ph.D. Program, laboratory of Dr. Brian A. Hemmings, at the Friedrich Miescher Institute, Basel, Switzerland. Research area: Structure and function analysis of Protein Phosphatase 2A

1992-1997 Master of Science in Food Technology, University of Applied Sciences Trier, Germany,

1986-1989 Comprehensive Secondary School for Science, Ludwigshafen, Germany.

## Professional Experience

| | |
|---|---|
| 1997- 1999 | Research Assistant in the mass spectrometry laboratory of Dr. Anthony G. Craig, department of Dr. Jean E.F. Rivier, the Clayton Foundation Laboratories for Peptide Biology at the Salk Institute, San Diego, CA, USA. |
| 1996- 1997 | MS thesis in the mass spectrometry laboratory of Dr. Anthony G. Craig, department of Dr. Jean E.F. Rivier, the Clayton Foundation Laboratories for Peptide Biology at the Salk Institute, San Diego, CA, USA. |
| 1989- 1992 | Chemical laboratory assistant in the biotechnology department of BASF AG, Ludwigshafen, Germany |
| 1986- 1989 | Apprenticeship as chemical laboratory assistant with BASF AG, Ludwigshafen, Germany |

## Technical skills

**Molecular biology**: Microarray analysis of gene expression, siRNA technology, quantitative real-time-PCR, genomic & cDNA cloning, SDS-PAGE, immuno blotting, Southern blotting, and immuno-precipitation

**Protein biochemistry:** Peptide purification, protein sequencing, gel chromatography, and HPLC

**Cell biology:** Cell culture, recombinant protein expression, insect cell culture (baculovirus expression system), and transient gene expression

***in vivo* work:** Gene targeting, injections, and biopsies

**Mass spectrometry**: Phospho-peptide & glycoprotein MS analysis using MALDI-TOF & ESI-iontrap mass spectrometey

## Meeting Presentations

| | |
|---|---|
| 2002 | EU Grant Meeting, Disease Insights from Single Cell Signaling<br>Engelberg, Switzerland<br>Oral presentation: Dissecting the function of PP2A regulatory PR55/B subunits |
| 2001 | EMBO Conference: Protein Phosphorylation and Protein Phosphatases, University of Marburg, Germany<br>Poster presentation: Dissecting the function of PP2A regulatory PR55/B subunits |
| 2001 | Biomed 2 Program Meeting, Protein Phosphorylation and Cancer, University of Vienna, Austria<br>Oral presentation: Targeted disruption of the PP2A regulatory PR55/B subunits |
| 2000 | Biomed 2 Program Meeting, The Regulation of Protein Phosphatase 2A in Signal Transduction, Cell Cycle and Malignant Transformation, University of Leuven, Belgium<br>Oral presentation: Protein phosphatase 2A: knocking out the PR55 genes |

# X. Publications

**K. Schmidt**, S. Kins, A. Schild, R. Nitsch, B. A. Hemmings and J. Goetz
Diversity, developmental regulation and distribution of murine PR55/B subunits of protein phosphatase 2A.
Eur. J. Neurosci. 2002;16(11):2039-2048

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S. W. Sutton, M. Akhtar, **K. Schmidt**, W. H. Fischer, W. W. Vale and A. G. Craig
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A. G. Craig, T. Norberg, D. Griffin, C. Hoeger, M. Akhtar, **K. Schmidt**, W. Low, J. Dykert, E. Richelson, V. Navarro, J. Mazella, M. Watkins, D. Hillyard, J. Imperial, L. J. Cruz, and B. M. Olivera.
Contulakin-G, an O-glycosylated invertebrate neurotensin
J. Biol. Chem. 1999, 274, 20: 13752-13759

## Manuscript in preparation

**K. Schmidt**, E. J. Oakeley, Viktor Zhang, Herbert Angliker and B. A. Hemmings
Transcriptional effects of the protein phosphatase inhibitor okadaic acid on promoter complexes using Affymetrix GeneChips