Signalling by Teneurin-1

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ii. ABBREVIATIONS

aa	amino acids
AEBSF	aminoethyl-benzene sulfonyl fluoride
ApoER2	apolipoprotein E receptor 2
BSA	bovine serum albumine
B. subtilis	*Bacillus subtilis*
BMP	bone morphogenetic protein
C. difficile	*Clostridium difficile*
DCC	deleted in colorectal cancer
EGF	epidermal growth factor
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
FLten-1	full-length teneurin-1
HRP	horseradish peroxidase
IDten-1	intracellular domain of teneurin-1
IPTG	iso propyl-β-D-thiogalactoside
MLten-1	medium-length teneurin-1
MW	molecular weight
Nb2a	mouse neuroblastoma cells
NSF	*N*-ethyl maleimide sensitive factor
odz	odd oz
opa	odd-paired
p75NTR	p75 neurotrophin receptor
SH3	sarc homology 3 domain
TGF	transforming growth factor
TNFα	tumour necrosis factor alpha

iii. SUMMARY

The work described in this thesis focuses on one member of the teneurin family: teneurin-1 (ten-1). The *chicken ten-1* gene encodes a 300kDa protein. The intracellular domain of ten-1 harbours proline rich sequences, which serve as docking sites for SH3-domain containing proteins, followed by a transmembrane domain, an extracellular domain containing several EGF-like repeats involved in protein dimerization and 26 YD repeats.

Regulated Intramembrane Proteolysis (RIP) initiates an event that involves the cleavage of type I and II transmembrane proteins, leading to the translocation of the cleaved intracellular domain into the nucleus to regulate transcription of target genes. Prominent proteins known to be activated through RIP are Notch, Amyloid Precursor Protein (APP), Sterol regulatory element-binding protein (SREBP) and others. Recently, evidence of a cleavage mechanism of this type has also been obtained for the teneurin family of proteins.

With the aim to identify binding partners of teneurin-1, yeast two-hybrid studies were performed. Both CAP/ponsin and MBD1 were found to interact with the intracellular domain of ten1 (IDten-1). In cell fractionation experiments, IDten-1 co-purified with CAP/ponsin and MBD1 in the nuclear matrix of U2OS cells. Moreover, when full-length teneurin-1 was expressed in cells in culture, the generation of IDten-1 could be detected, suggesting that this fragment is the result of an endogenous cleavage mechanism.

Based on these observations, we concluded that IDten-1 either alone, or in a complex with CAP/ponsin and MBD1 could be involved in the regulation of transcription of yet-to-be identified target genes. To identify such target genes the gene expression profile of cells induced to express IDten-1 was compared to control cells not induced to express this domain. This analysis revealed a particular transcript, here defined as XM_039676, the expression of which was highly upregulated in the presence of IDten-1. This gene represents the first candidate for a RIP-induced target gene of ten-1.

I. Introduction

Cell-cell and cell-extracellular matrix (ECM) interactions, in addition to ligand binding to a receptor, are of fundamental importance for cellular signalling. Surprisingly, despite the enormous complexity of our bodies, the repertoire of messages that pass between cells to coordinate the developmental processes is limited and relatively similar in kind. Among the molecules involved in the signalling network that orchestrates development, the teneurins emerged about eleven years ago as a novel family of proteins. Since then, elucidating teneurin function has been the objective of studies carried out in various laboratories.

I. 1. The discovery of Teneurins

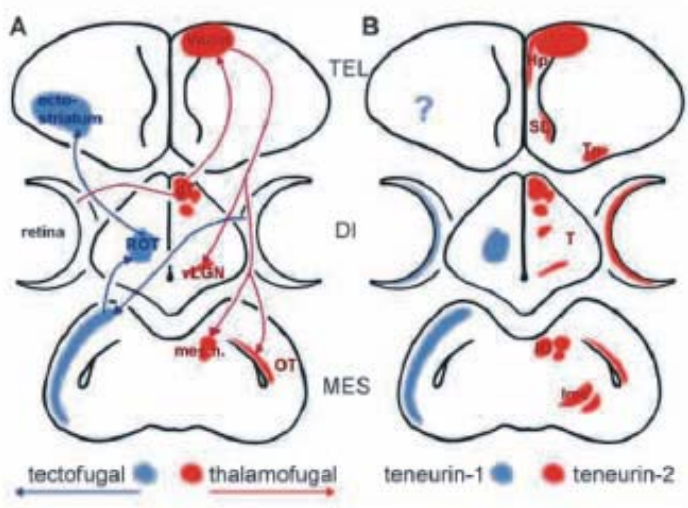
The teneurins comprise a family of glycosylated type II transmembrane proteins that were originally discovered in *Drosophila* in a search intended to identify orthologues of the vertebrate tenascins. However, *ten-a*, the first teneurin member to be characterized (Baumgartner and Chiquet-Ehrismann, 1993) and the second member, *ten-m/odd oz* (*odz*) (Baumgartner et al., 1994; Levine et al., 1994) turned out to be structurally and functionally distinct from the tenascins despite the high degree of conservation of their EGF-like repeats. Subsequently, the vertebrate orthologues of *ten-a* and *ten-m* were discovered. The nomenclature of the homologous proteins in different species is not standardized as different laboratories found and named members of this protein family independently. The actual term teneurin is mostly attributed to family members in chicken and man (Minet and Chiquet-Ehrismann, 2000; Minet et al., 1999). Oohashi et al. named the mouse proteins ten-m1, ten-m2, ten-m3 and ten-m4, the latter being identical to mouse DOC4 (Oohashi et al., 1999). DOC4 was identified as a downstream target of the transcription factor CHOP (C/EBP homology protein) (Wang et al., 1998). In rat, a family member was termed neurestin. It is most similar to teneurin-2 and was cloned by Firestein and Otaki in 1999 (Otaki and Firestein, 1999a). Two zebrafish orthologues,

highly related to the mouse ten-m3 and ten-m4, were therefore termed accordingly (Mieda et al., 1999).

Expression pattern of Teneurins

The striking common feature among all these family members appears to be their highly conserved neuronal expression. In *Drosophila* for example, expression of ten-m coincides with the location of neurons involved in receiving, transmitting and processing visual inputs from the eye to the brain just as was found for teneurin-1 in the case of chicken (Minet et al., 1999). In rat, neurestin/teneurin-2 was found to be highly expressed in the brain and also in other tissues, but at much lower levels (Otaki and Firestein, 1999a). Its expression overlaps with the timing of synapse formation of olfactory sensory neurons and glomeruli immediately before and after birth. For that reason, neurestin was proposed to serve as a target recognition molecule involved in producing the olfactory perception by directing neurons to correct sites to form synapses and glomeruli (Otaki and Firestein, 1999b). The murine *ten-m* genes also show overlapping expression patterns during development and in the adult brain (Zhou et al., 2003). By *in situ* hybridization of parasagital brain sections, ten-m1 (teneurin-1) positive signals were first seen at E15.5 in forebrain, midbrain, hindbrain, spinal cord and trigeminal ganglion. In the adult brain, ten-m1 expression is present in the cortex, thalamus, hippocampus and in the granular layer subregion of the cerebellum. In chicken, Northern blot analyses of the brain, show highest expression levels between stages E11 and E17 and lower levels in the adult brain. By *in situ* hybridization, the most prominent site of expression is the developing visual system. At E7, teneurin-1 is present in the retina, telencephalon, optic tectum and diencephalon (Rubin et al., 1999) and at E17, the hybridization signal is still concentrated within the developing visual system and within interconnected brain areas suggesting a role in neuronal connectivity (Minet et al., 1999). Indeed, immunohistochemistry studies showed teneurin-1 and 2 expressions to be refined to neurons belonging to specific pathways (Rubin et al., 2002). Teneurin-1 expression was found to

belong to the tectofugal pathway, responsible for colour discrimination and brightness, whereas teneurin-2 was found to belong to the thalamofugal pathway, involved in detection of movements and also known to modulate the tectofugal pathway (**figure 1**). Because these expression patterns coincide with periods of target recognition and synaptogenesis, teneurin-1 and -2 have been suggested to be a regulator in the development of appropriate synapses in the avian visual system (Rubin et al., 2002).



Taken from Rubin et al., *Development*, 2002.

Figure 1. The primary visual pathways of the chick and patterns of teneurin expression. **(A)** There are two primary visual circuits in birds, the tectofugal pathway and the thalamofugal pathway. **(B)** Teneurin-1 is expressed by neurons in at least two of the three major parts of the tectofugal pathway (expression in the telencephalon has not been examined). Teneurin-2 is expressed by the neurons of the thalamofugal visual pathway.

Domain architecture and molecular properties of Teneurins

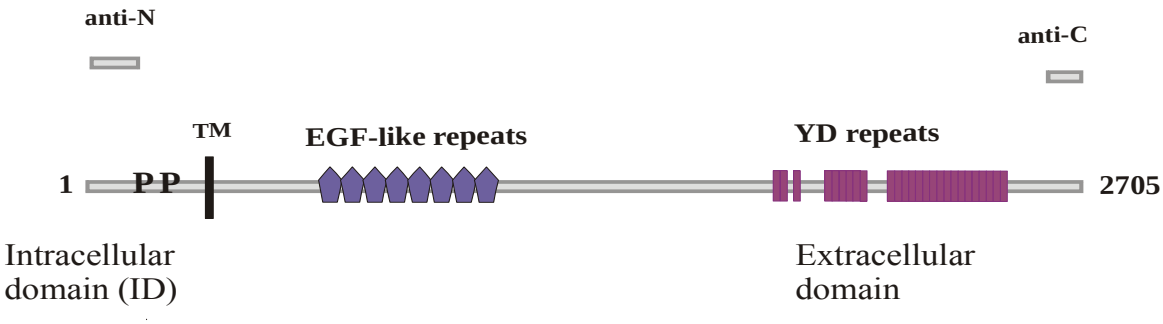


Figure 2 depicts the structural domains present in the teneurin-1 protein. The amino-terminal intracellular domain of teneurin-1 (IDten-1) contains a short sequence rich in prolines that can serve as docking sites for SH3-domain containing proteins (Mayer and Eck, 1995). Following the intracellular domain, the protein contains a hydrophobic stretch characteristic of a transmembrane domain and a long extracellular tail harbouring 8 consecutives highly conserved tenascin-type EGF-like repeats followed by 26 YD repeats.

In addition to the immunohistochemistry, northern blotting and in situ hybridization studies described above, recombinant chicken teneurin-1 and 2 proteins were also overexpressed to assay their molecular properties. *In vitro,* the YD repeats of teneurin-1 were reported to be important for carbohydrate binding and to promote neurite outgrowth when offered as a substratum, a property that can be inhibited by heparin (Minet et al., 1999). In a similar fashion, over expressed teneurin-2 in Nb2a neuroblastoma cells resulted in actin-containing teneurin-2-enriched filopodia and enlarged growth clones, which suggested an interaction of the cytoplasmic part of this protein with the cytoskeleton (Rubin et al., 2002; Rubin et al., 1999).

Interestingly, a very intriguing feature of the teneurins is that they are the exclusive proteins in eukaryotes containing short repeats of about twenty amino acids containing the previously mentioned dipeptide tyrosine-aspartate sequence, the YD-repeats. Elsewhere, this domain is only found in the predicted protein of the rearrangement hot spot (rhs) elements in *E. coli*. Highly

related sequences are also found in the wall-associated protein A (WAPA) of *B. subtilis* and more distant related sequences are present in the toxin A of *C. difficile* (Minet et al., 1999).

In other studies, recombinantly expressed mouse extracellular domains of ten-m1, ten-m2, ten-m3 and ten-m4 were able to form homo and hetero-dimers *in vitro* via the EGF-dimerisation unit, indicating that teneurins are dimers (Feng et al., 2002; Oohashi et al., 1999). Dimerisation could also be observed in the case of chicken teneurin-1 (Nunes et al., 2005). Taking into account all the different homo- and heterodimers that could form (Feng et al., 2002), in combination with the alternative splicing of teneurin transcripts (Tucker et al., 2001), a large variety of different molecular combinations are conceivable, which would significantly enhance the functional complexity of teneurin-ligand interactions.

The EGF-like repeats of chicken teneurin-2, when overexpressed in cell culture, do not appear to play a role in the homophilic interaction that leads to the aggregation of cells *in vitro*. Instead, it was proposed that the aggregation depended on the part of the molecule distal to the EGF-like repeats (Rubin et al., 2002). Apart from the studies by (Feng et al., 2002; Oohashi et al., 1999) and (Rubin et al., 2002) knowledge on the functional properties of the EGF-like repeats for the teneurin family of proteins is scarce to date. In contrast, the highly related EGF-like repeats of tenascins have been investigated more thoroughly. It was found that tenascin-C elicits repulsive (or anti-adhesive) effects on oligodendrocyte precursor cell migration through two distinct mechanisms; (1) reduced substrate adhesion and (2) a direct inhibition of cell migration that is independent of adhesion. The repulsive effect on oligodendrocytes was then mapped to the EGF-like repeats and to the alternatively spliced FNIII repeats of the protein (Kiernan et al., 1996). Moreover, neurites and growth cones were shown to be strongly repelled from areas coated with fragments containing the EGF-like domains (among others) of tenascin-R. Importantly, the binding site of the neuronal receptor for tenascin-R, the immunoglobulin superfamily adhesion molecule F3/11, was

localized to the EGF-like domain (Xiao et al., 1996). The repulsive effect of the EGF-like repeats could be further confirmed in an *ex-vivo* assay of the central nervous system. Outgrowth of embryonic and adult retinal ganglion-cell axons from mouse retinal explants was significantly reduced on homogeneous substrates containing tenascin-R or a bacterially expressed tenascin-R fragment comprising the EGF-like repeats (Becker et al., 2000). Later on, it was proposed that selected EGF-like repeats of tenascin-C elicit mitogenesis and EGFR autophosphorylation in an EGFR-dependent manner (the repeats being a novel EGFR-ligand class) (Swindle et al., 2001). In addition, the EGF-like repeats of tenascin-C were shown to be involved in EGF-receptor signalling of myofibroblasts that are present at the invasion front in colon cancer (De Wever et al., 2004). These cells are able to secrete two converging signals, namely the scatter factor/hepatocyte growth factor and tenascin-C. For proper pro-invasive and permissive signalling of SF/HGF, it is believed that the EGF-like repeats of tenascin-C contribute to the responses necessary for the transformation of a nonmigratory morphotype of the myofibroblasts to a migratory one, namely by inducing changes in RhoA and Rac expression.

Elsewhere than in tenascins, the equivalent domain mediates diverse functions. Over 100 cell surface and secreted molecules have been found to contain EGF repeats. In Notch, the EGF-like repeats are *O*-fucosylated to form the substrate of Fringe β1,3-*N*-acetylglucosaminyltransferases and are necessary for proper Notch signalling at developmental boundaries (Moloney et al., 2000) (Bruckner et al., 2000). Moreover, the hint that *O*-fucosylation is essential for Notch signalling in mammals came from studies demonstrating that mouse embryos lacking *O*-fucosyltransferase 1 die at midgestation with several defects (Shi and Stanley, 2003). Furthermore, the EGF-like repeats are also necessary for Notch interaction with delta and serrate during the development and patterning of the wing disc in *Drosophila* (Lawrence et al., 2000).

I. 2. Signal transduction by membrane proteins

Cells have learned to respond to various stimuli from their environment in a rapid and efficient manner by inducing changes in their gene expression program. This trans-membrane signalling is achieved by receptors or sensors, which are triggered by the stimuli. The signal is then relayed within a specific signalling cascade comprised of several effector proteins by mechanisms such as post-translational modifications (e.g. phosphorylation) or non-covalent interactions.

Despite the high complexity and diversity of multicellular organisms only a limited number of conserved signalling pathways are used repetitively in the different tissues and organisms throughout evolution to orchestrate the developmental processes. The Wnt/wingless-, BMP/TGF-beta-, sonic hedgehog-, receptor tyrosine kinase-, nuclear receptor-, JAK/STAT and Notch-pathway are some of the most prominent examples. The signal transducers and activators of transcription (STAT) are among the best-studied latent cytoplasmic signal-dependent transcription factors. The STAT transcription factors are activated by phosphorylation on conserved tyrosine and serine residues in their C-terminal domains following cytokine signalling or by various stress-induced stimuli. Janus kinases (JAKs) and mitogen-activated protein kinase (MAPK) are among the kinases that phosphorylate the STATs and allow them to dimerise and to translocate to the nucleus where they regulate gene expression (Ihle, 2001); reviewed in (Leonard and O'Shea, 1998).

The unexpected finding that mechanisms of regulated proteolysis can be involved in triggering activation of transcription factors by processing an inactive membrane-precursor form to generate its active soluble form has opened up an entirely new field of research on transmembrane proteins.

Regulated intramembrane proteolysis (RIP) (Brown et al., 2000; Kanehara and Akiyama, 2003) and regulated ubiquitin/proteasome-dependent processing (RUP) (Hoppe et al., 2001) are examples of the cleavage events described above. They are novel mechanisms added to the list of cellular strategies to mobilize membrane-anchored proteins for signalling purposes. In

the case of RIP, the signalling pathway starts by a mechanism where substrates are hydrolysed within the hydrophobic environment of the membrane by specific membrane-bound proteases. In the case of RUP, the proteolysis machinery involved is the proteasome. For teneurin-2, RUP was considered unlikely to be responsible for its proteolytic cleavage since proteasome inhibitors actually enhanced the amount of the cleaved intracellular domain (Bagutti et al., 2003).

Studies on RIP have started about seven years ago, with the discovery that cholesterol not only had a role in modulating the fluidity of plasma membranes, but also in association with sphingomyelin was responsible for the formation of plasma membrane rafts or caveolae. Rafts are storage sites for signalling molecules and cells need to maintain a constant level of cholesterol, in order to control homeostasis, integrity and function (Anderson, 1998; Simons and Ikonen, 1997). To achieve this task, RIP is necessary for the activation of SREBPs (Sterol regulatory element-binding proteins), which are membrane-bound transcription factors responsible for cholesterol homeostasis in plasma membranes, cells and blood of animals. SREBPs enhance the synthesis and uptake of cholesterol and fatty acids. When cells are replete with sterols, SREBPs remain bound to the membranes of the endoplasmic reticulum (ER) and are therefore inactive. Serbs are bound to the membrane by a multipass membrane protein called SCAP (SREBP cleavage-activating protein) that itself is bound to a small membrane protein called Insig. When cells are depleted of sterols, Insig dissociates from SCAP. This event allows SCAP to now guide SREBP to move from the ER to the Golgi complex. There, two proteases release the active portions of the SREBPs, which then enter the nucleus and activate transcription of target genes (Goldstein et al., 2002).

Another extensively studied endoplasmic reticulum transmembrane protein is the activating transcription factor 6 (ATF6), which is proteolytically activated in response to ER stress. Normally, the steps involved in the activation of membrane-anchored transcription factors are tightly regulated. For example, the transport of ATF6 from the ER to the Golgi apparatus and

that from the Golgi to the nucleus are distinct steps that can be distinguished by treatment with the serine protease-inhibitor AEBSF (Okada et al., 2003).

Since teneurins are transmembrane proteins (Oohashi et al., 1999; Rubin et al., 1999) and *Drosophila ten-m* was intriguingly found to be the first member of a pair-rule gene not encoding a classical transcription factor (Baumgartner et al., 1994), the hypothesis emerged that a regulated cleavage event taking place extracellularly may be involved in activation of the protein. As a consequence of that, liberation of an intracellular domain would ultimately signal to the transcription machinery. That could in turn help to explain the discrepancy observed in *Drosophila* between the mRNA and the protein expression pattern. In *Drosophila*, the *ten-m/odz* gene is expressed in odd-numbered segments of the developing embryo but the protein is found to be secreted (Baumgartner et al., 1994), most likely as a result of a cleavage event. So far, evidence for an extracellular cleavage at a furin site has been obtained for teneurin-2 (Rubin et al., 1999) but the physiological protease responsible for the liberation of the intracellular domain remains to be identified. The intracellular domain of teneurin-2 was shown to have a nuclear function in repressing zic-mediated transcription (Bagutti et al., 2003). *Zic* genes are the vertebrate homologues of the *Drosophila* zinc-finger transcription factor *opa* that was shown to cooperate with *ten-m* in the same signalling cascade (Baumgartner et al., 1994).

I. 3. Intramembrane-cleaving proteases (I-CLiPs)

I-CLiPs are multispanning, integral membrane proteins that have classical protease motifs within their putative transmembrane domains (Golde and Eckman, 2003; Weihofen and Martoglio, 2003). I-CLiPs cleave their substrates in the plane of the membrane, although the precise mechanism of peptide-bond hydrolysis is not fully understood. They can be grouped in three main families that differ in their mechanistic class and in the topology of their substrates (**see tables 1 and 2**) (Kopan and Ilagan, 2004). I-CLiPs regulate

diverse biological processes in a wide range of organisms from archaea to humans (Brown et al., 2000; Urban and Freeman, 2002).

Among the intramembrane-cleaving proteases, the discovery of presenilin (PS) in 1995 through positional cloning was particularly important to illuminate a key mechanistic feature of Notch signalling that remained elusive and controversial for many years (Rogaev et al., 1995). Of special interest, presenilin has been later shown to be involved in the production of amyloid beta peptides (Aβ) from amyloid precursor protein (APP) in Alzheimer's disease (Haass and De Strooper, 1999). Presenilins are multifunctional proteins and function as the catalytic subunit of the γ-secretase complex, an intramembrane aspartyl protease. In order to acquire a functional γ-secretase active site, presenilin goes through a conformational change induced by endoproteolysis. Furthermore, it has to be in complex with other co-factors such as nicastrin, anterior pharynx defective-1 (APH-1) and presenilin enhancer-2 (PEN-2) (Xia and Wolfe, 2003). In yeast, functional reconstitution of γ-secretase activity requires all four components (Edbauer et al., 2003; Kimberly et al., 2003; Luo et al., 2003; Takasugi et al., 2003).

Cervantes et al. and others have previously shown that presenilin homodimerises and suggested that a presenilin dimer is at the catalytic core of γ-secretase. Later on, they were able to demonstrate that the transmembrane domains of these proteins contribute to the formation of the dimer. Both presenilinase and γ-secretase activities were abolished by an in-frame substitution of the hydrophilic loop 1, which is a region that modulates the interactions within the N-terminal fragment/N-terminal fragment dimer. In addition, they also provided evidence that specific diaspartyl groups within the presenilin catalytic core of γ-secretase mediate the cleavage of different substrates (Cervantes et al., 2001; Cervantes et al., 2004).

In addition to Notch and APP, an increasing number of proteins were subsequently shown to be cleaved by γ-secretases in the last years. Intriguingly, these γ-secretase substrates turned out to be a set of diverse proteins, such as

e.g. ErbB4, E-cadherin, N-cadherin, CD44, Nectin-1α, syndecan-3, Delta, Jagged and ApoER2, implicating γ-secretase in normal cellular processing events (reviewed in (Medina and Dotti, 2003)).

Interestingly, the amino acids within the transmembrane domains of these substrate molecules share little homology suggesting that γ-secretase might not require a specific consensus sequence for activity (Struhl and Adachi, 2000) supporting the concept that co-factors are necessary for protease activity.

γ-secretase cleaves APP and other type I transmembrane proteins in the process of regulated intramembrane proteolysis, in which an obligatory first cleavage sheds most of the ectodomain and allows the substrate to undergo subsequent intramembranous cleavage.

Moreover, the so-called "spatial paradox" which refers to the apparent discrepancy between PS localisation (residing mainly in the endoplasmic reticulum, trans-Golgi network and intermediate compartments) and γ-secretase activity seems to be solved. Chyung and colleagues were the first to recently demonstrate that γ-secretase exists on the plasma membrane as an intact complex that accepts substrates and effects intramembrane cleavage (Chyung et al., 2004).

Related to the presenilin family are the signal-peptide peptidases (SPP) that remove membrane-associated signal peptides (Weihofen et al., 2002). SPP cuts type II transmembrane proteins, illustrating that presenilin-like proteases play a key role in intramembrane proteolysis of single-pass membrane proteins oriented in either direction (Xia and Wolfe, 2003). However, in contrast to presenilin that is not active unless part of a larger complex, SPP does not appear to require protein co-factors. In addition, because of its requirement for hepatitis C virus maturation and a possible immune modulatory role, SPP is also considered a potential therapeutic target (Martoglio and Golde, 2003).

In conclusion, the intramembrane cleaving proteases participating in RIP initiate similar cellular responses. By means of regulated proteolysis, membrane bound signalling proteins and transcription factors that are kept in a

dormant state, have the potential to be activated rapidly by certain stimuli to control gene expression. Nonetheless, one has to keep in mind that neither RIP nor RUP processes are restricted to the activation of transcription factors or to the regulation of nuclear import. For example, in the case of N-cadherin, the released intracellular domain associates with the transcriptional activator CREB binding protein (CBP) and promotes its export to the cytosol and degradation by the proteasome (Marambaud et al., 2003). Furthermore, the release of the EGF orthologue Spitz by Rhomboid proteolysis does not generate a transcription factor, but a secreted growth factor instead (Lee et al., 2001). In fact, all I-CLiPs discovered so far have important biological activities and are tightly regulated, but the means of control vary. They are all involved in cell signalling, but as mentioned above, some release specialized transcription factors (S2P), whereas others generate transcriptional modulators and/or contribute to degradation of their substrates (γ-secretase and SPP). Other I-CLiPs generate fragments that can act as immune system surveillance molecules (SPP) or secreted growth factors (Rhomboid) (Wolfe and Kopan, 2004).

Finally, it is of interest to note that several presenilin and γ-secretase substrates, such as e.g. DCC, ErbB4, Delta and p75NTR are known to influence neuronal structure and function, and are important for nervous system development (Koo and Kopan, 2004).

Table 1. Intramembrane-cleaving proteases

iCLiP family (mechanistic class)	Substrate topology*	Substrate	Substrate function
Aspartyl proteases			
Presenilin (γ-secretase)	type I	**(see table 2)**	**(see table 2)**
Signal-peptide peptidase (PSH3)	type II	MHC-class-I	Immune surveillance
Metalloproteases			
Site-2-protease	type II	SREBP	Sterol regulation
		ATF6	Unfolded protein response
Eep	type II	cAD1 pheromone	Pheromone release during conjugation in *E.faecalis*
Serine proteases			
Rhomboid	type I	Spitz, Keren and Gurken	Ligands for EGFR signalling in *Drosophila*
Aar A	type I	Unknown	Quorum sensing in *P.stuartii*

* type-I topology - amino terminus is lumenal/extracellular, carboxy terminus is cytoplasmic; type-II topology - amino terminus is cytoplasmic, carboxy terminus is lumenal/extracellular.
ATF6, activating transcription factor-6; EGFR, epidermal growth factor receptor; MHC, major histocompatibility complex; PSH, presenilin homologue; SREBP, sterol regulatory element binding protein; *E. faecalis*, *Enterococcus faecalis*; *P. stuartii*, *Providencia stuartii*.

This table has been modified. For reference information see Kopan and Ilagan, ***Nature Review***s, 2004.

Table 2. γ-Secretase substrates and their proposed biological functions

Substrate	Known role	Mechanism regulating ectodomain shedding/ γ-secretase cleavage	Function of released intracellular domain (ICD)
APP	Pathological role in Alzheimer's disease physiological role in transport/adhesion?	Constitutive? F-spondin ligand binding?	Forms transcription complex with FE65 and TIP60?
N-cadherin	Cell adhesion	Membrane depolarization; NMDA-receptor activation	Promotes CBP degradation
DCC	Netrin-1 receptor	Constitutive?	ICD-Gal4 fusion construct activates Gal-4 reporter-gene
Notch 1-4	Signalling receptor	Ligand binding	Transcriptional regulation

CBP, CREB (cAMP-responsive-element binding protein)-binding protein; ICD, intracellular domain; DCC, deleted in colorectal cancer; NMDA, *N*-methyl-$_D$-actetate;

This table has been modified. For reference information see Kopan and Ilagan, ***Nature Reviews***, 2004.

I. 4. Regulated intramembrane proteolysis in Alzheimer's disease and in Notch signalling

Alzheimer's Disease (AD), the most common age-associated neurodegenerative disorder is characterized by a loss of neurons and synaptic dysfunction in the cortex, hipoccampus and many other structures involved in judgment, memory and emotions. This is assumed to be an important cause for the cognitive impairment seen in patients. (Koo and Kopan, 2004; Selkoe, 2002; Terry et al., 1991). It is believed that abnormal processing of the amyloid precursor protein (APP) contributes to pathogenesis as an initiating factor due to the deposition of the amyloid peptide (residues 38-43 of the processed amyloid beta (Aβ) protein) in the form of plaques in the brain (Hardy and Selkoe, 2002; Katzman, 1986). A major accompaniment of this event is the activation of kinases in the neuronal cytoplasm, leading to

hyperphosphorylation of the microtubule-associated protein tau, and its polymerization into insoluble neurofibrillary tangles (**figure 3**) (Selkoe, 2004; Walsh and Selkoe, 2004). Aβ is generated normally throughout life by virtually all-mammalian cells. APP is a ubiquitous type I membrane glycoprotein that occurs as a heterogeneous group of polypeptides arising from alternative splicing, N- and O-linked glycosylation, phosphorylation, sulphation, glyosoaminoglycan addition and complex proteolysis. Aβ is released from APP through sequential cleavages by β-secretase (also called BACE-1), a membrane-spanning aspartyl protease with its active site in the lumen and γ-secretase. In addition, APP undergoes both constitutive and regulated secretory cleavages by certain metalloproteases referred to as α-secretases, principally believed to be ADAM 10 and ADAM 17. The α-secretase cleavage occurs primarily between residues 16 and 17 of the Aβ region of APP, and any precursors cleaved at this position cannot yield toxic Aβ peptides. Instead, processing by α-secretase followed by γ-secretase generates a smaller hydrophobic fragment referred to as p3, whose normal function and role in Alzheimer's disease (if any) is unclear.

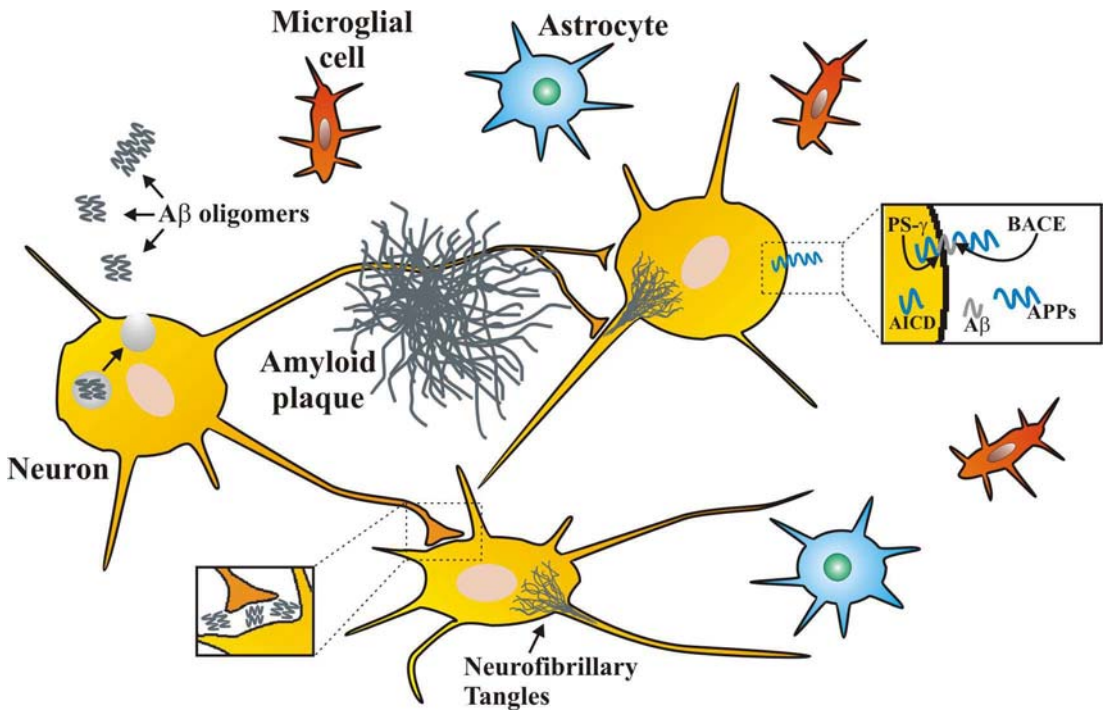
α-secretase processing releases the large APP ectodomain (APPs-α) from the cell. Several biological activities have been ascribed to this secreted derivative *in vitro*, including as a trophic or neuroprotective factor, a serine protease inhibitor and a cell-substrate adhesion molecule. Sequential processing by α-secretase and γ-secretase also releases the APP intracellular domain (AICD), a portion of which can bind to the cytoplasmic adaptor protein Fe65, and apparently help mediate aspects of nuclear signalling. However, there has been no consensus so far as to which transcripts may be regulated by complexes shown to contain AICD, Fe65 and the histone acetylase Tip60 (Selkoe, 2004).

Although the inherited form of Alzheimer disease accounts only for a minor fraction of reported cases, genetic studies led to the important finding that early-onset, dominant forms of familial AD (FAD) are caused by

mutations in APP, presenilin 1 (PS1) and presenilin 2 (PS2) proteins (Goate et al., 1991; Hardy and Allsop, 1991; Hendriks et al., 1992; Mullan et al., 1992) consistently associated with selective increases in Aβ42 peptides or total Aβ levels (Murayama et al., 1999).

However, the observations that presenilin modulates proteolysis and turnover of several signalling molecules have led to speculations that other pathways that are important in development may also contribute to neurodegeneration. Moreover, since the majority of AD cases do not carry presenilin mutations, it remains to be clarified whether other mechanisms contribute to the accumulation of Aβ. One such option would be for example the age-dependent reduction in Aβ clearance or degradation originating from accumulated insults throughout life. Mechanisms that are not related to Aβ, but primary related to presenilin activity instead, have been reviewed in (Koo and Kopan, 2004) and are considered as contributors to sporadic cases of AD.

Interestingly, a crucial determinant in Aβ generation is the targeting of the protein to the correct subcellular site. For example, amyloidogenic processing of the β-amyloid precursor protein depends on lipid rafts. APP within rafts appears to be cleaved by β-secretase whereas APP outside rafts undergoes cleavage by α-secretase (Ehehalt et al., 2003).



Adapted from Selkoe, *Nature Cell Biology*, 2004 and Walsh and Selkoe, *Neuron*, 2004.

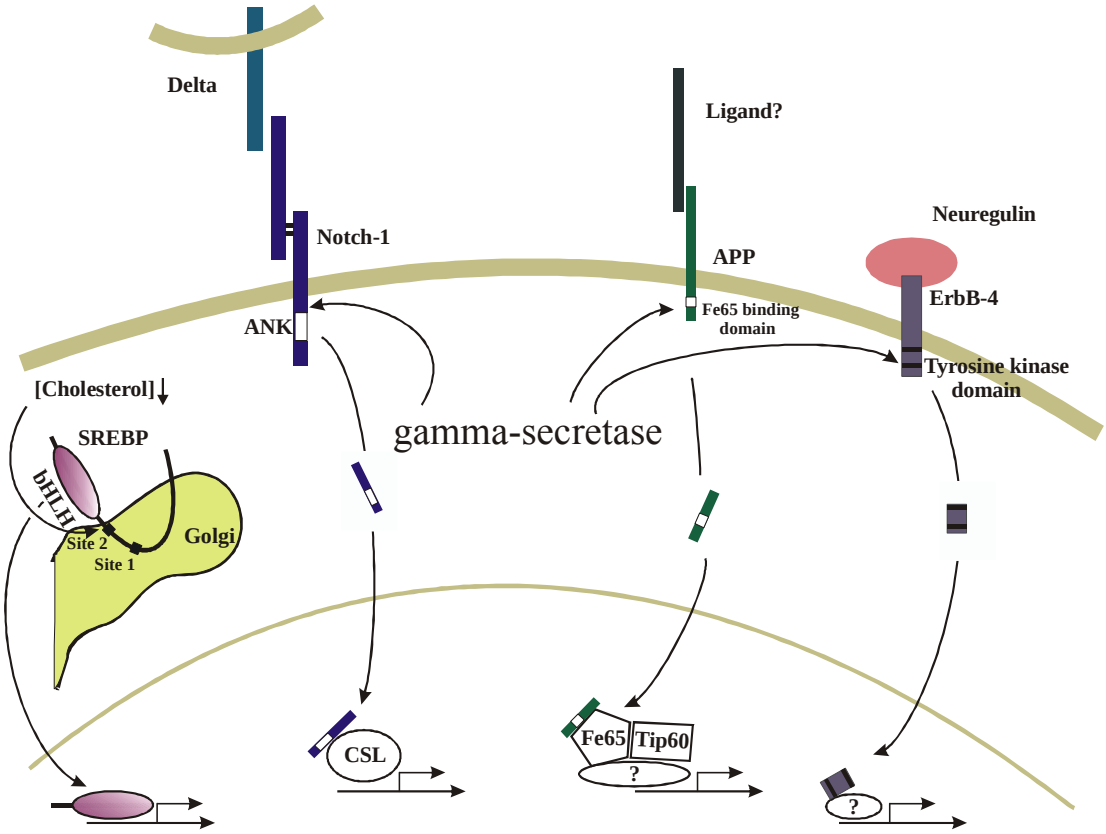
Figure 3. Model of key events underlying the pathogenesis of Alzheimer's disease. APP on the plasma membrane and in intracellular vesicles is cleaved by BACE and the presenilin-γ-secretase complex (PS-γ) to liberate the Aβ region. A portion of Aβ can oligomerize and is released into the interstitial fluid of the brain. These soluble oligomers may diffuse into synaptic clefts and interfere with synaptic function. Aβ can further polymerize into insoluble amyloid fibrils that aggregate into spherical plaques, resulting in dysfunction of adjacent axons and dendrites. The activation of kinases in the neuronal cytoplasm leads to the hyperphosphorylation of the microtubule-associated protein tau and its polymerization into insoluble filaments defined as neurofibrillary tangles. The different Aβ assembly forms may mediate diverse effects, such as decreased synaptic efficacy, distortion of axonal pathways, shrinkage of dendritic arbors, activation of microglia, free radical release and inflammatory changes that may contribute to neurotoxicity.

Notch signalling has been implicated in a wide variety of processes from cell-fate decisions, tissue patterning and morphogenesis to human diseases and cancer. In most cases, Notch signalling acts as a "gatekeeper against differentiation" because it blocks differentiation towards a primary differentiation fate and instead directs the cell to a second, alternative differentiation program or forces the cell to remain in an undifferentiated state. In addition to its classical role in lateral inhibition, recent findings demonstrate that Notch signalling is instructive, by actively promoting a particular cell fate. For instance in the nervous system, Notch promotes differentiation of glial cell types e.g. astrocytes, Schwann cells, Muller glial cells and radial glial cells (Hansson et al., 2004). Notch signalling has also been shown to be important in post-development processes, for example, in synaptic plasticity of the hippocampus (Wang et al., 2004) and in contributing to long-term memory formation in the adult brain of *Drosophila* (Ge et al., 2004).

Activation of Notch is accomplished by multi-step cleavage events. The receptor is synthesized as a 300 kDa precursor form in the endoplasmic reticulum and transported via the secretory pathway to the trans-Golgi compartment where it is cleaved by furin convertases at cleavage site 1 (S1). This cleavage leads to the formation of a functional heterodimeric receptor at the cell membrane (Blaumueller et al., 1997; Logeat et al., 1998). S1 cleavage is necessary for cell-surface expression and is not directly involved in the ligand-induced release of the active intracellular domain.

Ligand-receptor interaction occurs between cells in physical contact. Upon binding ligands of the delta/jagged/serrate/LAG-2 family, a TNF-α converting enzyme (TACE) mediates cleavage at site 2 (S2), at a consensus site 12 amino acids juxtaposed to the plasma membrane and removes the extracellular portion of the Notch heterodimer (Brou et al., 2000; Mumm et al., 2000). The S2 cleavage event probably serves as a trigger for an additional third cleavage. This latter cleavage is presenilin-mediated and efficiently removes the remaining membrane-anchored intracellular signalling domain at site 3 (S3).

A special family of transcriptional effector proteins, the CSL (human C promoter binding factor 1-CBF1/ fly Suppressor of hairless/ worm Lag-1) factors (Bray and Furriols, 2001) and the Mastermind (Mam)/Lag-3 co-activator (Petcherski and Kimble, 2000a; Petcherski and Kimble, 2000b) provide the link between Notch activation and gene regulation. Notch intracellular domain (NICD) translocates into the nucleus and assembles into a ternary complex with CSL, which binds specific regulatory DNA sequences and activates the expression of CSL/Notch target genes. For reviews see (Schroeter et al., 1998; Schweisguth, 2004; Struhl and Adachi, 1998). An illustration of these processes can be seen in **figure 4**.



Adapted from Ebinu and Yankner, *Neuron*, 2002.

Figure 4: Signalling by Regulated Intramembrane Proteolysis (RIP).
Several proteins known to signal by RIP are illustrated. Upon cholesterol depletion, SREBP is transported to the Golgi, where it undergoes intraluminal cleavage (site1) followed by transmembrane cleavage (site 2). Consequently, an N-terminal fragment is released that translocates to the nucleus and activates transcription of lipid

biosynthetic genes. Binding of the ligand Delta to its receptor Notch triggers intramembrane proteolytic cleavage by γ-secretase. This results in the release of the Notch intracellular domain (NICD), which then translocates to the nucleus where it associates with the CSL family of DNA binding proteins and transactivates gene expression. Constitutive γ-secretase cleavage of APP leads to the generation of the CTF-γ fragment. In overexpression systems, this fragment associates with the adaptor protein Fe65 in the nucleus to form an active transcriptional complex with the histone acetyltransferase Tip60. Activation of the receptor tyrosine kinase (ErbB-4) by its ligand neuregulin can trigger the proteolytic release of the ErbB-4 C-terminal fragment (ErbB-4/CTF).

I. 5. The nucleus

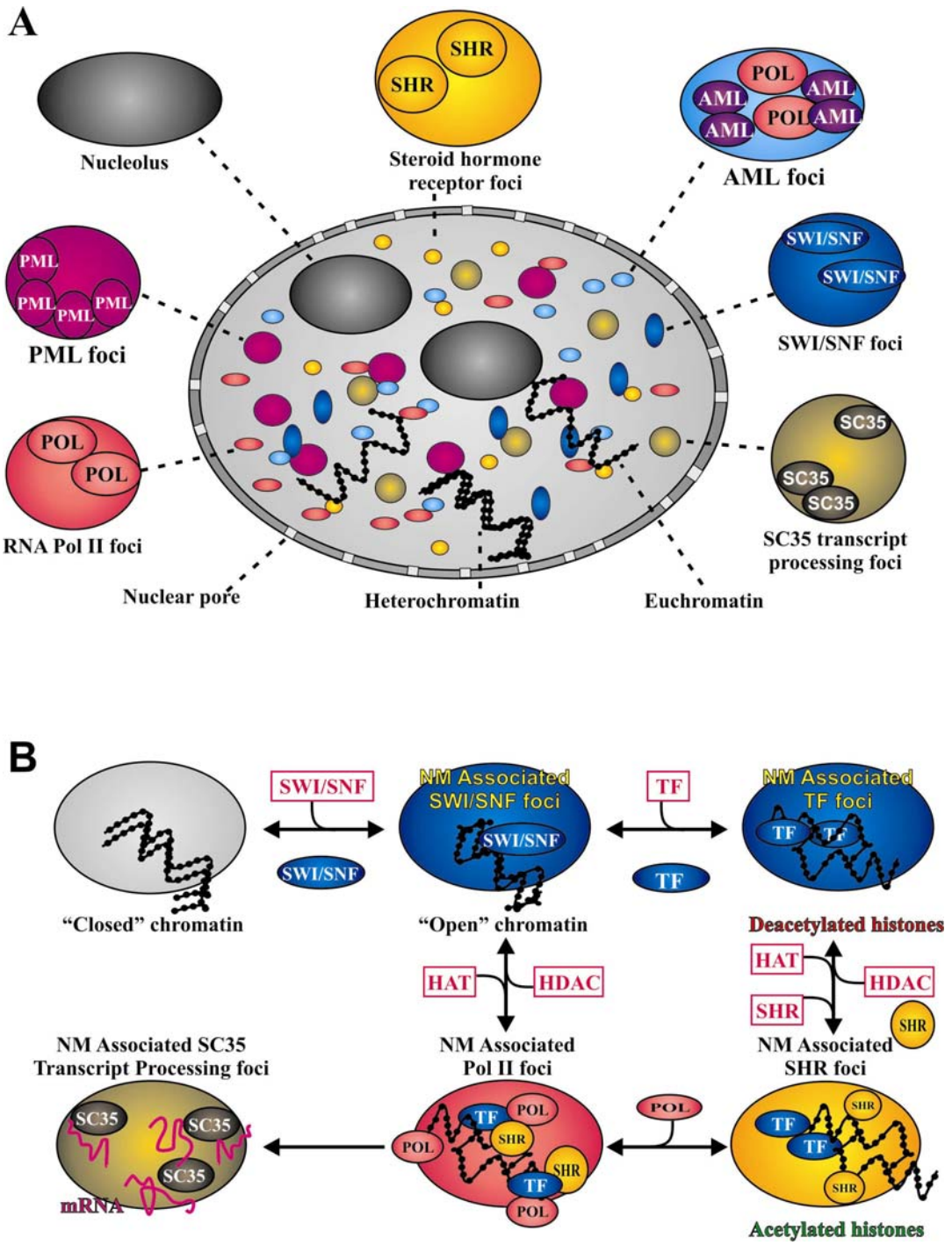
Because my work will focus to a large extent on elucidating the functions of the intracellular domain of teneurin-1 in gene regulation, the following section is dedicated to the nucleus, a highly organized cellular compartment.

In a didactic manner, the eukaryotic genome can be comprehended as a system running at different hierarchical levels, in which regulatory mechanisms are integrated at three levels (van Driel et al., 2003). The first one is the sequence, where all the yet-to-be deciphered information is stored in the form of linear organized transcription units, coding regions and regulatory sequences. At this level, developmentally co-regulated genes are sometimes organized in clusters, constituting individual functional units per se. Above the linear level, stands the second level, chromatin, a very complex platform where yet-to-be discovered mechanisms determine gene expression profiles, dictating entire chromosomal regions whether to be in a functional state or not. To modify gene expression at the chromatin level, factors contributing to histone modification, DNA methylation and a variety of repressive and activating mechanisms also come into play. Besides the rough distinction between euchromatin and heterochromatin, chromatin can exist in many more different structural and functional states, which exhibit different core histone compositions and post-translational modification of histones (the "histone code"). This regulatory level is combined with control mechanisms that switch individual genes in a cluster on and off, depending on the properties of the

promoter. Finally, the third level is the nuclear level, which includes the three dimensional spatial organization of the genome within the nucleus.

The architecture of the nucleus is closely related to genome function and the position of a gene inside the nucleus is important: some areas are repressive and some promote transcription. In order to impose these levels of control, the nucleus is compartmentalized into different foci and domains.

In recent years there seems to be an increasing interest on the roles of different nuclear compartments and subnuclear domains in the regulation of gene expression, signalling and cellular functions (Zimber et al., 2004). For the cytoplasm this has been long known. It is structured in a manner that allows different organelles to perform their highly specialized functions. In analogy, complex substructures are also found in the nucleus and specific functions are thought to occur in different domains with typical morphology. In some cases, these domains have been shown to be dynamic structures with rapid exchange of proteins between them and the nucleoplasm (Misteli, 2001). As a consequence of these nuclear dynamics, some special foci can become transiently nuclear matrix associated and influence the transcriptional status of genes by facilitating their interaction with repressors or activating proteins, represented in **figure 5**. For example, nuclear bodies (NBs) are a group of intranuclear (nucleoplasmic) structures, situated within the nuclear matrix (see I.6) that is mainly distinguishable by morphological and antigenic criteria. NBs include several species containing granular and fibrillar materials and can be arranged as "coiled bodies", vesicles, spheres, or doughnut-like structures. The nuclear matrix provides a structural substratum for NBs. A summary of NB appearance and composition is seen in **table 3** adapted from (Zimber et al., 2004). The importance of these subnuclear structures is exemplified by perturbations in their composition and organization that accompany the onset and progression of disease (Cook, 1999; DeFranco, 2002; Misteli, 2000; Penman, 1995; Stein et al., 2000; Stenoien et al., 2000).



Adapted from Stein, *Journal of Cell Science*, 2000

Figure 5: Panel A: Transcription of protein-encoding genes by RNA polymerase II occurs at a large number of foci (red ovals), and rRNA transcription by RNA polymerase I is restricted to one or more nucleoli (dark grey ovals). Both types of transcriptional foci are distinct from sites involved in DNA replication (Wei et al., 1998). Gene regulatory factors (e.g. AML, hormone receptors), chromatin-remodelling proteins (e.g. SWI/SNF), and processing factors (e.g. SC35) are organized into discrete subnuclear foci, a subset of which is associated with RNA polymerase II, depending on biological conditions. Chromatin-remodelling proteins mediate the transition between transcriptionally inactive "closed" chromatin

(heterochromatin) and active "open" chromatin (euchromatin) through a series of enzyme-mediated regulatory steps. The nuclear pore (open ovals) function in the nuclear import of regulatory proteins containing nuclear localization signals (NLSs). Intranuclear trafficking of proteins through subnuclear foci associated with the nuclear matrix involves specific nuclear-matrix targeting signals (NMTSs). PML domains (purple ovals) are specialized subnuclear domains that are rearranged in promyelocytic leukemias. SC35 domains (yellow ovals) contain RNA-splicing factors that generate mature mRNA transcripts.
Panel B: A model for dynamic modifications in the interactions of gene regulatory factors at subnuclear foci associated with the nuclear matrix. Subnuclear compartments containing inactive "closed" chromatin (grey ovals) are converted to "open" chromatin by SWI/SNF remodelling proteins. This opening of chromatin promotes accessibility of gene regulatory factors (e.g. transcription factors [TFs], dark blue; steroid hormone receptors [SHRs], golden brown) that recruit RNA polymerase II. Histone acetyltransferases and deacetylases (HDACs) mediate the reversible (de) acetylation of histones to influence nucleosomal organization. Nascent transcripts are processed into mature mRNAs at SC35 transcript processing foci (yellow oval).

Table 3. Major nuclear bodies, their number, diameter, molecular composition and known or proposed functions in interphase eukaryotic cells

	Number per cell (range)	Diameter (μm)	Major constituents (components)	Established or proposed functions
Splicing speckles[a]	25-30	0.8-1.8 (in IGCs)	pre-mRNA splicing factors	Storage sites of pre-mRNA splicing factors; correspond to interchromatin granule clusters (IGCs) and to perichromatin fibrils (PFs); involved in the storage of SR proteins and the assembly and/or the modification of pre-mRNA
Cajal bodies (and Gems)[a,b,c]	0-10	0.1-2.0	p80 coilin; fibrillarin; U1, U2, U4/U6, and U5 snRNPs; U7 snRNP for histone 3'-end processing; U3 and U8 snoRNPs for pre-rRNA processing; histone U1, U2 U3, U4 and U12 genes	snRNP biogenesis; trafficking of snoRNPs and snRNPs to nucleoli or to splicing speckles, respectively, snRNA modification
Nucleolus[a,b]	1-5	0.5-5.0	p80 coilin, fibrillarin Nopp 140; ARF, MDM2, p53; components of telomerase	rDNA transcription; rRNA biogenesis, metabolism; cell cycle regulation; cell lifespan
Perinucleolar compartment[b,c]		0.25-1.0	PTB/hnRNPI, and several other RNA-bindind proteins (KRSP); small RNAs transcribed by RNA polymerase III	RNA processing; accumulation of newly synthesized RNA (for further processing or retention?)
PML-NBs[a,b,c]	10-30	0.3-1.0	Sp-100; Daxx; CBP; HAUSP; p53; Rb; ...(sumoylation is needed) human MHC locus (chromosome 6) is preferentially associated with PML-NBs note: chromatin and RNA are not found in PML-NBs	transcriptional regulation (e.g.Pax3); replication and transcription of nuclear-replicating DNA viruses, genes and proteins (herpes viruses, adenoviruses and papova virus)
Cleavage bodies[a,b,d]	1-4foci	0.3-1.0	contain several factors that are necessary for cleavage and polyadenylation steps of mRNA processing	some cleavage bodies are adjacent to or overlap CBs; there may be distinct subclasses of these bodies

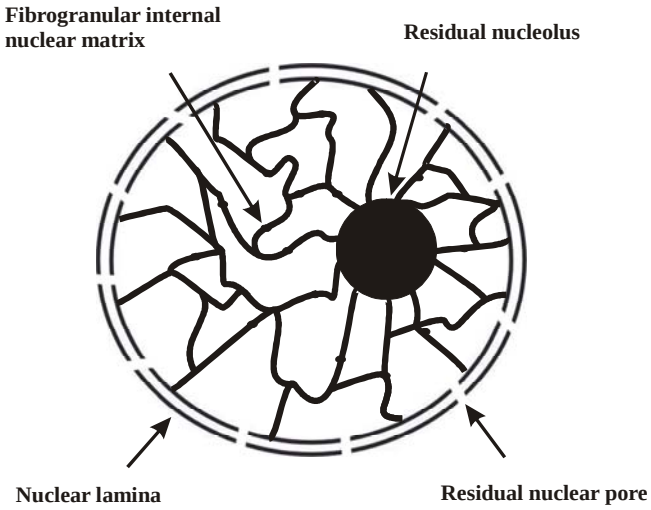
PML-NBs and cleavage bodies are often found juxtaposed to Cajal bodies/Gems. The significance of these associations is not known. [a]The number, size and integrity of these NBs depend on cellular activity (i.e., transcriptional), or cell cycle. [b]NBs that are more abundant in transformed and cancer cells (as compared to normal cells). [c]These NBs were shown to be dynamic structures and mobile within the nuclear space (as determined by fluorescence-imaging techniques in living cells). [d]NPD=nuclear compartments.

This table has been modified. For reference information see Zimber et al., *Cellular Signalling*, 2004.

I. 6. The nuclear matrix

The nuclear matrix can be defined as a “compartment” which provides the surface for the attachment of multienzyme complexes involved in RNA and DNA metabolism (Baskin, 1995; Jackson et al., 1981). The nuclear matrix can be considered as the backbone/skeleton of the nucleus.

Berezney and Coffey first coined the actual term "nuclear matrix" to define the residual nuclear structures observed after removal of DNA and histones by high-salt extraction and mild nuclease treatment in pioneering experiments in the early 1960s (Georgiev and Chentsov, 1960; Razin). Surprisingly, this harsh treatment still resulted in the retention of shape and morphological features of the cell nuclei (Berezney et al., 1979; Berezney and Coffey, 1974; Berezney and Coffey, 1975). Therefore, Berezney and Coffey's definition of the nuclear matrix was "the residual proteinaceous structure that remains after the nuclei are depleted of the nuclear membranes, histones, soluble nuclear proteins and nucleic acids" (**figure 6**).



Adapted from Razin (Razin).

Figure 6: Scheme of the nuclear matrix as observed by Berezney and Coffey. Treatment of isolated nuclei with nucleases, sequential extractions with low salt (0.2mM $MgCl_2$, 10mM Tris-HCl) and high salt (2M NaCl) solutions, extraction with 1% triton X100 followed by a second treatment with nucleases still retained a residual nuclear shape defined as nuclear matrix.

The attention of other scientists to this field of research was caught only 10 years after the initial discovery of the nuclear matrix. The interest was driven by the first demonstration of an association between active replication complexes and the nuclear matrix (Berezney and Coffey, 1975). Over the years, this concept has been supported by experimental evidence. Recent studies demonstrate that DNA replication initiates at domains overlapping with nuclear matrix attachment regions in the xenopus and mouse c-*myc* promoter (Girard-Reydet et al., 2004). The functional relationship between nuclear matrix and replication will be discussed later on.

Matrix preparations have been shown to contain 85-95% protein and 5-15% nucleic acids. (Nakayasu and Berezney, 1991) have identified the major rat liver nuclear matrix proteins to be lamins A, B, C, the nucleolar protein B-23/numatrin, residual protein components of core heterogeneous nuclear ribonucleoproteins (hnRNP) and matrins 3, 4, D-G, 12 and 13. Amongst the matrins, two were shown to be zinc- finger motif containing DNA-binding proteins.

Recently, more and more proteins are being discovered to be present in the nuclear matrix but its precise composition is still a subject of ongoing research. Some examples of nuclear matrix proteins will be cited below. Because many protocols initially relied on harsh extraction conditions (e.g. high salt-containing buffers) to reveal the nuclear matrix, a lot of criticism emerged concerning the physiological relevance of the structures observed (Pederson, 1998; Pederson, 2000). But the retention of a more or less similar structure after fairly different treatment protocols became an important argument in favour of the biological validity of the concept of the nuclear matrix.

With time, many different laboratories provided independent evidence of the existence of the nuclear matrix *in vivo*. For example, Bioluminescence Resonance Energy Transfer (BRET) was used to study the interaction of transcriptionally active proteins, including nuclear matrix proteins in living cells (Germain-Desprez et al., 2003). Importantly, chromatin was reported to be

effectively removed from nuclei at physiological ionic strength by formaldehyde crosslinking and nuclease digestion (Nickerson et al., 1997) and both DNA and histones could also be removed efficiently from the nucleus by nuclease digestion and the modification of primary amino groups with sulfo-NHS (Wan et al., 1999). Again, these treatments left behind what is called nuclear matrix.

Electron microscopic analyses carried out in the laboratory of Sheldon Penman were also seminal to the field. By using a protocol in which sharp increases in ionic strength in the extraction buffer were avoided a fine network of nuclear matrix filaments along with some dense bodies could be revealed on electronmicrographs. He was also the first to demonstrate that treatment with RNase A removed the core filaments, suggesting RNA's to be an integral part of these filaments (Fey and Penman, 1988; He et al., 1990; Nickerson and Penman, 1992; Penman, 1995). Finally, other variations of microscopy methods such as embedment-free electron microscopy (EF-EM) have overcome some limitations of heavy metal staining and aldehyde fixation. This technique was able to present a cellular scaffold as a purified, isolated, three-dimensional network with various thicknesses of filaments. With the adaptation of immunocytochemistry to EF-EM, the nuclear matrix can now be better studied (Gajkowska and Wojewodzka, 2003).

However, beyond this role as a scaffold and backbone that merely supports the nucleus, the nuclear matrix has also been assessed as a special nuclear compartment with specific functions in replication, transcription and RNA splicing. Examples to illustrate such functions are the lamins. These proteins not only sustain the nuclear envelope and participate in the formation of internal nuclear matrix filaments as structural proteins, but lamins can also be detected in replication foci speckles (Bridger et al., 1993; Moir et al., 1994). Using a truncated dominant negative form of lamin, it was found that coincident with the disruption of lamin organization, there was a dramatic reduction in DNA replication (Spann et al., 1997). Moreover, contrary to the commonly held view that replication begins at hundreds of dispersed nuclear

sites, studies done with primary fibroblasts showed that DNA synthesis was initiated at a limited number of foci that contain replication proteins, surround the nucleolus and overlap with lamin A/C structures (Kennedy et al., 2000).

Similar to DNA replication, processes of transcription and splicing were also shown to occur within specific foci or speckles (Carter et al., 1993; Jackson et al., 1993; Xing et al., 1993; Xing et al., 1995).

Interestingly, during apoptosis, the proteins of the nuclear skeleton components [term suggested by Razin which designates the nuclear lamina and the network of filaments supporting the internal nuclear matrix] are the first to be proteolytically cleaved. That, in turn, reinforces the concept that the nuclear skeleton is essential for maintenance of both the shape and the internal structural organization of eukaryotic cell nuclei. Several nuclear matrix proteins can serve as substrates for proteolytic cleavage during apoptosis and cleavage of lamin B, NUMA and nucleoporin TPR follow a specific temporal pattern (Dynlacht et al., 1999; Dynlacht et al., 2000).

In addition, it has been proposed that transcription factors are recruited to the nuclear matrix facilitating interactions with regulatory DNA elements (Samuel et al., 1998) and chromatin-modifying enzymes have also been found associated with the nuclear matrix (Davie and Hendzel, 1994; Sun et al., 2001).

As mentioned above, the exact composition of the high-salt-insoluble nuclear matrix compartment remains unclear. Besides containing exclusive and permanent matrix proteins (e.g. nuclear skeleton proteins), the nuclear matrix also contains proteins that are temporarily deposited there. These latter proteins are known to be regulated by posttranslational modifications induced by specific signalling cascades. For example, the promyelocytic leukaemia protein (PML) is a phosphoprotein found to be tightly associated with the nuclear matrix (Chang et al., 1995). In recent years, this protein has been extensively studied because of its role in causing acute promyelocytic leukaemia (APL). In this disease, the *PML* gene is disrupted by a translocation to the *RARA* gene on chromosome 17 resulting in aberrant expression of a fusion protein and disruption of PML bodies. PML is not only affected in leukaemia, but is

mislocalised and misregulated in solid tumours of various histological origins, indicating that PML and PML bodies might also be involved in the development of other tumour types (Zink et al., 2004). Loss of PML expression correlates with the invasiveness of malignant cells (Koken et al., 1995) and with tumour progression in prostate cancer, breast cancer and CNS tumours (Gurrieri et al., 2004).

The following groups of proteins are found to partition between the nuclear matrix and the soluble compartments of eukaryotic cell nuclei are:

1. Enzymes involved in DNA replication and postreplicative modifications e.g. DNA polymerase α, proliferating cell nuclear antigen (PCNA), etc
2. Enzymes involved in transcription and splicing of messenger RNA and proteins of nuclear RNP-particles e.g. RNA-polymerase II
3. Proteins of the mitotic apparatus such as NuMA and the CENPs B, C and F
4. DNA-binding proteins
5. Regulators of the cell cycle e.g. PML, cdk2 and cdc2
6. Proteins involved in the control of chromatin dynamics e.g. histoneacetyl-transferase and histone diacetylase
7. Receptors (e.g. oestrogen receptor) and receptor-binding proteins

Gene-nuclear matrix associations are also involved in regulating cell- and tissue specific gene expression. In the differentiation of rat osteoblasts for example, dramatic changes in nuclear matrix protein patterns occurred during transitions from the proliferation to the ECM maturation stage and from ECM maturation to mineralization (Dworetzky et al., 1990). Finally, the composition of the nuclear matrix not only changes in the course of cell differentiation as exemplified above, but also during malignant cellular transformation. Alterations in nuclear structure including dramatic changes in morphology are a typical characteristic of most cancers. Since the nuclear structure is maintained predominantly by the nuclear matrix, it is logical to assume that alterations in nuclear shape or structure that occur with neoplastic

transformation are accompanied by changes in nuclear matrix composition or architecture, or both (Konety and Getzenberg, 1999). Examples of nuclear shape changes associated with cancer have been reviewed in (Zink et al., 2004).

I.7. Aim of my work

The aim of my work was to gain a better understanding of teneurin-1 signalling and function. Using cell biological techniques, I assessed the model that teneurin-1 is cleaved in a Notch-like manner and that its intracellular domain translocates to the nucleus (see I.2). Furthermore, I was interested to investigate the involvement of the N-terminal domain of teneurin-1 in gene regulation and to characterize proteins that interact with it.

II. MATERIALS AND METHODS

II.1 Vector information

1. pCEP-Pu: (9.48 kb) provided by Prof. J. Engel, Biozentrum, University of Basel. This vector was used to clone cDNAs encoding the intracellular domain of teneurin-1 "IDten-1" (1-300aa) and the putative full-length teneurin-1 "FLten-1" (1-2705aa) into the NotI / XhoI restriction sites. Sequencing of the putative full-length construct revealed that it contained a mutation that generated a premature stop codon at 2161 bp, truncating the protein at 722 aa. This teneurin-1 cDNA encoding the protein with the premature stop codon was designated medium-length teneurin-1 "MLten-1" (1-721aa). Site-directed mutagenesis was used to remove the premature stop codon to generate a cDNA that expresses full-length teneurin-1 (for details see II.11.).

2. pCMV5-myc: (3.8 kb) provided by Yoshimi Takai, Osaka University Medical School, Japan. The vector already contained Ponsin-2 cDNA (Mandai et al., 1999) cloned into the EcoRI / SalI restriction sites.

3. p-CMX: provided by Ronald M. Evans, San Diego. The vector already contained PML cDNA encoding the intact protein (560aa) (Kakizuka et al., 1991).

4. pcDNA3: (5.4 kb) commercially available vector (Invitrogen). The amplified MBD1 PCR product was cloned into the HindIII and XhoI sites by Jacqueline Ferralli.

5. pIND: (5.0 kb) commercially available vector (Invitrogen). The intracellular domain of teneurin-1 was cloned into the NotI/ XhoI sites by Doris Martin. This construct was used to transfect EcR-293 cells for the generation of stable cell lines (for details see II.19.).

6. pQE30: (3.4 kb) commercially available vector (Qiagen) containing a 6xHis-tag. The PCR products corresponding to the first N-terminal 160 amino acids and the C-terminal last 334 amino acids of teneurin-1 were cloned into the KpnI / SphI sites.

7. p123T: (3.0 kb) commercially available vector (MoBiTec). It was used for cloning of PCR products. Correct insert size was verified by digestion using XhoI and XbaI restriction enzymes and analysis by 1% agarose-gel electrophoresis.

II.2 Bacterial Strains

1. *Escherichia coli* M15[pREP4]. This strain contains the pREP4 plasmid to allow glucose-regulated expression of constructs cloned into the pQE vector. Transformation with pQE was done in the presence of kanamycin at 25µg/ml and ampicillin at 100µg/ml.

2. *Escherichia coli* XL1-blue. recA$^-$ cloning strain used to host the ligation products in pKS (BlueScriptII KS+), p123T or pQE. Transformation was done in the presence of ampicillin at 100µg/ml or kanamycin at 25µg/ml, depending on the vector used.

3. *Escherichia coli* DH10. This strain harbours the Bacterial Artificial Chromosome RP11-424C23, which was ordered from CHORI (Children's Hospital Oakland Research Institute). Transformants were selected by plating on plates containing chloramphenicol at 20µg/ml.

II.3 Extraction of plasmid DNA

1. Small scale purification (Mini-Prep): DNA extraction was performed using the commercial kit from Qiagen "QIAprep Spin Miniprep Kit" according to the manufacturer's instruction. Mini-preps were done with the overnight culture grown in 2ml LB medium containing the appropriate antibiotic(s).

2. Medium scale purification (Midi-Prep): Larger scale DNA extraction was performed using the commercial kit from NUCLEOBOND "Nucleobond AX PC100" according to the manufacturer's instructions. Midi-preps were done with the overnight culture grown in 100ml LB medium containing the appropriate antibiotic(s).

3. DNA isolation from BAC clones: A rapid alkaline lysis miniprep method was used, consisting of a modification of a standard Qiagen-Tip method. All

recommendations on how to handle the clones were taken from: http://bacpac.chori.org/home.htm

II.4 Ligation

Rapid ligation reactions used the molar ratio mixture of 3:1 (insert:vector) and were done using the Rapid DNA Ligation Kit (Roche) based on the manufacturer's instructions. All reactions had a final volume of 20µl.

II.5 Transformation of bacteria

1. Preparation of competent cells (Ariane Minet, PhD thesis, 2000)

- inoculate 10ml LB-medium with a single colony of *E.coli* XL Blue and grow at 37^0C o/n
- transfer o/n culture into 100ml warm LB-medium and grow at 37^0C until OD_{600}= 0.94
- centrifuge 2 x 50ml bacteria culture at 2500g (4500rpm Sorval minifuge) at 4^0C for 5 minutes
- carefully resuspend the pellet in 2 x 10ml ice-cold solution I and incubate on ice for 10 minutes
- centrifuge at 2500g for 5 minutes
- carefully resuspend the pellet in 2 x 2ml ice-cold solution II
- aliquot (50µl or 100µl) and freeze immediately at -70^0C for storage

Solution I: (pH 5.8) 30mM KOAc, 50mM $MnCl_2$, 100mM $RbCl_2$, 10mM $CaCl_2$ and 15% glycerol - sterile filter (0.22µm)

Solution II: 10mM MOPS pH 7.0, 75mM $CaCl_2$, 10mM $RbCl_2$ and 15% glycerol - sterile filter (0.22µm)

2. Transformation

- thaw a vial containing 50µl of competent XL-Blue/Gold cells on ice
- add the entire ligation reaction (see II.4.) directly to the competent cells
- incubate on ice for 30 minutes
- heat shock at 42^0C for 1.5 minutes
- put back on ice for 2 additional minutes

- plate cells on LB-plates containing the appropriate antibiotics (for calculation of the concentration consider approximately 20ml per plate)
- incubate plates at 37^0C overnight

Note 1: If screening by blue/white selection, allow the LB plates to reach room temperature and only then add both IPTG (10µl of 100mM stock) and X-gal (30µl of 2% solution). Note 2: If propagating plasmid DNA, follow the same protocol as above but instead of adding the ligation reaction to the competent cells, add 1ng of the DNA to be propagated.

II.6. DNA digestion

Plasmid DNA and PCR products were digested in a buffer containing the adequate restriction enzyme(s), double-distilled water and BSA, if required. Incubation time and temperature varied according to the enzyme(s) used. Samples were analyzed by running an aliquot of the digestion products in an agarose gel containing 0.5µg/ml of ethidiumbromide, followed by visualization with a UV transilluminator.

II.7. Agarose gel eletrophoresis

Agarose gels were prepared in various concentrations (0.7%, 0.8% and 1.0%) by weighing agarose (Agarose Electrophoresis Grade Ultra Pure - Invitrogen) in TBE buffer and boiling the mixture in a microwave oven. Before casting the gel ethidiumbromide was added to the mixture as it had cooled down. Loading-buffer was added to the samples and gels were run at 50 to 100V for approximately one hour. To visualize the DNA, an UV transilluminator was used.

10x TBE buffer: 1M Tris-HCl; 1M boric acid and 20mM EDTA.
6x loading buffer: 0,25% bromophenolblue; 0,3% cyanol xilene and 30% glycerol.

II.8. Isolation and Purification of DNA fragments from agarose gels

The DNA fragments were excised from the gel using a razor blade under UV light. The DNA in the gel pieces was extracted following the instructions of the Qiagen "QIAquick Gel Extraction Kit".

II.9 DNA sequencing

DNA sequencing was a service provided in house and was carried out using BigDyeTerminators (Applied Biosystems) with a GeneAmp PCR system 9700 thermocycler (Applied Biosystems). Samples were processed in an ABI PRISM 3700 DNA Analyser (Applied Biosystems).

II.10 Polymerase Chain Reaction (PCR)

PCR reactions consisted of 1µl of DNA template, 0.4µl of polymerase high fidelity (Expand High Fidelity PCR System) or long template (Long Template Polymerase) both from Roche, 1µl of a 10mM dNTPs mixture (Eppendorf), 2.5µl of a 10x $MgSO_4$ containing buffer (Roche), 1µl of each primer (20mM) and bi-distilled water to a final volume of 25µl. Reactions were run in an Eppendorf Mastercycler Personal as well as a Gradient PCR machine (Eppendorf).

For most of the reactions, the PCR was programmed as follows, unless otherwise indicated:

1) 94^0C 0:03:00
2) 94^0C 0:00:30
3) 55^0C 0:00:30
4) 72^0C 0:03:00
5) repetition of steps 2 to 4 for 30 cycles
6) 04^0C until analyses

Note: For nested-PCR's the products of the initial PCR reaction were diluted 1:10 and used as template for a subsequent PCR.

II.11 Cloning

1. Correcting the premature stop codon found in the pCEP vector containing the so-called "Full-length teneurin-1 # 5"

The pCEP-vector containing full-length teneurin-1 # 5 (Minet; not published) was sequenced and found to have a mutation generating a stop codon at 2161 bp, truncating the protein at 722aa. Thus the original construct encoded a teneurin-1 protein with a truncation just after the 7^{th} EGF-like repeat. The cDNA region containing the premature stop codon was excised using PmlI and NotI and a series of PCRs were performed in order to correct the mutation by site-directed mutagenesis.

After sub-cloning the 2.5bp fragment back into the pCEP vector using the PmlI and Not I restriction sites, sequencing was used to identify clones where the mutation had been successfully removed. Subsequently, the clone expressing the truncated protein was termed medium-length (ML) teneurin-1, as the protein encoded roughly half the size of the full-length (FL), and the new corrected construct was defined as FL. Briefly, the region to be corrected was used as a template to design primers that would generate a sequence where the corresponding stop codon was exchanged. In the first PCR reaction amplified, primer forward A was used with primer reverse D to amplify a 2618bp fragment and in the second PCR reaction primer forward C was used with primer reverse B to amplify a 380bp fragment. Finally, the last PCR reaction put both products together using primers A and C and amplified a fragment of approximately 2500bp which was sub-cloned back into the pCEP vector and checked if it contained the right sequence. The following sequence correction was made:

^{2158}GGGTAGTGT^{2167} into ^{2158}GGGAAGTGT^{2167}

^{721}K*E^{723} into ^{721}KCE^{723}

Primers used are defined as follows: **A** (5' CTGCACTCACACTTCCCGTCTTTGCA 3'); **B** (5' AAAGACGGGAAGTGTGAGTGCAG 3'); **C** (5'AGGTGTTCCGTCTATT GC3'); **D** (5' AGAGCTCGTTTAGTGAACCG 3').

2. Cloning of putative splice variants of the XM_039676 gene

To confirm if the different bands observed in the nested PCR were spliced forms of the XM_039676 transcript, they were cloned and sequenced using the following protocol:

- excise band(s) from gel and purify according to the standard protocol from QIAquick Spin Handbook (Qiagen)
- ligate 7.5µl of the insert directly into 0.5µl of p123T vector
- transform into XL-Gold competent cells and seed in LB^+ after addition of X-gal and IPTG following standard protocol
- grow colonies overnight. Check for the presence of the insert by digestion with XhoI and XbaI enzymes

II.12 RNA extraction for Microarray Analysis

Before starting to work with RNA, all materials were treated with DEPC water to inactivate RNAses. For this experiment 15ml Corex tubes (n°8441) were treated

overnight with DEPC water, followed by autoclaving. Total RNA was extracted from 10cm cell culture dishes using the following protocol:

- remove medium and wash cells once with PBS^-

Homogenization:

- lyse cells by adding 5.5 ml Trizol-reagent (Life Technologies) directly into the dish. Immediately pipette up and down several times

Phase Separation:

- incubate sample at RT for 5 minutes
- add 1.1 ml of Chloroform (do one sample at a time)
- shake by hand for 15 seconds
- change to DEPC-treated Corex tubes
- incubate sample at RT for 2 - 3 minutes
- using the Sorvall SS-34 rotor, centrifuge samples with 12000g at 2 - 8^0C for 15 minutes
- keep oil phase for protein, proceed with the aqueous phase

RNA Precipitation:

- transfer aqueous phase to new Corex tubes
- add 2.75 ml of isopropyl alcohol and mix
- incubate sample at RT for 10 minutes
- using the Sorvall SS-34 rotor, centrifuge samples with 12000g at 2 - 8^0C for 12 minutes

RNA Wash:

- remove supernatant
- wash pellet by adding 6 ml of 75% ethanol
- vortex and freeze at -20^0C if stopped here, otherwise only vortex
- using the sorvall SS-34 rotor, centrifuge samples with 7500g at 2 - 8^0C for 5 minutes

Redissolving the RNA:

- dry RNA pellet (air dry) for 10 minutes (no centrifugation)
- dissolve RNA in 100 µl RNAse free water by pipetting the solution a few times up and down
- incubate at 55 - 60^0C for 10 minutes

- store at -20^{0}C overnight until used or proceed for RNA re-purification using RNA Clean up following manufacturer's protocol (RNAeasy; Qiagen).

Note: RNA concentration should be > 1.5µg/µl with an A260:280 ratio > of 1.9 determined using either 10mM Tris or TE pH 7.5

II.13 cDNA generation

cDNA for mRNA expression analysis by RT-PCR was generated using either random or oligo-dT primers and ThermoScript (15U/µl) from the ThermoScript™ RT-PCR System (Invitrogen) following instructions of the manufacturer.

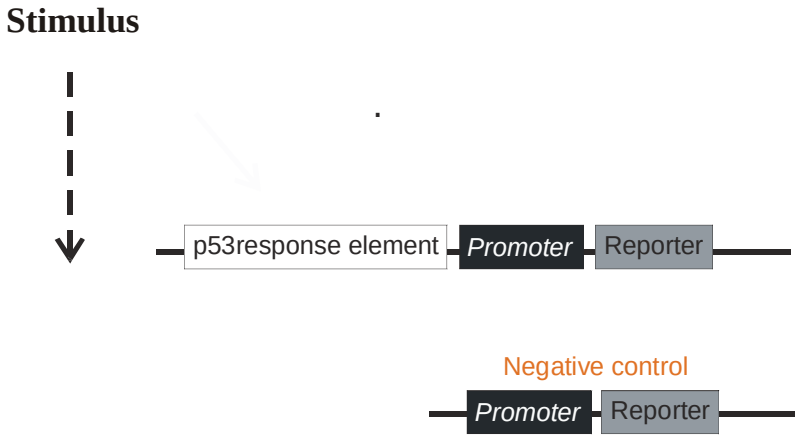
II.14 Microarray analysis

Microarray hybridisation and assistance with data mining was a service provided by the institute. Briefly, total mRNA (>5µg) was extracted from cells that stably express IDten-1 and from vector control cells. The mRNA was reverse-transcribed into cDNA using the genechip one-cycle cDNA Kit from Affymetrix and then *in vitro* transcribed into cRNA in the presence of biotinylated UTP using the Affymetrix IVT labelling Kit. After fragmentation, the antisense cRNA samples were hybridised to Affymetrix U133A GeneChips, washed, stained with streptavidin-phycoerythrin (Molecular Probes) and scanned in an Affymetrix GCS 3000 scanner. Expression data were generated using the RMA package from Bioconductor (Irizarry et al., 2003) and imported into GeneSpring 6.2 (Silicon Genetics, Santa Clara, CA, USA). In total, 12 chips were used to produce data in duplicate and from separate days, for the statistical analyses. The experiment consisted of a time course of 0, 6, 12 and 24 hours after induction of IDten-1 protein expression with ponasterone. Samples in which IDten-1 expression was induced were compared to control samples expressing the empty pIND vector. Genes were pre-filtered whose expression value was ≥ 100 units in at least one condition with a fold change of ≥1.5 relative to the time point 0. Fold change lists were combined and genes were further considered that passed at least a 1-Way ANOVA ($p \leq 0.05$). To minimise the effect of multiple testing errors, a Benjamini and Hochberg correction to the ANOVA was performed. A Tukey *post*-hoc test was then applied in order to identify the origin of the significant effects within the time course. The lists containing the genes that passed the Tukey *post*-hoc test are not shown in this thesis. Moreover, a 2-Way ANOVA test was applied to refine the analysis. Ho

(null hypothesis) was that treatment with ponasterone has no effect on the kinetics of gene expression over time. This hypothesis was rejected when the interaction P-value was ≤ 0.05. Finally, genes present in the 1-Way and in the 2-Way ANOVA lists were functionally classified using the following website: http://fatigo.bioinfo.cnio.es/. FatiGO stands for Fast transference of information using Gene Ontology (Ashburner et al., 2000) and is a web-based interactive tool that classifies an input text file containing, for example, affymetrix probeset IDs into categories according to biological processes, molecular function or cellular components. This tool was developed in the Bioinformatics Department of the Spanish National Cancer Center.

II. 15 Luciferase Assays

Reporter assays were performed using a commercially available Mercury Pathway Profiling detection system (Clontech) that allows expression of luciferase under the control of the p53 promoter. The Luciferase Assay System is commercially available from Promega. Briefly, U2OS and CEF cells were seeded in 6 well dishes and were transfected after 12 hours either with the IDten-1 plus pSV-β-galactosidase or with the vector control plus pSV-β-galactosidase. pSV-β-galactosidase is used to normalize samples as internal control. After 24 hours cells were lysed in a special reporter luciferase buffer and processed as described in (Bagutti et al., 2003).



II.16 Antibody generation

N-and C-terminal sequences of the chicken teneurin were used to derive primers to amplify PCR products (see details for N-terminal primer-set sequences and C-terminal primer-set sequences below). PCR products were cloned into the 6XHis-Tag

pQE-30 expression vector (Qiagen) using the KpnI and SphI restriction sites. This vector was transformed in XL-blue competent cells in order to propagate the DNA. The isolated purified DNA was then used to transform the M15 [pREP4] *E.coli* strain. Expressed fragments were transformed into bacteria to yield recombinant protein, which was purified on a Nickel column (Qiagen). Purified recombinant protein fragments were injected into 4 rabbits (RCC Ltd, Switzerland), giving rise to two antisera against the N-terminus and two antisera against the C-terminus of teneurin-1 (termed anti-A, anti-B, anti-C anti anti-D, respectively). For sake of simplicity, the antibody termed anti-B was designated anti-N in the subsequent work. All antisera were purified on a protein-A column (Affi-Prep Protein A Matrix- BIO-RAD) in order to concentrate immunoglobulins of the IgG subtype (see II.17 for details). Constructs were amplified using the following primer sets:

01-N terminus (first 160aa):

CT_Nt_SphI_5 (5' ATTCAGGCATGCATGGAGCAGATGGACTGC 3')

CT_NtS_Kpn_3 (5' GCTCCCGGTACCGTCATTCTCCCCATCAGAC 3')

02-N terminus (first 300aa):

CT_Nt_SphI_5 (5' ATTCAGGCATGCATGGAGCAGATGGACTGC 3')

CT_NtL_Kpn_3 (5' ACTCAGGGTACCGCACTTCCAGTTGCAACAC 3')

03-C terminus (last 334aa):

CT_Ct_SphI_5 (5' CCAAACGCATGCATATGGAAACACCTGAATGC 3')

CT_Ct_KpnI_5 (5' CGTAGGTACCTTACCTTCTGCCTATTTCAC 3')

Anti-teneurin-1 antibodies and antibodies from commercial sources are:

Antibody	Company/supplier	WB dilution	IF dilution
HA (12CA5)	Roche	1:100	1:100
PML (PG-M3)	Santa Cruz	1:1000	1:1000
Myc (9E10)	Santa Cruz	1:1000	1:1000
β-galactosidase	3prime5prime	not tested	1:500
BrdU	PharMingen	not tested	1:500
Acetyl-histone H4	Upstate	1:100	1:100
Flag (M2)	Stratagene	1:1000	1:1000

Ponsin	Upstate	1:1000	1:300
MBD1	Abgent	1:100	1:300
phospho-p53	Santa Cruz	1:500	not tested
phospho-MAPK	cell signaling	1:1000	not tested
Tubulin	Sigma	1:200	not tested
N-terminus teneurin-1	RCC	1:300	1:300
C-terminus teneurin-1	RCC	1:300	1:300
goat anti rabbit FITC-conjugated	Alexa	not applicable	1:1000
goat anti mouse rhodamine-conjugated	Alexa	not applicable	1:1000

II.17 Affinity Chromatography of Teneurin-1 antibodies

Affi-Prep protein A (BIO-RAD) consists of highly purified protein-A covalently coupled to a polymer matrix gel and was used to bind immunoglobulins, thereby enriching the polyclonal anti-teneurin-1 antibodies. The system used MAPs buffer that increases protein-A's capacity to bind IgGs and IgMs from many different species. The following antibodies were purified using this method: anti-A and anti-B (both against the N- terminus of teneurin-1) plus anti-C and anti-D (both against the C-terminus of teneurin-1). In addition, the pre-immune serum from rabbit B, which was used as control in immunostaining experiments, was also purified.

Purification protocol based on manufacturer's instructions and (Harlow and Lane, 1999)

- dilute serum which contains approximately 10mg/ml of polyclonal antibody in binding buffer in a ratio of 1:2 (serum:buffer)
- filter diluted serum through a 0.45 or 0.8µm filter before loading onto the column (with a syringe adaptor)
- pack appropriate volume of beads onto the column (calculate approximately 1ml of beads for 1ml of serum before dilution). Bead loading capacity for rabbit IgG is 9-16 mg/ml and 5-7 mg/ml for IgM
- equilibrate column applying binding buffer until the eluate reaches the pH of 9.0
- load the serum (diluted and filtered) onto the column

- let it flow through very slowly, collect the flow through
- pass the flow through over the column again
- wash with 10-15 bead volumes of binding buffer (apply 0.5ml at a time) until the OD_{280} has decreased
- elute with 15 bead volumes of elution buffer (apply 0.5ml at a time)
- collect the eluate in 0.5ml volumes in eppendorf tubes containing 1/10 column volume of 1M Tris-HCl pH 8.0. Mix gently
- identify the IgG-containing fractions by absorbance reading at 280nm ($1OD_{280}$ = approximately 0.8mg/ml). Combine antibody-containing fractions, run 2µg of total protein on a SDS-PAGE gel and stain with Coomassie blue. Pure heavy and light chain bands should be seen.

MAPs buffer: Binding Buffer: (3.2M NaCl, 1.6M Glycine pH 9.0). Elution Buffer: (100mM NaCitrat pH 3.0)

II.18 Cell culture

Primary cell culture of Chicken Embryo Fibroblasts (CEF): Fertilized eggs were grown in a humidity and temperature-controlled incubator until the desired developmental stage was reached. CEF cells were derived from 11-day-old embryos using the following protocol:

- rinse eggshell with 70% ethanol and break it open
- transfer embryo directly into a 10cm plate containing a physiological solution such as PBS- and immediately dissect the head off
- transfer the body to a clean dish and dissect the skin with sterile forceps
- place the skin in a Falcon tube containing 10ml of a trypsine/EDTA solution (T/E)
- incubate at 37^0C for one hour to facilitate cell dissociation
- pass cells through a cell strainer (Falcon, nylon 70µm) and pipette up and down a few times before seeding them into a new 10cm dish.
- cultivate cells in DMEM containing 10% FCS
- split cells at 70% confluence

Cos-7 (green monkey kidney), HT1080 (human fibrosarcoma), U2OS (human osteosarcoma) cells and HEK 293 (human embryonic kidney) cells: All

immortalized cells lines described above were maintained in DMEM with the addition of 10% FCS, unless otherwise specified. Cells were grown in 10cm cell culture dishes and always split at 80% confluence.

EcR-293 cells (Invitrogen): commercially available from Invitrogen. EcR-293 cells stably express the ecdysone receptor from the pVgRXR vector. They were maintained in DMEM with the addition of 10% FCS and 25µg/ml of zeocin.

II.19 Generation of stable cell lines

Briefly, EcR-293 cells were transfected with the pIND vector containing the construct of interest (encoding IDten-1, MLten-1 or FLten-1). For the current study an ecdysone inducible system was used to generate stable cell lines designated EcR-IDten-1, MLten-1 or FLten-1 (EcR-FLten-1 was generated by Marianne Brown-Luedi). As control, stable cell lines containing the empty vector were also generated, and termed "pINDvector". In order to induce protein expression, a synthetic analogue of ecdysone (ponasterone) was added to the medium at a final concentration of 10µM. To generate inducible stable cell lines expressing the various forms of teneurin-1 this protocol was followed:

- Thaw and cultivate EcR-293 cells in DMEM + 10%FCS supplemented with 25µg/ml of zeocin
- Cultivate cells for a few generations before transfection
- Transfect cells with pIND vector containing the gene of interest (see II.21.)
- The following day, add G-418 (400µg/ml) to the media in order to select for the transfected cells
- Cultivate cells until they reach 70% confluency and then split cells at several dilutions into 10cm plates in order to obtain growth of single clones. (suggested dilutions are 1:40, 1:60, 1:120, 1:240, 1:480)
- Once growth of single colonies is visible, pick and transfer each individual colony to a 24-well plate.
- Cultivate clones further and assay for protein expression (e.g. by Western blotting)

Note: Positive clones are propagated further and stocks are frozen.

II.20 Splitting, freezing and thawing of cells

Splitting: At 80 % confluence, cell layers were rinsed once with PBS^- (heated to 37^0C in a water bath) and trypsinised with a solution consisting of a mixture of trypsin/EDTA to disrupt cell-cell and cell-substratum interactions. The trypsin was inactivated by the addition of DMEM to the plate.

Freezing: cells were treated as above, and collected by centrifugation. The pellet was resuspended in cold freezing media (normal medium supplemented with FCS and DMSO). Recipe for 10ml: 6ml DMEM, 3ml FCS and 1ml DMSO. Cells were then transferred into cryo-tubes (Nunc), placed in a Styrofoam box and frozen at -80^0C. Note: for long-term storage cryo-tubes should be transferred to liquid nitrogen.

Thawing: cells were thawed in a water bath at 37^0C and quickly transferred to a Falcon tube containing 10 - 15 ml of DMEM + 10% FCS. After centrifugation the supernatant was discarded and the cell pellet was resuspended in DMEM +10%FCS. Cells were seeded into cell culture dishes and grown in an incubator at 37^0C + 5% CO_2.

II.21 Transfection of plasmid DNA into cells

Exogenous DNA was delivered into cells with the lipid based FuGENE 6 transfection reagent (Roche) using the ratio 3:1 (µl reagent/µg DNA) following the manufacturer's procedure.

II.22 Protein extraction from cell culture

Cells were cultivated until they reached 80% confluence. Medium was removed and after one wash with PBS^- the cells were directly lysed in the dish on ice for several minutes either in RIPA buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 50mM NaF and 0.5mM Na_3VO_4) or NP40 buffer (50mM Tris-HCl pH 7.4, 250mM NaCl, 5mM NaF, 5mM EDTA, 0.1% NP40 complemented with protease inhibitors cocktail from Roche). With a cell scraper, the cell lysate was transferred to an eppendorf tube and centrifuged at 11000rpm for 5 minutes at 4^0C to pellet cell debris. The supernatant was kept at -20^0C until use.

Protein concentration in each lysate was determined using the BIO-RAD Protein Assay Kit following their specifications.

II.23 Western blotting

All Western blots described in this thesis used the minigel system from BioRad and were run in a BIO-RAD 3000/300 or a Pharmacia Electrophoresis EPS 3500 power supply. Polyacrylamide Gel Electrophoresis (PAGE) gels were transferred to PVDF membrane using a Semi-Dry blotting apparatus from Millipore.

- Pour the desired percentage of acrylamide solution using the BioRad minigel system and allow to polymerise
- place the polymerised gels into the running chamber, fill with running buffer and load samples into wells
- run each gel applying 25mA for the stacking front and increase to 35mA at constant currant as soon as the samples reach the separating front
- if no transfer to PVDF membrane is needed, stain gel with GELCODE Blue Stain (Pierce)
- if needed, transfer proteins from mini-gel to PVDF membrane using the semi-dry blotting apparatus
- first soak 6 pieces of Whatman paper (3MM) in blotting buffer and place 3 pieces onto the anode side of the apparatus
- moisten the PVDF membrane for one minute in methanol and then leave it in blotting buffer until the “sandwich” is ready to be assembled
- transfer the membrane on top of the Whatman papers
- carefully transfer the acrylamide gel on top of the membrane
- complete the "sandwich" by placing the additional 3 pieces of Whatmann paper on top of everything. (use a glass rod to roll over the surface for the removal of air bubbles)
- apply a constant current of 45mA per minigel for 1 to 2 hours depending on the size of the protein to be transferred
- stain the gel with amidoblack (0.1% amidoblack, 40% isopropanol, 1% acetic acid) and take a picture of the transferred proteins
- block the membrane with 3% skin-milk in TBS-T for one hour

- incubate primary antibody at the appropriate dilution in blocking buffer for one hour at RT or 4^0C overnight in a humid chamber
- wash the membrane 3x in TBS-T (10 minutes each)
- incubate the secondary antibody for 45 minutes at RT
- wash as above and incubate the membrane with ECL detection solution (Amersham Life Science) for 1 minute, then drain the excess ECL and place the membrane between two pieces of transparent plastic inside the film cassette
- in the dark room, expose a film on top of the membrane and develop the film

Running gel: 2.5ml of lower buffer, adequate volume of acrylamide from a 30% solution (BioRad), 30µl of ammonium persulfate (20% ammoniumpersulfate w/v), 10µl of TEMED (Fluka) and distilled water up to 10ml.
Stacking gel: 1.25ml of upper buffer, 0.5ml of acrylamide from a 30% solution (BioRad), 15µl of ammonium persulfate (20% ammoniumpersulfate w/v), 10µl TEMED (Fluka) and distilled water up to 5ml.

percentage of separating gel	3%	5%	6%	7.5%	10%	12.5%	15%	20%
acrylamide (30%) in ml	1.0	1.7	2.0	2.5	3.3	4.2	5.0	6.7

Blotting buffer: 48mM Tris/HCl pH 9.2, 39mM glycine, 1.3mM SDS, 20% methanol
10x Running buffer: 250mM Tris/HCl, 1.9M glycine and 35mM SDS
4x Loading buffer: 200mM Tris pH 6.8, 4% SDS, 17.4% glycerol and 2.8M β-mercaptoethanol
4x Lower buffer: (pH 8.8) 1.5M Tris/HCl and 13.9mM SDS
4x Upper buffer: (pH 6.8) 0.5M Tris/HCl and 13.9mM SDS

II.24 Immunofluorescence

For the visualization of cells by immunofluorescence, cells were seeded onto special 35 mm 4-well dishes (Greiner). After approximately 12 hours, the general protocol described below was followed:

- transfect cells with desired constructs
- after approximately 24 hours, aspirate the medium

- rinse cells once with PBS^-
- fix cells with 4% paraformaldehyde in PBS^- for 15 minutes at 37^0C
- if cells need to be permeabilized (e.g. for nuclear staining) incubate with 0.1% Triton X-100 in PBS^- for 5 minutes
- then rinse two times with PBS^-/ otherwise rinse three times with PBS^- directly after the paraformaldehyde fixation
- block with 3% goat serum in PBS^- for 15 minutes (1ml per dish at RT)
- apply first antibody diluted in blocking buffer (use 70µl per well)
- leave for one hour at RT or 4^0C overnight in a humid chamber
- wash 3 x with PBS^- (10 minutes each)
- apply secondary antibodies goat anti-rabbit FITC conjugated and/or goat anti-mouse rhodamine conjugated at 1:1000 (70µl per well) note: when staining of DNA is desired, Hoechst can be diluted (1:200 - 1:500) in the solution together with the secondary antibodies.
- leave one hour in the dark
- wash as before
- carefully aspirate each well
- apply 10µl mounting medium (Moviol, from Calbiochem) per well
- carefully place a coverslip on top of each well and avoid air-bubbles
- store in the dark at 4^0C for a while (one hour) until visualization by microscopy

II.25 Immunoprecipitation and co-immunoprecipitation

1. Co-immunoprecipitation from cell culture extracts

The following protocol was used:

- Cos-7 cells were plated in a 6 well dish and transfected with IDten-1 and ponsin
- after 24 to 48 hours wells containing approximately $2.5x10^6$ cells each were frozen on dry ice, thawed and scraped in 300µl of PBS^- complemented with proteinase inhibitors (complete cocktail tablets from Roche)
- centrifuge cell extract at 14'000rpm, discard pellet containing debris and proceed with supernatant
- add 150µl RIPA buffer to the supernatant to obtain a final volume of 450µl

- add 9µl (approximately 6 µg) of anti-N antibody. As control, one can add to a parallel sample an unrelated antibody (in this case the pre-immune serum of the anti-N antibody)
- rotate cell extract for 90 minutes at 4^0C
- add 30µl of protein-A Sepharose beads (4 Fast Flow; Amersham) pre-washed in RIPA buffer. The specificity of the antibody for the protein was checked by incubating a part of the sample with beads only. This step ensures that the protein of interest in not being pulled down by the beads, but only by the antibodies.
- rotate for 60 minutes at 4^0C
- collect beads by centrifugation, take an aliquot of the supernatant (unbound fraction)
- wash beads 2x with PBS^-
- add 50µl of SDS-PAGE sample buffer to the beads, boil (shake occasionally) for 5 minutes at 95^0C
- pellet the beads by centrifugation in a mini-centrifuge
- analyze samples by immunoblotting

2. Immunoprecipitation of endogenous teneurin-1

Brain, heart and liver tissues were dissected from an E17 embryo and processed as follows:

- disrupt tissues on ice in an dounce homogenizer in RIPA buffer with 15 to 20 strokes using a tight pestle
- transfer homogenate to an eppendorf tube and centrifuge at full speed for 5 minutes at 4^0C ; proceed with the supernatant
- measure protein concentration with a Bradford assay
- take the equivalent to 500µg of total protein and complete to a final volume of 500µl with RIPA buffer.
- pre-clear lysate with protein-A sepharose beads for 20 to 30 minutes at 4^0C
- spin beads down using a mini-centrifuge. Discard them and to the supernatant, add 6µg of anti-N antibody. Rotate on a rotating device overnight at 4^0C
- next day, add 40µl of protein-A beads washed in RIPA buffer to precipitate the immunocomplexes and rotate for additional 4 hours at 4^0C

- spin beads down using a mini-centrifuge and take an aliquot of the supernatant (unbound fraction)
- wash beads 3x with PBS^-
- add 50µl of SDS-PAGE sample buffer to the beads, boil for 5 minutes at 95^0C
- collect beads by centrifugation in a mini-centrifuge
- analyse samples by immunoblotting. Note: Because the anti-N antibody was used both for immunoprecipitation and detection, incubation with a secondary antibody was replaced by incubation with protein A-HRP (1:2500) from ICN/CAPPEL instead.

II.26 Preparation of the nuclear matrix

1. Immunofluorescence: The methodology described in Nickerson et al. was followed (Nickerson, 1998). Note: Prepare three times the amount of extraction buffer as compared to the cytoskeleton and digestion buffers.

Cos-7 cells were seeded into four-well (35/10mm; Greiner) immunofluorescence dishes in DMEM 10% FCS. At 70% confluence cells were co-transfected with: IDten-1 plus MBD1 or IDten-1 plus PML. Approximately 24hs later, cells were washed twice in PBS^- and further treated as follows:

- on ice, extract cells by applying 70µl/well of cytoskeleton buffer for 3-5 minutes. This step removes soluble cytoplasmic and nucleoplasmic proteins
- on ice, apply 70µl/well of extraction buffer for 5-7 minutes. This step removes histone H1 and cytoskeletal proteins
- treat dishes with 70µl/well of digestion buffer (31^0C; 30-50 minutes). This step removes the DNA and remaining histones
- wash preparation twice with 70µl/well of extraction buffer (5 minutes per wash)
- fix with 100µl/well of 4% paraformaldehyde (>20 minutes)
- wash dishes three times in PBS^- (10 minutes each)
- block with 1ml per dish of 3% goat serum in PBS^- (15 minutes)
- incubate each well with 70µl of blocking buffer containing the primary antibodies diluted: anti-N(1:350), anti-PML(1:100), anti-MBD1(1:300), anti-PISN(1:300). Treat overnight at 04^0C
- wash dishes as before

- incubate each we`ll with 70µl of blocking buffer containing the secondary antibodies diluted: goat anti-rabbit FITC conjugated and goat anti-mouse rhodamine conjugated (1:1000). Treat for 1h in the dark at room temperature. To check if the treatment removed heterochromatic DNA stain with Hoechst (1:200) simultaneously with the secondary antibody. Staining should be negative.
- wash dishes as before and mount coverslips with 10µl/well Moviol (Calbiochem). Leave in the dark until analyses.

Cytoskeletal Buffer

Aliquots stored at -20^{0}C:

10mM PIPES pH 6.8, 300mM sucrose, 100mM NaCl, 3mM $MgCl_2$, 1mM EGTA

To be added just prior to use:

0.5% Triton X-100, 80u/ml RNAseOUT^{TM} Ribonuclease inhibitor (Invitrogen), 0.2mg/ml PMSF

note: other protease inhibitors except for EDTA can be added if proteolysis is suspected.

Extraction Buffer

Aliquots stored at -20^{0}C:

10mM PIPES pH 6.8, 250mM ammonium sulfate, 300mM sucrose, 3mM $MgCl_2$, 1mM EGTA

To be added just prior to use:

80u/ml RNAseOUT^{TM} Ribonuclease inhibitor (Invitrogen), 0.2mg/ml PMSF

Digestion Buffer

Aliquots stored at -20^{0}C:

10mM PIPES pH 6.8, 300mM sucrose, 50mM NaCl, 3mM $MgCl_2$, 1mM EGTA

To be added just prior to use:

0.5% Triton X-100, 80u/ml RNAseOUT^{TM} Ribonuclease inhibitor (Invitrogen), 0.2mg/ml PMSF, 200u/ml RQ1 RNase-Free DNase (Promega)

Triton Stock: 10%(w/v) Triton X-100. This is a 20x stock solution which is frozen in aliquots at -20^{0}C.

PMSF Stock solution is 20mg/ml in isopropanol, stored at room temperature.

2. Western blotting: The methodology described in Lee and Skalnik was followed (Lee and Skalnik, 2002).

Cos-7 cells were plated in 6 well dishes in DMEM 10% FCS. At 70% confluence cells were transfected with PML and co-transfected with IDten-1 and MBD1.

Approximately 24hs later, cells were fractionated as follows:

- remove supernatant and wash twice with cold PBS^-
- add 200µl CSK buffer complemented immediately before use with proteinase inhibitors, DTT and triton (see concentrations below)
- leave on ice in a shaking device for 5 minutes
- spin down at 5000g for 3 minutes
- keep supernatant (corresponds to the soluble fraction) to be loaded onto the gel later
- resuspend pellet in digestion buffer which is composed of CSK buffer added by 30 units of RQ1 RNase-Free DNase (Promega) (volume to be added should be tested empirically, but 50µl is a good start)
- incubate at 37^0C for 20 minutes
- add ammonium sulphate to a final concentration of 0.25M to the solution (e.g. 12.5 µl from a 1M stock into 50 µl)
- leave on ice for 5 minutes
- spin down at 5000g for 3 minutes
- keep supernatant (corresponds to the chromatin fraction) to be loaded onto the gel later
- resuspend pellet in CSK buffer containing 2M NaCl (volume to be added should be tested empirically, but 50µl is a good start)
- leave on ice for 5 minutes
- spin down at 5000g for 3 minutes
- keep supernatant (corresponds to the washing fraction) to load onto the gel later
- resuspend pellet in 8M urea to solubilize the nuclear matrix fraction (again, the volume to be added depends on how concentrated your protein of interest will be in this fraction).
- load an equivalent amount of protein determined by Bradford assay of each sample fraction and check where your protein of interest co-fractionates by SDS-PAGE and Western blotting.

Cytoskeletal Buffer

Aliquots stored at -20^{0}C:

10mM PIPES pH 6.8, 300mM sucrose, 100mM NaCl, 3mM $MgCl_2$, 1mM EGTA

To be added just prior to use:

leupeptin, aprotinin and pepstatin (1μg/ml each), 1mM PMSF, 1mM DTT, 0.5% Triton X-100

II.27 BrdU incorporation assay

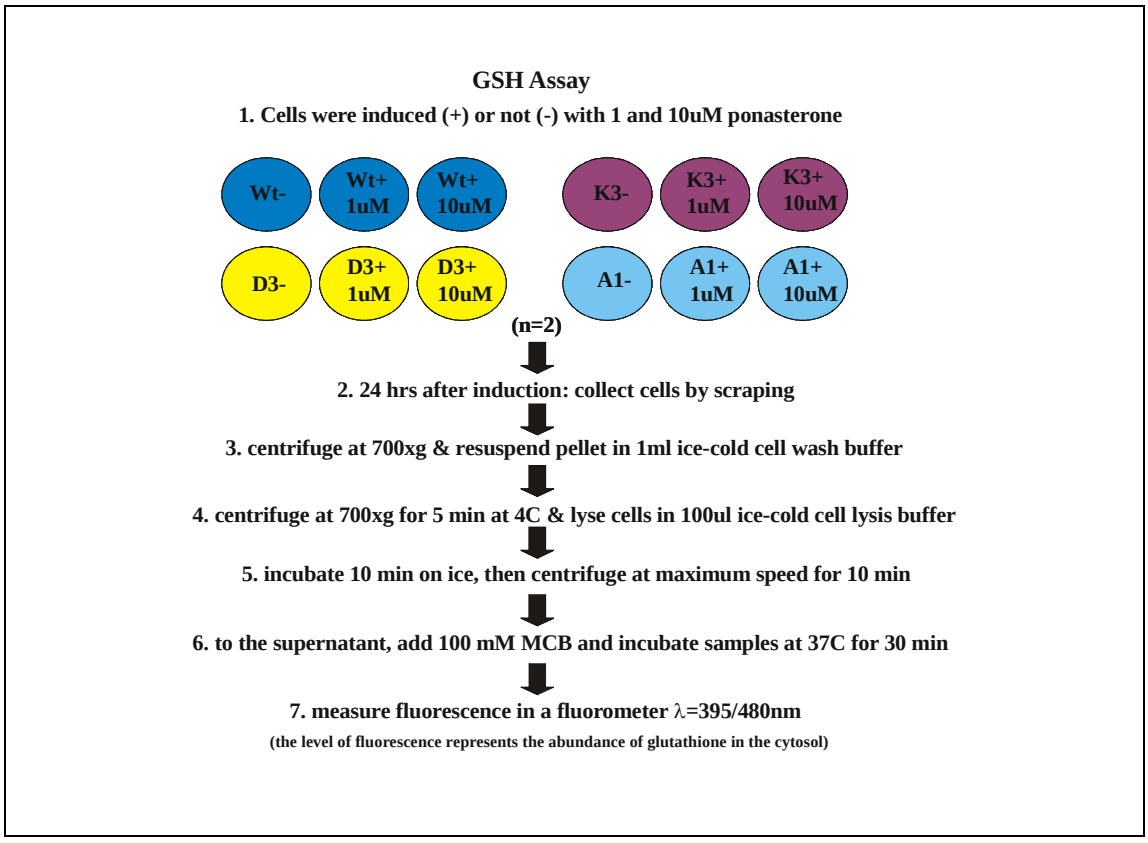
The methodology described in Lebrin et al. was followed (Lebrin et al., 2001). BrdU is an analogue of thymidine, allowing the detection of DNA synthesis in proliferating cells. Added to the media, cells incorporate BrdU in place of thymidine during DNA synthesis (S-phase of the cell cycle). Cells that could be visualized as positive for both β-galactosidase (β-gal; used as internal control for transfected cells) and BrdU were counted as positive for BrdU incorporation. BrdU and β-gal positive cells were detected by indirect immunofluorescence using a Zeiss Axiophot microscope (Carl Zeiss Ltd.) connected to a 3CCD camera (Sony). Per slide, at least 100 β-gal positive cells in five different fields were counted and each β-gal positive cell was checked for having or not incorporated BrdU.

- plate CEF/U2OS cells onto (35/10mm) immunofluorescence dishes in DMEM 10% FCS
- at a confluency of approximately 50%, co-transfect cells with β-gal plus IDten-1 or with β-gal plus empty vector pCEP-pu
- add BrdU to a final concentration of 20μM into the media and incubate cells for a further 17 +/- 3 h or until 80% confluency
- fix cells in 3.7% paraformaldehyde for > 20 minutes at RT
- rinse once with PBS^-
- treat cells with 2N HCl for 30 minutes at RT to render the chromatin accessible
- wash 5x with PBS^-
- block unspecific sites with 3% BSA in PBS^- for 1h at RT
- incubate primary antibodies (anti-β-gal 1:500, anti-BrdU 1:500), overnight at 4^{0}C
- wash 3x with PBS-T (10 minutes each)

- incubate secondary antibodies (goat anti-rabbit FITC conjugated, goat anti-mouse rhodamine conjugated at 1:1000) and DAPI (1:200) for 1h in the dark
- wash as above and mount coverslips with moviol
- keep slides in the dark until visualization

II.28 Glutathione assay

The methodology described in the Manufactorer's instruction was followed (ApoAlert™ Glutathione Detection Kit - Clontech). The concept behind this method as specified by Clontech is that in healthy cells, glutathione acts as a redox buffer. However, in Jurkat and some other cell types, the plasma membrane contains an ATP-dependent glutathione (GSH) transport system that is triggered by the initiation of apoptosis. When GSH is actively pumped out of the cell, the cytosol is shifted from a reducing to an oxidizing environment (Wang et al., 2000). An overview of the experimental set up is presented below:



II.29 Nuclear and cytoplasmic extraction of proteins

The protocol by Timchenko et al. was followed (Timchenko et al., 1997). In order to enrich for nuclear and cytoplasmic proteins, chicken tissues were disrupted on ice with buffer A in a dounce homogeniser with 15 to 20 strokes using a tight pestle. Tissue debris was collected by centrifugation at 5000rpm at 04^0C for 10 minutes. The pellet was washed once in buffer A and the supernatant was frozen as the cytoplasmic fraction. Nuclei were then incubated in buffer B on ice for 30 minutes, and centrifuged as before to separate the supernatant containing the nuclear extract from the nuclear pellet containing the nuclear matrix.

Buffer A: 25mM Tris HCl pH 7.5, 50mM KCl, 2mM $MgCl_2$, 1mM EDTA and 5mM DTT

Buffer B: 25mM Tris HCl pH 7.5, 0.42M NaCl, 15mM $MgCl_2$, 0.5mM EDTA, 1mM DTT and 25% sucrose

note: add protease inhibitor cocktail (Roche) in all buffers

III. RESULTS

PART 1. **The intracellular domain of teneurin-1 interacts with MBD1 and CAP/ponsin resulting in subcellular codistribution and translocation to the nuclear matrix**

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The intracellular domain of teneurin-1 interacts with MBD1 and CAP/ponsin resulting in subcellular codistribution and translocation to the nuclear matrix

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Abstract

Teneurin-1 is a type II transmembrane protein expressed in neurons of the developing and adult central nervous system. To investigate the intracellular signaling of teneurin-1, we searched for proteins interacting with its intracellular domain. One of the proteins identified is the c-Cbl-associated protein CAP/ponsin, an adaptor protein containing SH3 domains. This interaction results on one hand in the recruitment of the soluble intracellular domain of teneurin-1 to the cell membrane enriched in CAP/ponsin. On the other hand, it leads to the translocation of CAP/ponsin to the nucleus, the major site of accumulation of the intracellular domain of teneurin-1. The second interacting protein identified is the methyl-CpG binding protein MBD1. In the nucleus, the intracellular domain of teneurin-1 colocalizes with this transcriptional repressor in foci associated with the nuclear matrix. We propose that these interactions are part of a specific signaling pathway. Evidence for cleavage and nuclear translocation of the intracellular domain has been obtained by the detection of endogenous teneurin-1 immunoreactivity in nuclear speckles in chick embryo fibroblasts. Furthermore, in the nuclear matrix fraction of these cells as well as in cells expressing a hormone-inducible full-length teneurin-1 protein, a teneurin-1 fragment of identical size could be detected as in cells transfected with the intracellular domain alone.

Keywords: ten-m; odz; Signaling; Nuclear matrix

Introduction

Multicellular organisms rely on cell–cell interactions that regulate and coordinate morphogenesis and differentiation during development. One important cell membrane protein involved in the determination of the segmental body pattern of *Drosophila* is the pair-rule gene *ten-m/odz* [1,2]. Teneurin-1 to -4 are the homologous proteins in vertebrates [3]. Their expression patterns imply a function during tissue interactions in limb development [4,5] as well as during the development of the nervous system [6,7]. Teneurins are type II transmembrane proteins [6,8]. They dimerize through their extracellular domains within the region containing EGF-like repeats [8]. The teneurin-specific extracellular region containing YD repeats seems to confer heparin binding activity to teneurin-1 and is able to promote neurite outgrowth when offered as a substratum [9]. Interestingly, the chromosomal location in humans harboring the teneurin-1 gene has been localized to Xq24/25, a region implied in an X-linked mental retardation syndrome [9]. Furthermore, the expression patterns of teneurin-1 and teneurin-2 in the developing avian visual system correspond with periods of target recognition and synaptogenesis [10]. All of these data indicate that teneurins are important in regulating the differentiation of neurons and may play a role in specifying neuronal circuits [10].

Recently, regulated intramembrane proteolysis (RIP) has emerged as a novel signaling mechanism in which transmembrane proteins are cleaved, resulting in the production

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of a soluble intracellular domain that translocates to the nucleus and regulates transcription (for a review, see [11]). We have previously demonstrated that teneurin-2 represents such a membrane-anchored transcription factor and that upon proteolytic cleavage its intracellular domain translocates to the nucleus where it is capable of influencing zic-mediated transcription of a reporter construct containing the apolipoprotein E promotor [12]. Zic genes are the vertebrate homologues of the *Drosophila* transcription factor gene *opa* which has been shown to act epistatically to the *Drosophila* teneurin gene *ten-m* and therefore cooperates with *ten-m* in the same signaling cascade [1].

Since all teneurins are highly conserved and share a common domain structure, we assume that they signal by similar mechanisms and postulate that the intracellular domain of teneurin-1 has important signaling functions. We, therefore, searched for proteins that interact with the intracellular domain of teneurin-1. Through the identification of binding proteins, we expected to get an indication of the signaling pathways affected by this domain. Using a yeast-two-hybrid screen, we demonstrate that c-Cbl-associated protein CAP/ponsin [13] and methyl-CpG binding protein MBD1 [14] interact with the intracellular domain of teneurin-1. We confirm these interactions in cell cultures and demonstrate that teneurin-1 is proteolytically processed and that the cleaved intracellular domain of teneurin-1 colocalizes with these interacting proteins in nuclear matrix-associated speckles.

Materials and methods

Constructs

The full-length teneurin-1 cDNA encoding amino acids 1–2705, a medium length construct ending in the seventh EGF-like repeat (amino acids 1–721), and the intracellular domain encoding amino acids 1–300 [9] were cloned into pCEP-Pu (kindly provided by J. Engel, Biozentrum, University of Basel). Each of these constructs was supplied with an N-terminal Flag-tag (MDYKDDDDK) resulting in the constructs FLten-1, MLten1, and IDten-1 used for transfection into eukaryotic cells (cf. Fig. 1A). The inserts of FLten-1 and IDten-1 were also subcloned into the ecdysone-inducible vector pIND (Invitrogen) for transfection into 293-EcR cells (Invitrogen).

The following parts of the intracellular domains of teneurin-1 and teneurin-2 were cloned into pNLexA (OriGene Technologies, Inc.): the full intracellular domains ID1 and ID2 encompassing amino acids 1–300 of teneurin-1 [9] and amino acids 1–376 of teneurin-2 [6], respectively, as well as the N-terminal and C-terminal halves of each of the intracellular domains ID1N, ID1C, ID2N, ID2C encompassing amino acids 1–160 and 161–300 in the case of teneurin-1 and 1–168 and 169–376 in the case of teneurin-2. The construct ID1C was further subdivided into two parts ID1Ca and ID1Cb encompassing amino acids 161–240 and 241–300 (cf. Figs. 2A and B).

The mouse MBD1 insert identified in the yeast-two-hybrid screen (see below and Results) was amplified using the following primers 5′ ATAAGCTTCCACCATGTACCCTTATGATGTGCCAG3′/5′ATCTCGAGCTACAAAACTTCTTCTTTCAACTG3′ derived from the library plasmid pJG4-5 and the PCR product was cloned into the mammalian expression vector pcDNA3 (Invitrogen) after restriction with *Hin*dIII and *Xho*I. This resulted in a construct encoding at the N-terminus an HA-tag and a few amino acids encoded by the library plasmid pJG4-5 (MYPYDVPDYASPEFGTS) followed in frame with amino acids 27–636 of the full-length mouse MBD1 sequence which can be found in the GenBank/EMBL databases (accession number NM_013594). Thus, this construct encodes, with the exception of the first 26 amino acids, the entire MBD1 protein.

A myc-tagged full-length expression plasmid for ponsin (ponsin-2 in pCMV5-myc as described in [15] was a kind gift of Yoshimi Takai (Osaka University Medical School, Japan) and a myc-tagged PML (p-CMX-PML [16,17]) was kindly supplied by Ronald M. Evans, San Diego.

Antibodies

His-tagged proteins encoding the N-terminal 160 amino acids and the C-terminal 334 amino acids were expressed in bacteria (pQE30; Qiagen) and purified on a Nickel column (Qiagen) according to the manufacturer's procedure. The purified fragments were injected into rabbits to raise the antisera termed anti-N and anti-C. The IgG fractions of these sera were purified over Protein A Sepharose (Amersham) according to standard procedures. From commercial sources, we obtained anti-myc antibody (9E10, Santa Cruz), anti-Flag antibody (M2, Stratagene), anti-HA antibody 12CA5 (Roche), anti-PML (PG-M3, Santa Cruz), anti-acetyl-histone H4 polyclonal antibody (Upstate), anti-MBD1 (ABGENT), Alexa594- and Alexa488-conjugated goat anti-mouse and goat anti-rabbit IgG (Molecular Probes), and horseradish peroxidase coupled anti-mouse and anti-rabbit IgG (Soccochim).

Yeast-two-hybrid screen

Yeast two-hybrid screens using the cytoplasmic domains of teneurin-1 and teneurin-2 (ID1C and ID2C) as baits were carried out with the DupLEX-A™ system (OriGene Technologies, Inc.) using a whole mouse 19-day embryo DupLex-A cDNA library (OriGene Technologies, Inc.) according to their user's manual (Version 2.9 8/98).

Cell cultures and transfections

HT1080 fibrosarcoma, U2OS osteosarcoma, and COS-7 green monkey kidney cells were routinely maintained in

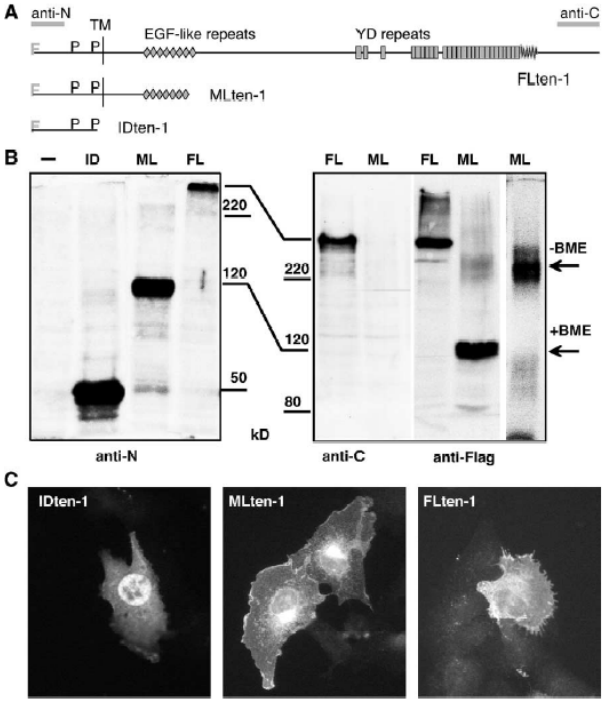

Fig. 1. Expression of teneurin-1 constructs in cultured cells. (A) Structural models of the full-length teneurin-1 (FLten-1), medium length teneurin-1 (MLten-1), and intracellular domain teneurin-1 (IDten-1) used for transfection of HT1080 and COS-7 cells. The grey F at the N-terminus indicates the FLAG-tag, P stands for polyproline stretches, and TM for the transmembrane domain. The EGF-like repeats and the YD repeats in the extracellular domain are labeled. Grey bars indicate the sequences used to express protein fragments in *E. coli* for the production of antisera anti-N and anti-C. (B) Immunoblots of a 7.5% gel (left panel) and a 6% gel (right panel) with the antibodies indicated of cell extracts of non-transfected COS-7 cells (−) or COS-7 cells transfected with IDten-1 (ID), MLten-1 (ML), and FLten-1 (FL) run under reducing conditions with the exception of the last lane showing a dimer of MLten-1 run under non-reducing conditions (arrow, −BME). (C) Immunostaining of HT1080 cells transfected with the indicated constructs using anti-N reveals cytoplasmic and nuclear staining of the soluble intracellular domain (IDten-1) and ER/Golgi staining and cell surface staining of the medium length (MLten-1) and full-length teneurin-1 (FLten-1) constructs.

DMEM medium supplemented with 10% FCS. Primary chick embryo fibroblasts (CEFs) were obtained by trypsinization of the skin from 11-day chick embryos. They were cultured in DMEM with 10% FCS. 293-EcR cells were transfected with either the empty vector pIND, or the constructs encoding FLten-1 or IDten-1 and clones were isolated that produced the respective proteins upon induction with ponasterone as described in the manual of the supplier of the cells and the expression vector (Invitrogen). These cells were termed EcR-FLten-1, EcR-IDten-1 and EcR-pIND, respectively.

For transient transfections, the cells were seeded in 6-well plates or 35-mm dishes containing four internal wells for immunostaining (Greiner). Twelve hours later, they were transfected with the indicated expression vectors (1 μg of each vector) by using FUGENE-6 (3 μl per μg of vector DNA; Roche). 24 h after transfection, the cells were rinsed with PBS and processed either for gel electrophoresis followed by immunoblotting, for immunoprecipitation, or for immunofluorescence staining.

Immunoblots, co-immunoprecipitation, and immunofluorescence

Cell layers of transfected COS-7 cells, EcR-FLten-1, and EcR-IDten-1 were directly lysed in RIPA buffer (150 mM NaCl; 50 mM Tris–HCl pH 8.0; 1% NP-40; 0.5% deoxycholic acid; 0.1% SDS; 50 mM NaF; 0.5 mM Na_3VO_4), subjected to SDS–PAGE, and blotted to PVDF membranes. The membranes were incubated with the appropriate primary antibodies (anti-N and anti-C diluted

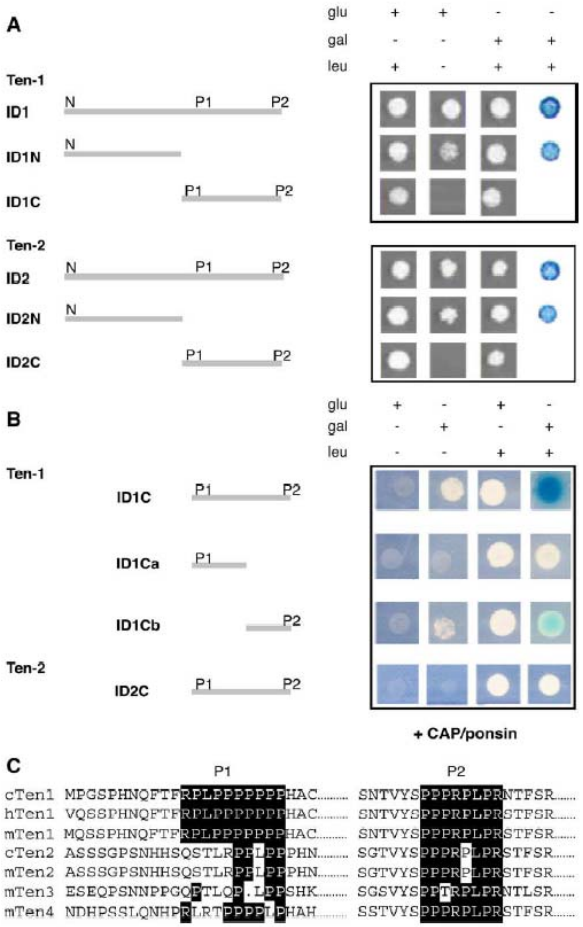

Fig. 2. Interaction of IDten-1 with CAP/ponsin in a yeast two-hybrid screen. (A) The intracellular domains of teneurin-1 (Ten-1) and of teneurin-2 (Ten-2) and an N-terminal fragment (ID1N/ID2N) and C-terminal fragment (ID1C/ID2C) of each teneurin were tested as fusion proteins with LexA as baits for a yeast two-hybrid-screen. Yeast transformed with the constructs indicated to the left (together with a beta-galactosidase construct under a LexA promotor) were grown on plates containing glucose (glu) or galactose (gal) in the presence or absence of leucine (leu) as indicated by +/−. Since ID1, ID1N, ID2, and ID2N resulted in yeast growth in the absence of leucine (leu) and to beta-galactosidase expression in the presence of galactose as revealed by the blue color production in the presence of X-gal in the panel on the right, they could not be used for further screenings. Constructs ID1C and ID2C could be used for further experiments. (B) Co-transfection of ID1C but not ID2C with a transcriptional activator construct fused to the third SH3 domain of CAP/ponsin resulted in the growth of yeast in the absence of leucine and in the production of beta-galactosidase, demonstrating an interaction between ID1C but not ID2C with CAP/ponsin. Separation of ID1C into two parts ID1Ca and ID1Cb demonstrated that the third SH3 domain of CAP/ponsin interacted with the fragment containing the second (P2) but not the first (P1) polyproline sequence. (C) Sequence alignments of chicken (cTen1), human (hTen1), and mouse (mTen1) teneurin-1, chicken (cTen2) and mouse (mTen2) teneurin-2, mouse (mTen3) teneurin-3, and mouse (mTen4) teneurin-4 of the two polyproline-rich segments P1 and P2 present in all intracellular domains.

at 1:350) followed by peroxidase-coupled secondary antibodies (diluted at 1:2000) and detection using a chemiluminescence kit (ECL, Amersham).

For co-immunoprecipitation, cell layers of transfected COS-7 cells were frozen on dry ice to break up cells, which were then scraped into 300 μl of PBS including

proteinase inhibitors (Complete proteinase inhibitor cocktail tablets; Roche). The cell extract was centrifuged at 14,000 rpm.

To the supernatant, 150 μl of RIPA buffer was added followed by immunoprecipitation with 8 μg of anti-N antibody and incubated in a rotating device for 90 min at 4°C. Then 30 μl of protein A Sepharose beads (4 Fast Flow; Amersham) washed in RIPA buffer was added. After 60 min of rotation at 4°C, the beads were collected by centrifugation and washed three times with PBS. Bound proteins were eluted by boiling in 50 μl SDS–PAGE sample buffer and analyzed by immunoblotting. The eluate was run in two parallel lanes on a 10% SDS–PAGE and blotted to PVDF membranes. As control, instead of anti-N, anti-C antibodies were added and the sample was processed as the others. The immunoprecipitated teneurin-1 was detected with anti-FLAG (diluted at 1:1000) and the co-precipitated ponsin by anti-myc (diluted at 1:1000) antibodies followed by secondary antibodies as described above.

For immunofluorescence staining, CEF and HT1080 cells in 35-mm dishes with four internal wells (Greiner) were fixed with 4% paraformaldehyde in PBS, washed 3 times in PBS, and blocked with 3% goat serum in PBS. The cells were incubated overnight at 4°C with primary antibodies diluted 1:100 in 3% goat serum in PBS. The dishes were washed three times with PBS and the appropriate secondary Alexa594- and Alexa488-conjugated antibodies were applied at a 1:1000 dilution for 1 h at room temperature. The dishes were washed three additional times with PBS and the coverslips were mounted with Moviol (Calbiochem). Finally, samples were examined using a Zeiss Axiophot microscope (Carl Zeiss Ltd.) connected to a 3CCD camera (Sony).

Subcellular fractionation and preparation of nuclear matrices

Transfected COS-7 cells, EcR-FLten-1, EcR-IDten-1, and CEF cells were fractionated into a soluble fraction, a chromatin fraction, a 2 M NaCl wash, and the nuclear matrix fraction following the procedure described in [18]. Of each fraction, an equal amount of protein determined by a Bradford assay (Bio-Rad) was analyzed on immunoblots with anti-PML (PG-M3; 1:100), with anti-acetylated histone H4 (1:1000), with anti-N (1:350) for IDten-1, with anti-HA (1:100) for MBD1, and with anti-myc (1:1000) for CAP/ponsin as described above.

Nuclear matrices were prepared for immunostaining of COS-7 cells co-transfected with either IDten-1 and PML, or with IDten-1 and MBD1 following the procedure described in [19]. The attached cells were extracted in the culture dish for the removal of all soluble cytoplasmic and nucleoplasmic proteins. Furthermore, the DNA was removed by digestion and the remaining nuclear matrix washed and fixed in 4% paraformaldehyde and processed for immunostaining as described above.

Results

Characterization of teneurin-1 expression in cell cultures

First, we characterized the full-length as well as shorter versions of ectopically expressed teneurin-1 in cell culture using antibodies developed against N-terminal (anti-N) and C-terminal (anti-C) fragments of teneurin-1. On immunoblots shown in Fig. 1B, we found that transfection of full-length teneurin-1 (FLten-1) in COS-7 cells resulted in the expression of a band of about 300 kDa monomer size detected by the anti-N and anti-C as well as by the anti-FLAG tag antibodies. Transfection of medium length (MLten-1) resulted in a band migrating at 120 kDa under reducing conditions and in a dimer of 240 kDa under non-reducing conditions. This observation is in agreement with the work done by Feng and colleagues with recombinantly expressed extracellular domain of Tenm-1 [20]. MLten-1 was recognized by the anti-N and anti-FLAG antibodies but not by anti-C since it does not contain the C-terminus of the intact protein. Finally, transfection of the shortest construct IDten-1 resulted in a monomer migrating at 50 kDa. The subcellular localization of the teneurin-1 proteins was analyzed by immunofluorescence. Expression of the intracellular domain alone (IDten-1) resulted in the presence of the protein throughout the cytoplasm as well as in an accumulation in the nucleus as can be seen from the staining obtained with anti-N in Fig. 1C. The medium length protein truncated after the EGF-like repeats (MLten-1) and the full-length protein (FLten-1), both of which include the transmembrane domain, were detectable in the ER/Golgi compartment as well as in the cell membrane (Fig. 1C). These expression patterns are consistent with the one described previously for teneurin-2 [12] supporting that the nuclear translocation of the intracellular domains of teneurins is a general phenomenon for this protein family.

CAP/ponsin binds to the intracellular domain of teneurin-1

In order to investigate possible functions of the intracellular domain, we performed a yeast two-hybrid screen to identify interacting proteins (Fig. 2). Since expression of the entire intracellular domain (ID1) as a bait resulted in the activation of the LexA promotor-driven reporter genes independent of an interaction with a prey, such a construct could not be used for the screening (Fig. 2A). We therefore separated the intracellular domain into two halves and expressed the N-terminal ID1N and the C-terminal ID1C independently. While ID1N still activated transcription, ID1C did not and could thus be used as a bait for the screening of an embryonic brain cDNA library. Interestingly, the same was the case for teneurin-2, implying that this activity might be a genuine feature of the N-terminal parts of the intracellular domains of the teneurins (Fig. 2A).

Using ID1C as bait, we isolated an interacting clone that encoded the third SH3 domain of CAP/ponsin located at the

C-terminus of this protein (Fig. 2B). This SH3 domain-containing protein is a cytoskeleton-associated adaptor protein reported to have diverse functions such as participation in insulin signaling [21,22] and cell–cell adhesion [23,15]. Since SH3 domains are known to bind polyproline motifs, we considered the two motifs P1 and P2 present in the intracellular domain of teneurin-1 as possible binding sites for CAP/ponsin. An alignment of these polyproline stretches between teneurin-1 orthologs and other teneurin paralogs is shown in Fig. 2C. This alignment shows that these sequences are 100% conserved between orthologs but not between paralogs. P2 is more similar among paralogs than P1 and shows only a single amino acid difference between teneurin-1 and teneurin-2 or -3 but is exactly the same as teneurin-4. To narrow down the binding site, we created two new constructs ID1Ca and ID1Cb separating P1 and P2. Interestingly, it was the bait containing the highly conserved P2 site that interacted with CAP/ponsin, suggesting that the exchange of a single proline for a leucine is enough to abolish this interaction since the corresponding teneurin-2 bait did not interact with CAP/ponsin (Fig. 2B).

To confirm that teneurin-1 also interacts with intact CAP/ponsin when expressed in cell cultures, we co-transfected a myc-tagged full-length CAP/ponsin construct together with IDten-1 and compared the subcellular distribution of the two proteins in co-transfected cells with cells transfected with either construct alone (Figs. 3A–G). While IDten-1 shows the strongest staining in the nucleus of transfected COS-7 cells (Fig. 3A), CAP/ponsin is mainly associated with the cytoskeleton and clearly excluded from the nucleus (Fig. 3B). Interestingly, co-transfection of the two constructs results in an indistinguishable staining pattern for the two proteins and CAP/ponsin now accumulates in the nucleus together with IDten-1, while IDten-1 partly redistributes to the cell periphery rich in CAP/ponsin (Figs. 3C and D, arrowheads). As shown in Figs. 3E–G, the redistribution of CAP/ponsin to the nucleus is clearly dependent on the presence of IDten-1 since in the co-transfected culture cells expressing only CAP/ponsin do not reveal any nuclear staining, while cells with both proteins show CAP/ponsin localized to the nucleus in conjunction with IDten-1 (Figs. 3E–G). Co-staining of the transfected cells with phalloidin to reveal the actin cytoskeleton did not reveal any difference in stress fiber formation or the general cell morphology or cell spreading neither in ponsin, nor in co-transfected cells when compared to the un-transfected counterparts (not shown).

To further confirm the interaction between IDten-1 and CAP/ponsin, co-immunoprecipitation experiments were performed from extracts of COS-7 cells transfected with both constructs. As shown in Fig. 3H, the immunoprecipitated IDten-1 detected by the anti-Flag was able to co-precipitate CAP/ponsin detected by the anti-myc antibody. Control immunoprecipitates with anti-C antiserum did not contain IDten-1 or CAP/ponsin. The same was true using control preimmune serum (data not shown). Thus, we conclude that upon co-expression of teneurin-1 and CAP/ponsin, the two proteins bind to each other and colocalize within a cell.

IDten-1 is enriched in nuclear matrix structures and binds to MBD1

In our yeast two-hybrid screen, the nuclear methyl CpG binding protein MBD1 was found to interact with IDten-1 (Fig. 4A). Because of the distinct nuclear staining of IDten-1 observed before, we wanted to investigate whether this accumulation was taking place at specific subnuclear structures, as e.g., the nuclear matrix. Furthermore, to confirm whether MBD1 interacts with IDten-1 in cells in culture, we tested whether the two proteins co-purify. We performed sequential extractions of cells co-transfected with IDten-1 and MBD1 or transfected with the nuclear matrix associated protein PML [24]. As shown in Fig. 4B, a small portion of IDten-1 is present in the soluble fraction while the majority accumulates in the nuclear matrix, the exclusive site where MBD1 and PML could be detected. IDten-1 is absent from the chromatin and the 2 M NaCl wash fractions containing acetylated histone H4. Consistent with the immunocytochemistry revealing CAP/ponsin in the nucleus together with IDten-1 (Fig. 3), CAP/ponsin can be co-purified in the nuclear matrix fraction as well (Fig. 4).

For immunolocalization studies, we co-transfected cells with IDten-1 and a construct expressing MBD1 or PML, respectively. A day later, we performed extractions of the cells, which removed all cell components except the nuclear matrix [19]. As shown in Fig. 5, the staining of IDten-1 colocalized with MBD1 in all double-transfected cells (Figs. 5D–I), whereas the overlap between IDten-1 and PML was only observed in a few selected cells and the patterns of these two proteins were mostly distinct (Figs. 5A–C). Such an occasional codistribution with PML could reflect the fact that a colocalization is only observed during a specific phase of the cell cycle as has been reported before for other proteins [25,26]. Thus, IDten-1 in the nucleus reveals a codistribution with MBD1 supporting a physical interaction between the two proteins. To exclude that the staining patterns for IDten-1, PML, and MBD1 are an artifact of over-expression, we stained cells for the endogenous proteins. In each case, we could observe a similar speckled nuclear staining (Figs. 5J–L).

Teneurin-1 cleavage of the intracellular domain occurs in cell cultures

In mRNA isolated from primary chick embryo fibroblasts, we were able to detect teneurin-1 transcripts by RT-PCR (not shown). We, therefore, tested these cells for the presence of the endogenous protein. Interestingly, teneurin-1 staining of CEFs with anti-N revealed a strong nuclear staining as compared to the staining with the preimmune serum (Fig. 5J), implying that in these cells the endogenous teneurin-1 protein is cleaved and the intracellular domain

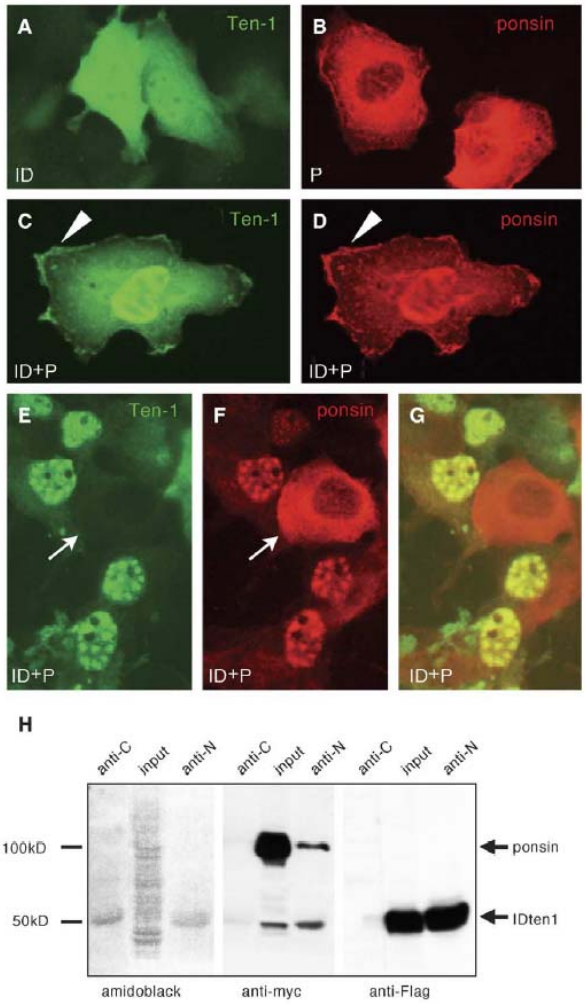

Fig. 3. Interaction of IDten-1 with CAP/ponsin in cell culture. HT1080 (A–D) and COS-7 (E–G) cells were transfected with the intracellular domain of teneurin-1 (ID) or CAP/ponsin (P) or both (ID + P) and stained for teneurin-1 with anti-N (A, C, and E) and for CAP/ponsin with anti-myc (B, D, and F). The overlap of E and F is shown in G. Note the colocalization of IDten-1 and ponsin both in the nucleus and at the cell membrane (arrowheads). In the same culture, cells expressing only CAP/ponsin do not reveal any nuclear staining (arrow), while cells with both proteins show CAP/ponsin localized in the nucleus together with IDten-1. (H) Cell extracts of COS-7 cells co-transfected with IDten-1 and CAP/ponsin (input) were precipitated with anti-C and anti-N antibodies and analyzed on immunoblots. The left panel shows amidoblack staining of the transferred proteins, the right panel reveals the immunoprecipitated IDten-1 by anti-Flag reactivity, and the co-precipitated CAP/ponsin is revealed on the middle panel by anti-myc.

accumulates in the nucleus. This could be confirmed by the isolation of cell lines that were induced with ponasterone to produce either full-length teneurin-1 (EcR-FLten-1) or the intracellular domain (EcR-IDten-1). Immunoblots of cell extracts revealed the expression of the full-length teneurin-1 in EcR-FLten-1 cells after ponasterone induction, and the

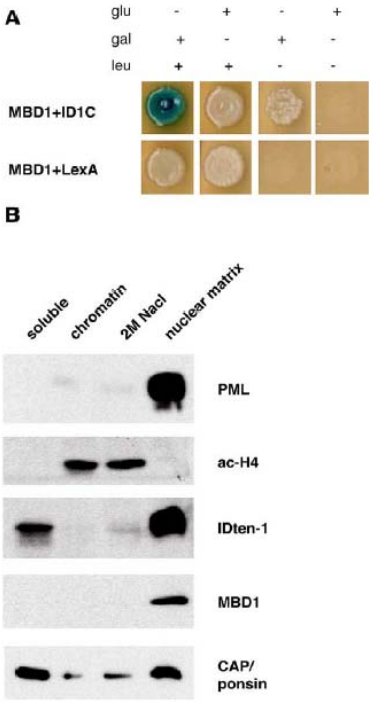

Fig. 4. Interaction and co-purification of IDten-1 with MBD1 and CAP/ponsin. (A) Yeast transformed with the constructs indicated to the left (together with a beta-galactosidase construct under a LexA promotor) were grown on plates containing glucose (glu) or galactose (gal) in the presence or absence of leucine (leu) as indicated by +/−. Co-transfection of MBD1 fused to a transcriptional activator domain with ID1C as a LexA fusion construct but not with LexA alone resulted in the growth of yeast on plates containing glucose in the absence of leucine. Beta-galactosidase expression developed in the presence of galactose as revealed by the blue color production in the presence of X-gal proving an interaction between ID1C and MBD1. (B) COS-7 cells were either transfected with PML or co-transfected with IDten-1 and MBD1 or IDten-1 and CAP/ponsin and sequentially fractionated into a soluble, chromatin, 2 M NaCl and a nuclear matrix fraction. Samples were analyzed on immunoblots for the presence of the proteins encoded by the transfected constructs as well as for the presence of acetylated histone H4 as a marker for chromatin, using anti-PML, anti-acetylated H4 (ac-H4), anti-N for IDten-1, and anti-HA for MBD1. This revealed that PML and MBD1 were exclusively found in the nuclear matrix fraction whereas some of IDten-1 was soluble, but the major fraction of IDten-1 was co-purifying with MBD1 in the nuclear matrix fraction. CAP/ponsin showed a similar distribution as IDten-1 and was mainly found in the soluble and the nuclear matrix fraction.

full-length protein could be detected by antibodies against the C- as well as the N-terminus of the protein (Fig. 6A). In contrast to the antibody against the C-terminus, the anti-N antiserum recognized additional bands of which one major band co-migrated with the intracellular domain expressed by EcR-IDten-1 cells after ponasterone induction (Fig. 6A, arrows). Interestingly, this was the only protein band that could be retrieved from the nuclear matrix fraction of EcR-FLten-1 cells after ponasterone induction (Fig. 6B). The same accumulation of the intracellular domain in the nuclear matrix fraction was observed for EcR-IDten-1 cells and for the endogenous teneurin-1 in CEF cells (Fig. 6B). Thus, in EcR-FLten-1 cells, a full-length teneurin-1 protein is expressed, a fraction is cleaved, and the intracellular domain is released and can be isolated in the nuclear matrix fraction.

Discussion

Teneurins have been implicated in many different functions, such as development and regeneration of the central nervous system [6,7], the formation of neuronal circuits [10], limb morphogenesis [4,5], or the ER stress response [27]. We recently presented evidence that teneurin-2 could function as a membrane-anchored transcription factor and showed that cleavage of its intracellular domain was induced by homophilic teneurin-2 interactions [12]. In this paper, we investigated whether a similar proteolytic processing occurs in teneurin-1 and what the teneurin-1 signaling mechanism might be.

In a first step addressing teneurin-1 signaling, we identified proteins interacting with its intracellular domain with the expectation that binding partners with previously described functions could help to elucidate the mechanism of action of teneurin-1. One of the binding partners we found was CAP/ponsin. In the yeast two-hybrid screen, we isolated the third SH3 domain of CAP/ponsin and confirmed in eukaryotic cells that not only this isolated domain, but the entire CAP/ponsin, was able to interact with the intracellular domain of teneurin-1. CAP/ponsin is a highly interesting adapter protein with a function in insulin signaling [22] as well as cell–cell and cell–matrix adhesion [23,15]. CAP/ponsin contains three SH3 domains and a sorbin homology domain [21]. The third SH3 domain of CAP has been shown to interact with yet another adapter protein Cbl and with its sorbin homology domain with flotillin in lipid rafts and thereby CAP is able to traffic cbl to lipid rafts [22]. Cbl is not only important for insulin signaling and the regulation of glucose transport but also regulates Rac and actin assembly [28]. In addition, Cbl functions as an E3 ubiquitin ligase and thereby regulates degradation of receptor tyrosine kinases (for reviews, see [29,30]). Since IDten-1 is able to bind to the same SH3 domain of CAP as Cbl, it could be possible that these two proteins compete with each other and therefore influence their respective signaling pathways. Clearly, a large fraction of IDten-1 accumulates in the nucleus of a cell and is able to carry CAP/ponsin with it to this new location. Intriguingly, a very strong nuclear staining with anti-ponsin antibodies has been observed before in the mouse mammary tumor cell line MTD-1A, but the significance of this was

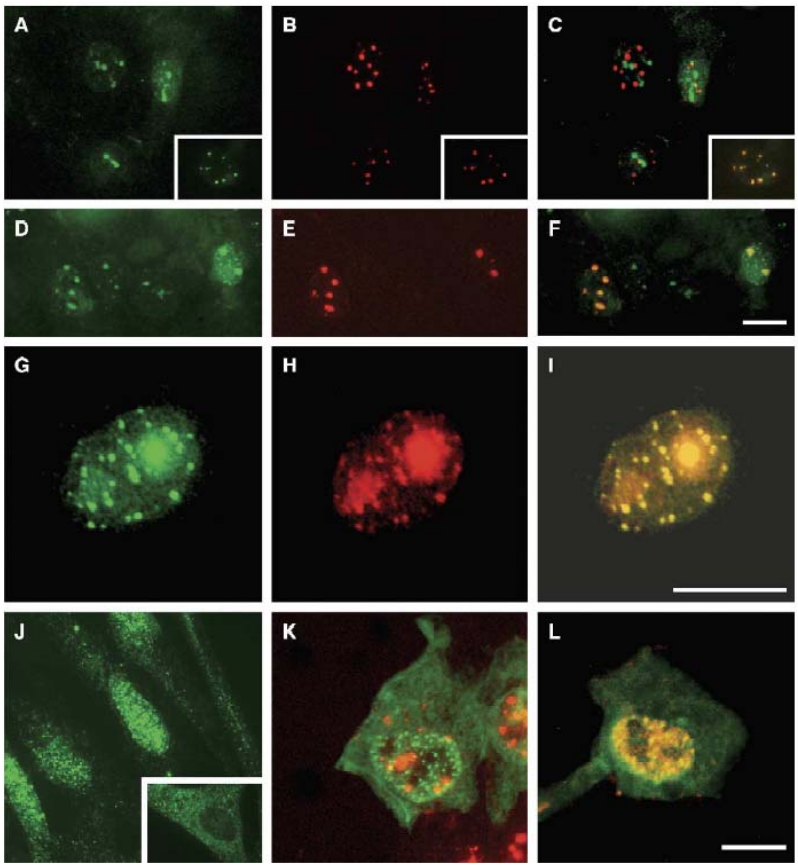

Fig. 5. Codistribution of IDten-1 with MBD1 in the nuclear matrix. (A–C) Cos-7 cells were co-transfected with IDten-1 and PML. After extraction of the cells to visualize the nuclear matrix, they were fixed and stained for IDten-1 with anti-N (A) and with anti-PML (B), and the overlap is shown in C. Most of the cells displayed distinct patterns for PML and IDten-1 staining, but occasionally a cell nucleus with a colocalization could be found as is shown in the insert of A–C. (D–I) Cos-7 cells were co-transfected with IDten-1 and MBD1. IDten-1 staining revealed by anti-N antibody (D and G) and MBD1 staining revealed by anti-HA antibody (E and H) and overlaps (F and I) demonstrate a strict colocalization of the two proteins. (J) Staining of endogenous teneurin-1 in chick embryo fibroblasts with anti-N reveals a dotted pattern in the nuclei while the preimmune serum (insert) excludes nuclei. (K) Double labeling of endogenous PML (red) in HT1080 cells and of transfected IDten-1 (green) shows distinct dots in the nucleus. (L) Double labeling of endogenous MBD1 (red) in HT1080 cells and of transfected IDten-1 (green) reveals overlapping nuclear speckles in yellow. Scale bars, 5μm.

unclear [15]. The results present in this paper indicate that this staining could be of physiological relevance and ponsin could have a function in the nucleus as well. Furthermore, CAP was shown to bind to the focal adhesion kinase [23]. The second and third SH3 repeats of ponsin were shown to interact with l-afadin and the first and the second SH3 domains with vinculin [15]. These CAP/ponsin-interacting proteins are localized at cell–cell and cell–matrix adhesion sites and their binding to ponsin appears to be mutually exclusive [15]. Thus, the interaction of CAP/ponsin with many proteins known to regulate cell adhesion and the actin cytoskeleton supports a function in these processes. Since we have shown that IDten-1 interacts with the last SH3 domain and it is known that vinculin binds to the first ones [15], it may be possible that both proteins bind simultaneously to ponsin and thereby teneurin-1 could get recruited to vinculin-rich cell–cell adhesion sites. This may be particularly important for the intact teneurin-1 protein present as a

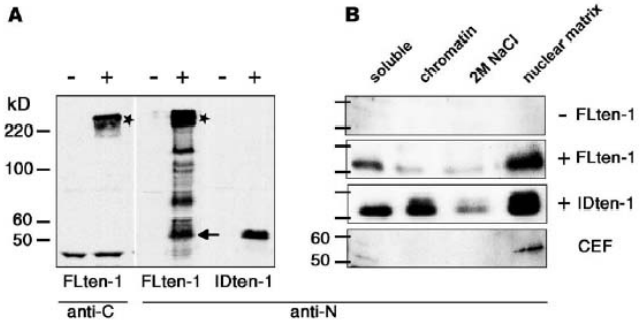

Fig. 6. Expression and cleavage of full-length teneurin-1. (A) Extracts were prepared from EcR-FLten-1 and EcR-IDten-1 cells cultured in the absence (−) or presence (+) of ponasterone as indicated. Immunoblots show expression of the full-length protein recognized by anti-C and anti-N (stars) after induction with ponasterone. Anti-N recognizes in addition smaller teneurin-1 fragments of which the lowest band co-migrates with the induced soluble intracellular domain in EcR-IDten-1 cells (arrow). (B) Fractionation and nuclear matrix preparation of un-induced (−) and induced (+) EcR-FLten-1 cells and EcR-IDten-1 cells and of CEF cells. Immunoblots show the co-purification of the N-terminal teneurin-1 fragment with the nuclear matrix as is seen in induced EcR-IDten-1 cells. A fragment of the same size can be detected in the nuclear matrix fraction of CEF cells. To the left of each blot, the positions of the 50-kDa and 60-kDa markers are indicated.

transmembrane protein that could be sequestered to cell–cell interaction sites.

We have identified a part of the intracellular domain of teneurin-1 containing the polyproline stretch PPPRPLPR as the binding site for CAP/ponsin. This sequence is 100% conserved in chicken, mouse, and human teneurin-1, and there is only a single amino acid difference in teneurin-2 or -3. However, teneurin-2 did not interact with CAP/ponsin, demonstrating the selectivity of SH3 domains in ligand recognition. Since teneurin-4 contains exactly the same sequence at the same position of its intracellular domain, the possibility exists that CAP/ponsin interacts with teneurin-4 as well. Since CAP/ponsin belongs to a family of four related proteins including vinexin, ArgBP2, and nArgBP2 [31], it might be possible that other family members bind to other teneurin proteins.

The second interacting protein of IDten-1 described in the manuscript is methyl-CpG-binding protein 1, MBD1. As the name implies, this protein binds to methylated DNA in heterochromatin and is known for its function in transcriptional repression (for a review, see [14]). It interacts with other heterochromatin-associated proteins such as the histone H3 methylase Suv39h1 [32], H3-K9 methylase SETDB1 [33], the p150 subunit of chromatin assembly factor 1 [34], and the methylpurine-DNA glycosylase MPG involved not only in transcriptional repression but also with a role in DNA repair [35].

We found that IDten-1 colocalizes with MBD1 in foci associated with the nuclear matrix. An increasing number of publications report on an important physiological function of the nuclear matrix in DNA organization, transcription, replication, RNA synthesis, and splicing as well as serving as binding sites for steroid hormone receptors (for a review, see [36]) and other transcription factors such as p53 [37] or Runx factors [38]. MBD1-deficient mice have deficits in adult neurogenesis and display impaired spatial learning [39]. Furthermore, neural stem cells isolated from these mice exhibit reduced neuronal differentiation. Since teneurin-1 is known to be specifically expressed in the developing and adult nervous system [8], it is conceivable that MBD1 together with IDten-1 participate in the regulation of gene transcription in neurons. Support for a nuclear function of teneurin-1 was obtained from the identification of endogenous teneurin-1 immunoreactivity in nuclei of chick embryo fibroblasts and the co-fractionation of a band migrating slightly above 50 kDa recognized by the anti-N antiserum in their nuclear matrix fraction. Furthermore, in cells that can be induced to express full-length teneurin-1 by hormone addition, we were able to detect a teneurin-1 fragment corresponding to the intracellular domain, which co-fractionated in the nuclear matrix. In our future studies, we aim to elucidate the cleavage mechanism of teneurin-1 and ultimately the role of its intracellular domain in the regulation of gene expression.

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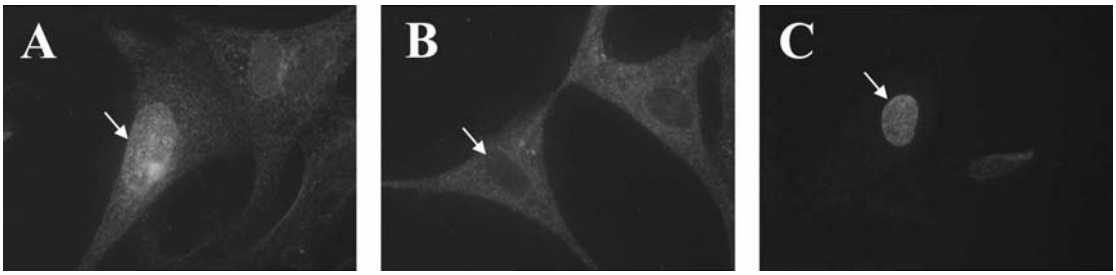
Supplementary data not shown in the publication:

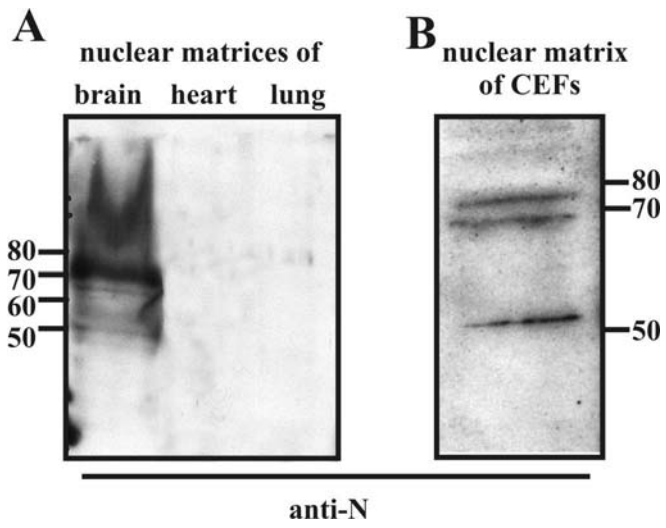
To corroborate the finding of the CEF nuclear staining we decided to look for the presence of the cleaved endogenous teneurin-1 in the nuclear matrix of chicken cells by immunocytochemistry. Antibody against the N-terminus of teneurin-1 "anti-N" was able to reveal the same staining pattern regardless if cells were treated or not to uncover their nuclear matrix (**supplementary figure 1**).

Subsequently, Western blotting of nuclear matrix fractions of CEF cells was used to confirm the presence of the putative endogenous teneurin-1 not only in cultured chicken cells (**supplementary figure 2B**), but also in chicken tissues. Nuclear matrix fractions derived from total brain lysates, revealed the presence of a 75kDa band, at much weaker levels, also the 55kDa band with the "anti-N" antibody, in contrast to the nuclear matrix fractions of heart and lung, where no teneurin-1 could be detected (**supplementary figure 2A**).

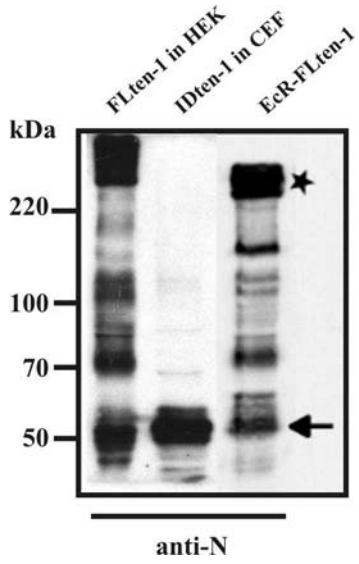
Finally, clones induced to express full-length teneurin-1 (EcR-FLten-1) or HEK-293 cells transiently transfected with FLten-1 were extracted and assayed by Western blotting using the "anti-N" antibody. Noteworthy, in both cell lines, a similar pattern of teneurin-1 cleavage was observed, including the teneurin-1 fragments of 55kDa and 75kDa (**supplementary figure 3**).



Supplementary Figure 1. Indirect immunofluorescence of teneurin-1. Staining using the "anti-N" antibody in CEF cells untreated **(A)** or treated **(C)** to uncover their nuclear matrix revels a nuclear signal. **(B)** Untreated cells stained with pre-immune serum are shown as control. Pictures were taken at 100x magnification.



Supplementary Figure 2. Endogenous teneurin-1 in chicken tissue and chicken cells. Immunoblots of the nuclear matrix fraction of chicken brain, heart and lung lysates **(A)** and from the nuclear matrix fraction of CEF cells **(B)**. Both in brain and in CEF lysates, bands of similar molecular weight were detected that may correspond to endogenous teneurin-1.



Supplementary Figure 3: Cleavage of transiently transfected full-length teneurin-1 in HEK-293 cells. HEK-293 cells were transiently transfected with a vector encoding the full-length teneurin-1 (FLten-1). CEF cells were transfected with IDten-1 and stable clones were induced to express full-length teneurin-1 (EcR-FLten-1). Extracts were probed in Western blotting with anti-N antibody. Star represents the position of full-length teneurin-1. The arrow represents the 55kDa band and other cleaved fragments are seen.

Part 2. **Investigation of teneurin-1 signalling**

Samantha M. Nunes, Karen Choi and Ruth-Chiquet Ehrismann

Based on studies in *Drosophila*, where *ten-m1* was found to be secreted and mutants displayed segmentation defects typical of mutations in pair-rule genes, the hypothesis emerged that teneurins may be proteolytically cleaved in a Notch-like fashion after being synthesized as a membrane-bound transcription factor. In a first step, the protein would be cleaved extracellularly as shown for teneurin-2 (Rubin et al., 1999). An additional cleavage still to be characterized would then release the intracellular domain that is thought to translocate to the nucleus and affect gene expression as shown for teneurin-2 (Bagutti et al., 2003).

Since the cleavage mechanism of teneurin-1 full-length protein has not yet been elucidated, I decided to generate stable clones of different forms of the protein for studies in cell culture. EcR-IDten-1, EcR-MLten-1 and EcR-FLten-1 expression was driven by a promoter that responded to an activated ecdysone hormone receptor. This allowed the expression of these different forms of teneurin-1 at lower and closer to physiological levels than the use of the strong CMV-promoter-driven expression of IDten-1 in pCEP-Pu that also mimics the putative cleavage product of the full-length protein. Importantly, this system also allowed teneurin-1 gene expression to be switched on and off when required.

Some of the experiments with these constructs are described in the publication of Part 1 of the Results section. Additional experiments that did not fit in that context are described below. Although at present not mature enough for submission to a peer-reviewed journal, these experiments will serve as a basis for follow-up studies.

III. 1 Immunoprecipitation of the endogenous teneurin-1

In a first step trying to understand the cleavage of teneurin-1, we attempted to detect the endogenous form of the protein. Preliminary Northern

blot analyses of teneurin-1 in chicken had revealed that at the developmental stages tested, expression in the brain starts at E5, gradually increases to a higher level by E11 and reaches its plateau at E17. The adult brain also shows expression of teneurin-1, which is not the case for other adult organs such as liver and lung (Minet et al., 1999).

For immunoprecipitation, brain, heart and lung from chicken embryos (E11) were used as sources of positive and negative controls respectively for the presence or absence of endogenous teneurin-1 protein. Tissues were homogenized in RIPA buffer and the equivalent of 500µg of total protein was processed for immunoprecipitation with "anti-N" antibody followed by Western blot detection of precipitated bands. In agreement with the above-mentioned report (Minet et al., 1999), a 75kDa protein band was immunoprecipitated only from brain tissue (**figure 1A**). Interestingly, the presence of a 75kDa protein has been observed previously in immunoblots of nuclear matrices of CEF cells and of chicken brain tissue (**cf. supplementary figure 2**). Moreover, using a different polyclonal antibody directed against the N-terminus of teneurin-1 called "anti-A" for precipitation, the same protein band was detected by the "anti-N" antibody on Western blots (**figure 1B**). As control for both immunoprecipitations, pre-immune serum was added to the protein lysate instead of the anti N-terminus antibodies and processed in the same way. In addition, Western blotting using an antibody against the "C-terminus" of teneurin-1 "anti-C" revealed no signal, indicating that the immunoprecipitated protein was not the full-length protein, but a processed form instead (not shown).

Apart from chicken tissues, it was tested whether teneurin-1 could be immunoprecipated from the cortex of mice using the chicken "anti-N" antibody. Although teneurin-1 shows high homology between these two species, the chicken antibody did not seem to be able to immunoprecipitate the mouse teneurin-1 protein (not shown). However, on Western blots of total lysate of cortex a 75kDa protein band was detected, which was different from the 80kDa band in total lysate of heart particularly prominent in the blot with

the "anti-A" antibody (**figure 2**). At present it cannot be distinguished whether this 80kDa band represents a distinct proteolytic fragment of teneurin-1 present in heart or crossreactivity with an unrelated protein expressed in that tissue. The detection of a 75 kDa band in chicken brain lysates (as well as possibly in mouse brain) suggests that teneurin-1 may undergo constitutive cleavage in the extracellular domain *in vivo*.

III.2 Effects of IDten-1 overexpression on the rate of cell proliferation

I noticed that cell lines expressing IDten-1 seeded at the same density as control cells without IDten-1 expression displayed a lower cell number after several days in culture. Therefore, it was postulated that this effect might be due to a decrease in proliferation and an increase in differentiation in the presence of IDten-1. To exclude that the phenomenon was due to increased apoptosis, advantage was taken of an *in vitro* assay that monitors the cellular redox status. In this assay cytosolic glutathione (GSH) levels, which are reduced in cells that are in early stages of apoptosis are measured (Wang et al., 2000). Stable clones of EcR-IDten-1 (D3 and A1), EcR-IDten-2 (K3) and Wt 293-EcR cells were seeded in 6-well dishes and after 12 hours, IDten-1 protein expression was induced in half of the wells with ponasterone. 12 hours later, monochlorobimane (MCB) was added to the medium and its incorporation into cellular cytosolic glutathione was measured using a fluorometer. No significant differences in GSH levels were detected between induced and non-induced cells (**figure 3**). This suggested that expression of IDten-1 was unlikely to have an impact on the growth rate of cells by inducing global changes in their redox status and programmed cell death.

To quantify a possible reduction in the proliferation rate after induction of IDten-1 expression, the number of cells in S-phase of the cell cycle was monitored using a BrdU incorporation assay. To do this, beta-galactosidase (βgal) was co-transfected either with the IDten-1 construct or with the empty pCEP-vector to compare BrdU incorporation of the transfected cells. Indeed,

overexpression of IDten-1 in U2OS cells caused a reduction of approximately 30-40% in BrdU and βgal-positive cells as compared to vector transfected cells indicating that the presence of this domain is capable of reducing the capacity of cells to proliferate at their normal rate. Similar results were obtained when BrdU incorporation was assessed in primary chick embryo fibroblasts (CEF) cultures transfected to express IDten-1 (**figure 4**). Therefore, it can be speculated that the nuclear form of IDten-1 facilitates the transition from cell proliferation to cell differentiation (see discussion).

III.3 Analyses of the involvement of IDten-1 in signal transduction

Since cells expressing IDten-1 displayed a reduced rate of proliferation as measured by BrdU incorporation assays, it was investigated whether this correlated with the level of expression and activity of selected cell cycle regulators involved in proliferation. A first candidate that was checked was the p53 tumour suppressor protein, which is known to have a direct role in the control of cell proliferation (Komarova et al., 1997). As an indirect readout of cellular p53 activity, advantage was taken of a p53-responsive luciferase reporter system (see details in Material and Methods). **Figure 5** shows the relative luciferase values corresponding to p53 activation of overexpressed IDten-1 in both U2OS cells (a,b) and in primary CEF cells (c,d). Considering the experimental variations (see error bars), only a subtle reduction in p53 activity was detected in cells transfected with the IDten-1 as compared to the cells transfected with the vector alone.

Western blotting using anti-p53 phospho-Ser^{392} antibodies in EcR-cells induced to express IDten-1 revealed that phosphorylation at this site of p53 remained unaltered (**figure 6**). However, that does not exclude that other sites known to be phosphorylated in p53 (e.g. serine 6, 9, 15 and 20) could be affected. Finally, the effects of IDten-1 on other signalling pathways known to control cell proliferation were investigated. The phosphorylation status of p44/p42 MAPK and PKB were analysed by Western blotting and neither activity was altered in cells expressing IDten-1. Thus, further experiments are

required to shed light onto the factors and pathways that are induced and may affect the rate of proliferation in response to the expression of IDten-1 (see discussion).

III.4 Microarray Analysis of the Intracellular Domain of teneurin-1 (IDten-1) expressing cells

Microarray analysis was performed to investigate the possible roles of the IDten-1 domain in gene regulation and to identify its potential target genes. The experiment consisted of a time course of 0, 6, 12 and 24 hours after induction of IDten-1 expression with ponasterone. Since the induction of IDten-1 relied on the addition of ponasterone, one concern was that the non-physiological effects of this drug or the activation of the ponasterone receptor could trigger the changes in gene expression independently of IDten-1 expression. To address this point, wild type EcR-293 cells were transfected with the empty vector pIND to generate the stable EcR-pIND "empty vector" control cells. These cells allow the identification of possible effects resulting from the addition of ponasterone to the medium.

In the microarray experiment, EcR-IDten-1 samples were compared with control EcR-pIND samples as a screen for differentially expressed genes. **Figure 7** shows the accumulation of IDten-1 protein after the induction of its expression in the stable EcR-cell line C6 at the times indicated. N-terminal anti-teneurin-1 antibody "anti-N" detected a band by Western blotting that migrated at the expected molecular weight (55kDa). Similar results were obtained with other stable cell lines (**cf. figure 16**). Furthermore, immunofluorescence using "anti-N" showed that IDten-1, but not MLten-1, was predominantly localised to the nucleus of EcR cells following induction by ponasterone **(figure 8)**. In parallel samples, total mRNA was extracted from these cells at the time points shown in figure 7 for the microarray analysis. Probes were prepared and Affymetrix U133A GeneChips^{TM} were hybridised by the FMI microarray facility. After pre-filtering the data (see details in Materials

and Methods), the following analysis was performed on these data with the assistance of Edward Oakeley:

1-Way ANOVA analysis

In addition to following the fold change of genes, we wanted to be sure that changes observed were robust and reproducible. The fold change lists were then combined and a 1-Way analysis of variance (ANOVA) test was performed (see details in Materials and Methods II.14). 263 genes passed this test and were therefore differentially expressed between the control and the treated samples (**figure 9**). The Affymetrix annotations of these 263 genes are shown in the appendix supplied at the end of this thesis. This set of genes was classified using the following website (http://fatigo.bioinfo.cnio.es/) - see details in Material and Methods II.14. The chart presented in **figure 10** lists the percentage of genes attributed to a certain Gene Ontology function. It shows that the largest group of genes that change their expression levels are within the group of genes classified as nucleic acid binding.

2-Way ANOVA test

In order to refine the analysis, a 2-Way ANOVA test was performed, which allowed testing the interaction of two parameters at a significance level. The 2-Way ANOVA tested the interaction of time and treatment with ponasterone (see details in Materials and Methods II.14). This test reduced the original list to a total of 39 genes. A list containing the Affymetrix annotations is provided in the appendix of this thesis. The expression kinetics of these 39 transcripts as well as their classification using "Fatigo" (http://fatigo.bioinfo.cnio.es/) is shown in **figures 11 and 12**. Interestingly, nucleic acid binding genes and transcription factor activity genes are now representing over half of all genes identified (**figure 12**). Ideally, an IDten-1 target gene would remain unaltered in the control pIND sample and respond by either being up - or downregulated in the presence of IDten-1. Strikingly, the expression values for the AB033066 gene shown in **figure 13** precisely

matched these criteria and this gene was therefore chosen for further investigation.

III.5 Identification and characterization of a gene induced by IDten-1

The 3'UTR sequence of the AB033066 gene was used for a blast search against the NCBI database from PubMed to find a full-length cDNA and the corresponding protein. It was possible to obtain an alignment of known EST's as well as cDNAs covering the entire coding region that support the existence of the AB033066 gene transcript. Furthermore, the program also predicted that two consecutive transcripts (AB033066/AK125718 and AK094821) might be joined together to form a single longer transcript called XM_039676 (**figure 14**). To test this prediction, RT-PCRs were performed using primers derived from transcript AK094821 and transcript AK125718 indicated in figure 14. A single band of approximately 1100bp spanning the two transcripts could be amplified (**figure 15A**), confirming the computer-based prediction that these transcripts are part of one single gene. From now on, this gene will be called XM_039676.

To confirm the results of the microarray analysis Reverse Transcription RT-PCR was performed on mRNA isolated from the same EcR-IDten-1 clone C6. As shown in **figure 15 B**, in the presence of IDten-1 the expression levels of the XM_039676 transcript are strongly induced whereas tubulin, which was selected as internal control based on its unaltered expression profile throughout the experiment, remains unaffected. To exclude that induction of XM_039676 is a cell clone specific phenomenon, mRNA from two other clones that can be induced to express IDten-1 B2 and C13 was extracted, reverse transcribed and RT-PCR was performed as described above. The results of this analysis are shown in **figure 16**. All clones expressing IDten-1 also contain elevated levels of XM_039676 transcripts. Therefore, induction of XM_039676 consistently correlates with IDten-1 expression and remains an interesting candidate as a teneurin-1 target gene.

No experimental data or publications are available about this protein or gene. To explore possible functions of this newly identified putative IDten-1 target gene, the Scansite database (http://scansite.mit.edu/) was used to check if the XM_039676 gene product could reveal any conserved protein domains that would hint at its function. An ATPase/AAA and a BROMO domain were predicted to be present in the KIAA1240 protein. The entire protein is supposed to be encoded by 35 exons giving rise to a protein with a calculated molecular weight of about 161 kDa (**figure 17**).

III.6 Promoter identification

In order to test whether XM_039676 is directly controlled by IDten-1 we tried to identify the promoter sequence to generate a reporter system (e.g. luciferase) for measuring the activation of XM_039676 by teneurin-1.

To identify the promoter, an 8 kb sequence upstream of and including the beginning of the putative first exon of XM_039676 was selected and blasted against the mouse genome with the expectation to find similar regulatory DNA sequences and/or exons between these two species. This search revealed two highly homologous sequences present in the mouse genome corresponding to the predicted first exon plus an additional 150 bp sequence about 4 kb upstream (**figure 18**). Such a high degree of homology suggested that the latter might represent an additional exon. We therefore postulate that the promoter of the XM_039676 gene might be located 5' to either of these putative first exons. The cloning of the two potential promoter regions in front of a luciferase gene will allow the generation of a reporter system for use in future experiments where activation by IDten-1 can be experimentally tested.

III.7 Figures

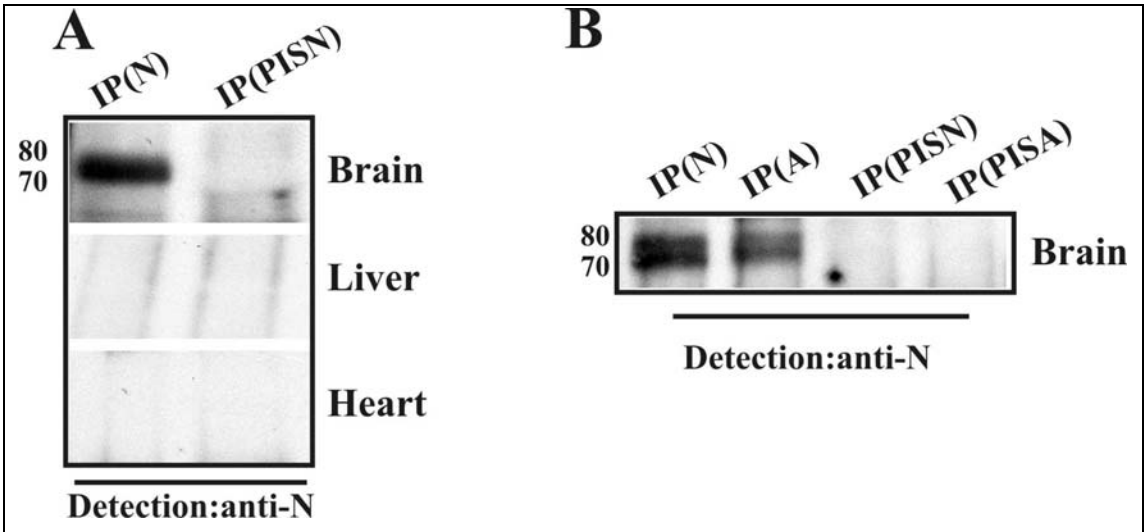


Figure 1. Immunoprecipitation of teneurin-1 from chicken tissues. (A) Immunoprecipitation followed by Western blotting with "anti-N" antibody detects a protein with apparent molecular weight of 75kDa from chicken brain extracts. **(B)** Immunoprecipitation using another antibody against the N-terminus "anti-A" followed by detection with "anti-N" recognizes the same protein band. IP: immuno-precipitation, PIS: pre-immune serum.

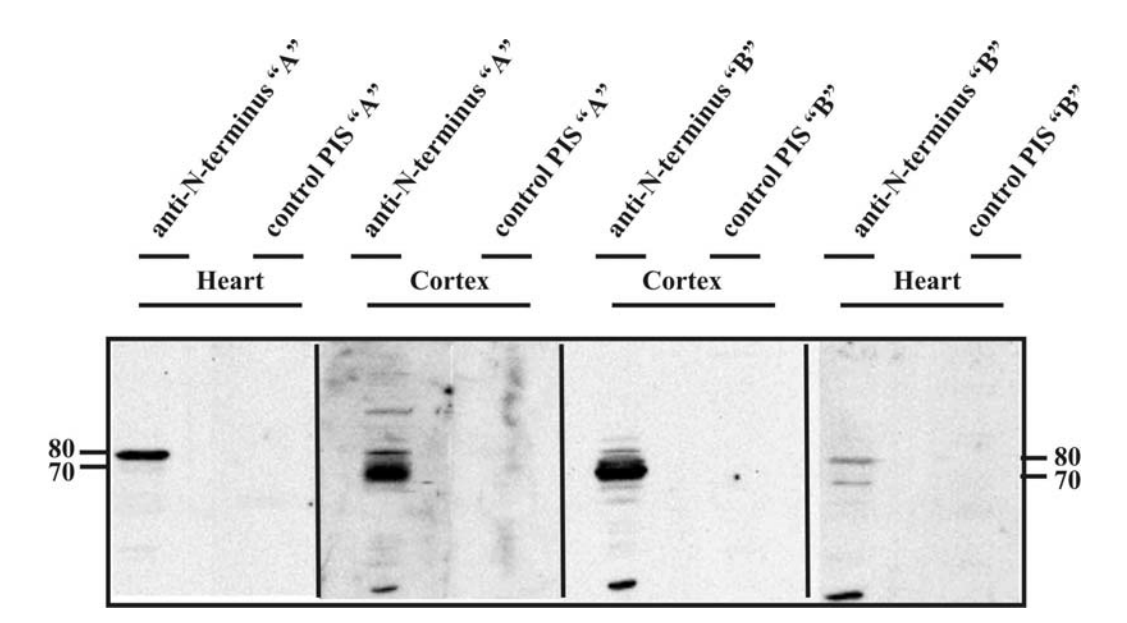


Figure 2. Endogenous teneurin-1 in mouse tissues. Immunoblots of mouse heart and cortex analysed by both antibodies against the N-terminus of the chicken teneurin-1 "anti-A" and "anti-B". These antibodies are able to recognize the 75kDa protein band in the cortex. In the heart, an 80kDa signal is detected. Pre-immune serum (PIS) was used as control.

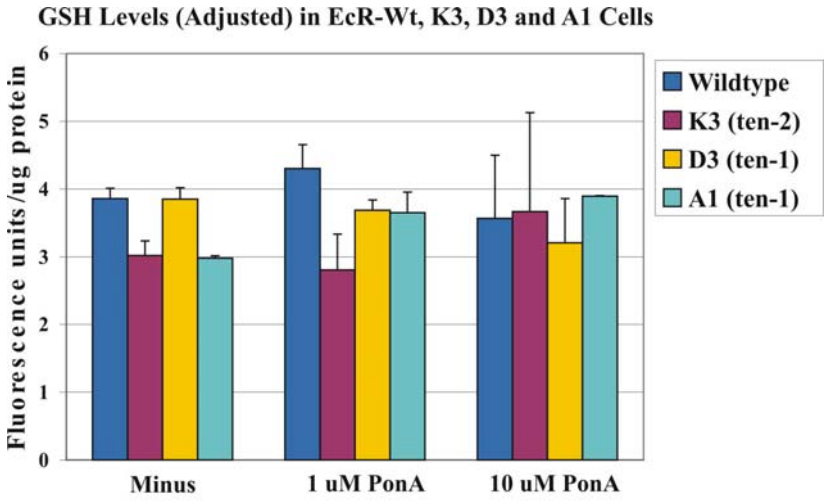


Figure 3. Determination of GSH levels in stable clones expressing IDten-1 and IDten-2. Arbitrary fluorescence readings representing the GSH levels in stable IDten-1 (D3,A1), IDten-2 (K3) and wild type cells with and without addition of different concentrations of ponasterone. Error bars represent the standard deviations of replicates.

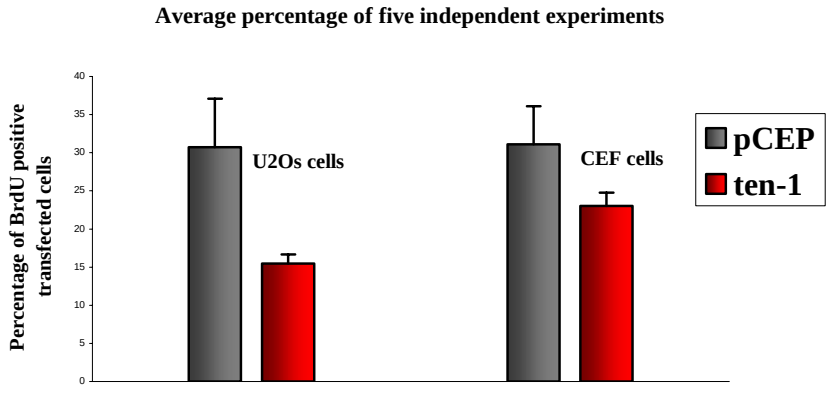


Figure 4. Measurement of the rate of cell proliferation in IDten-1 overexpressing cells. Comparison of levels of BrdU incorporation in U2OS and CEF cells transfected either with empty vector control or with IDten-1. IDten-1 transfected cells show a reduction in proliferation rates seen by fewer BrdU-positive cells as compared to control cells. Results are derived from five independent experiments in which three of them were performed in U2OS cells and two performed in CEF cells. Error bars represent the standard deviations of replicates.

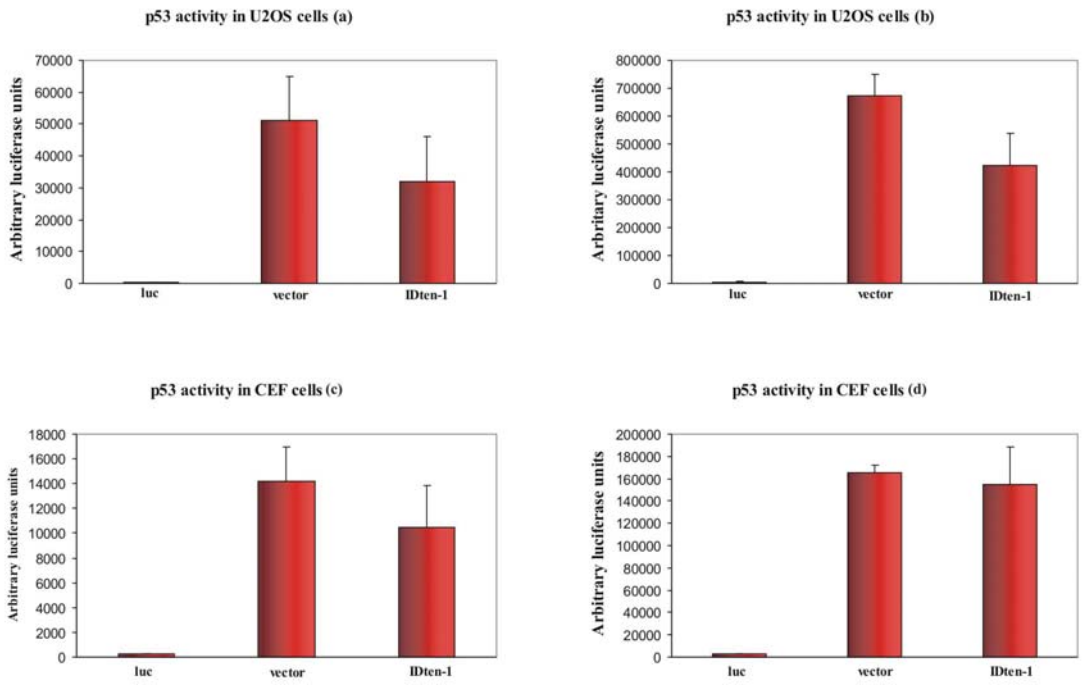


Figure 5. Determination of p53 activity in IDten-1 overexpressing cells by luciferase assay. Results are derived from four independent experiments, each done in duplicate. The first two were performed in U2OS cells (a, b) and the last two in primary CEF cells (c, d). Subtle changes in p53 activation could be detected when U2OS and CEF cells were transfected with empty vector as control and compared with cells transfected with IDten-1. Considering the experimental variations these changes are not significant.

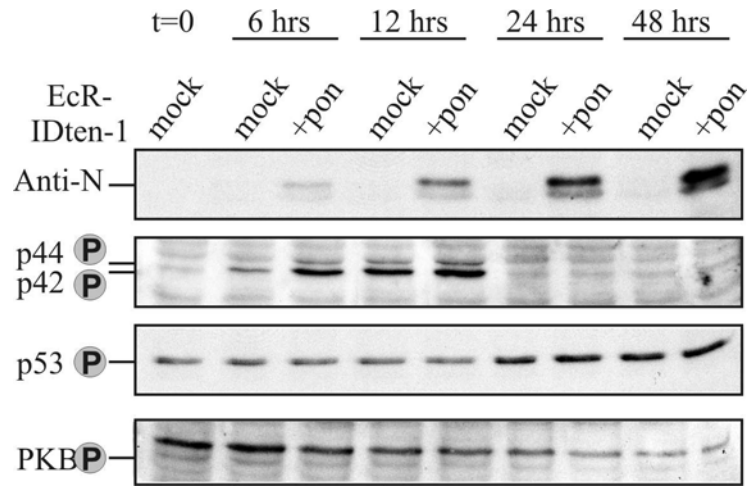


Figure 6. Effects of IDten-1 overexpression on phosphorylation of MAPK, p53 and PKB. Ponasterone was added over a time course of 48 hours to EcR-IDten-1 cells and extracts were prepared at the indicated times. Induction of IDten-1 protein and levels of phosphorylated p44/p42, p53 and PKB were assessed by immunoblot.

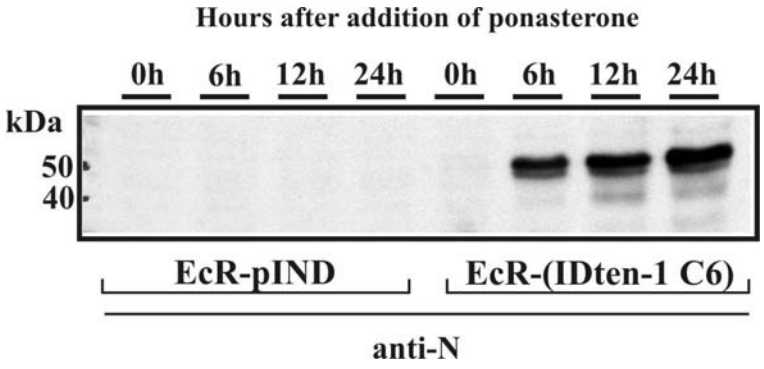


Figure 7. Time course of IDten-1 induction in cells used for microarray analysis. Extracts were prepared at the indicated times from empty vector control cells (EcR-pIND) and IDten-1 C6 cells induced with ponasterone and probed for IDten-1 expression by Western blotting. mRNA from each clone was isolated at the defined time points and used for microarray analysis. Note that empty vector control cells are negative for IDten-1 expression.

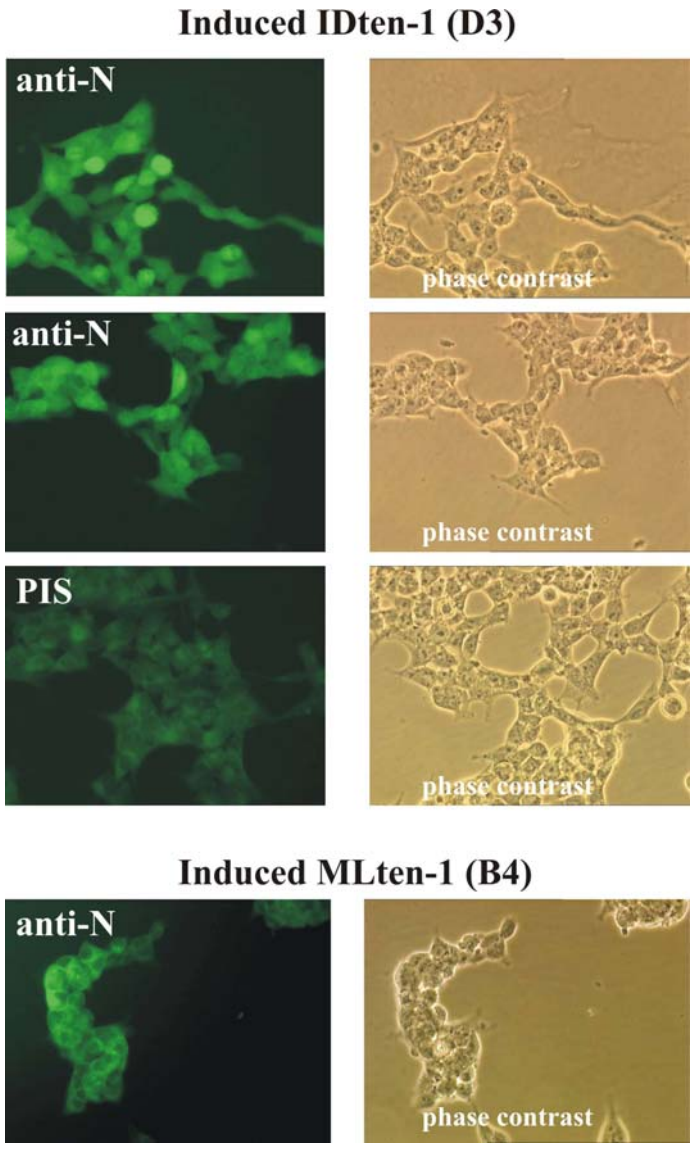


Figure 8. Immunofluorescence of stable clones induced to express teneurin-1. Staining by indirect immunofluorescence using "anti-N" antibodies reveals that IDten-1 expression is predominantly nuclear (upper panels) whereas pre-immune serum (PIS) gives no staining. MLten-1 expression results in a nuclear excluded pattern (lower panels).

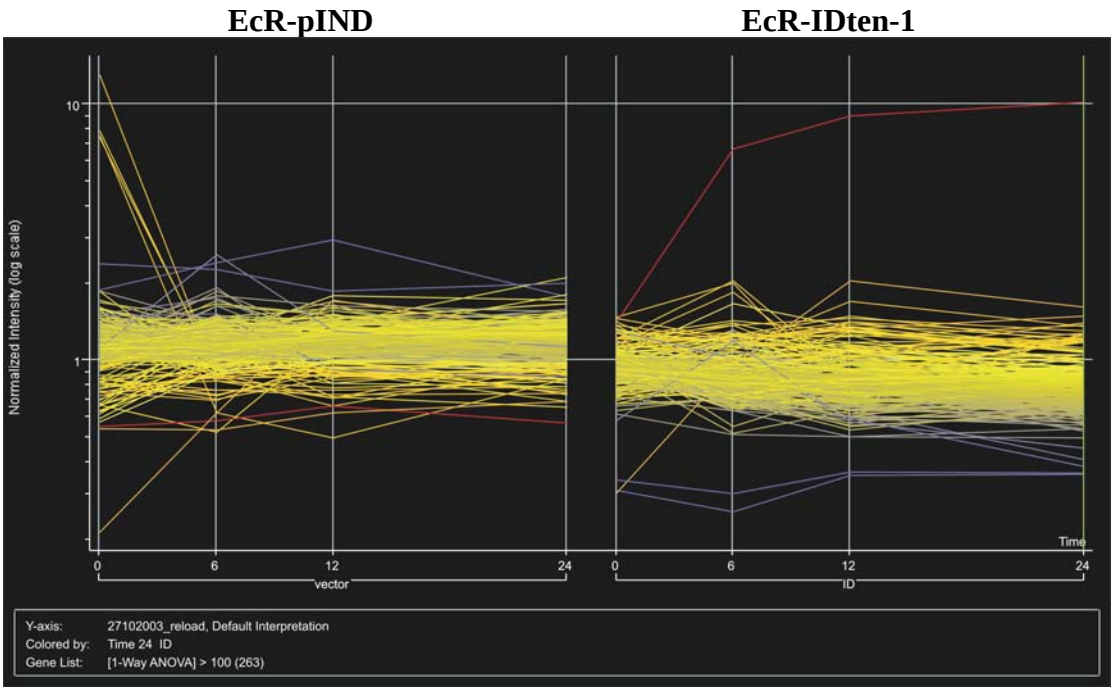


Figure 9. Expression kinetics of genes affected by the expression of IDten-1. The graphics depicts the expression kinetics of the 263 genes that passed a 1-Way ANOVA test over a 24-hour time course. Colour information is based on the time point ID 24h.

Figure 10. Classification of the 263 genes after analysis with FatiGO. FatiGO: a web tool for finding significant associations of Gene Ontology terms with groups of genes. *Bioinformatics 2004 20: 578-580*

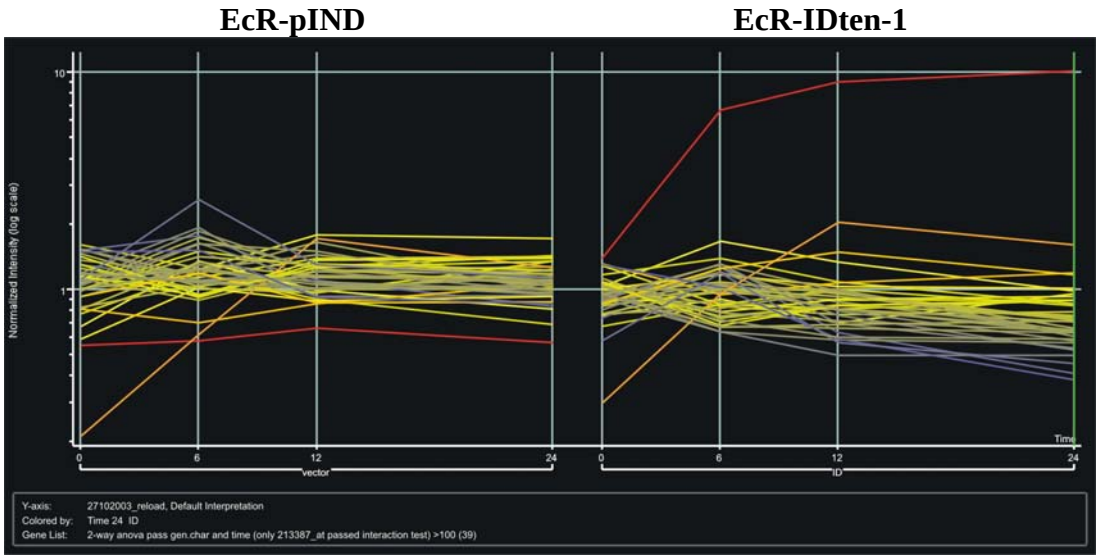


Figure 11. Expression kinetics of genes affected by the expression of IDten-1. The expression kinetics of the 39 genes that passed a 2-Way ANOVA test over a 24-hour time course is shown. Colour information is based on the time point ID 24h.

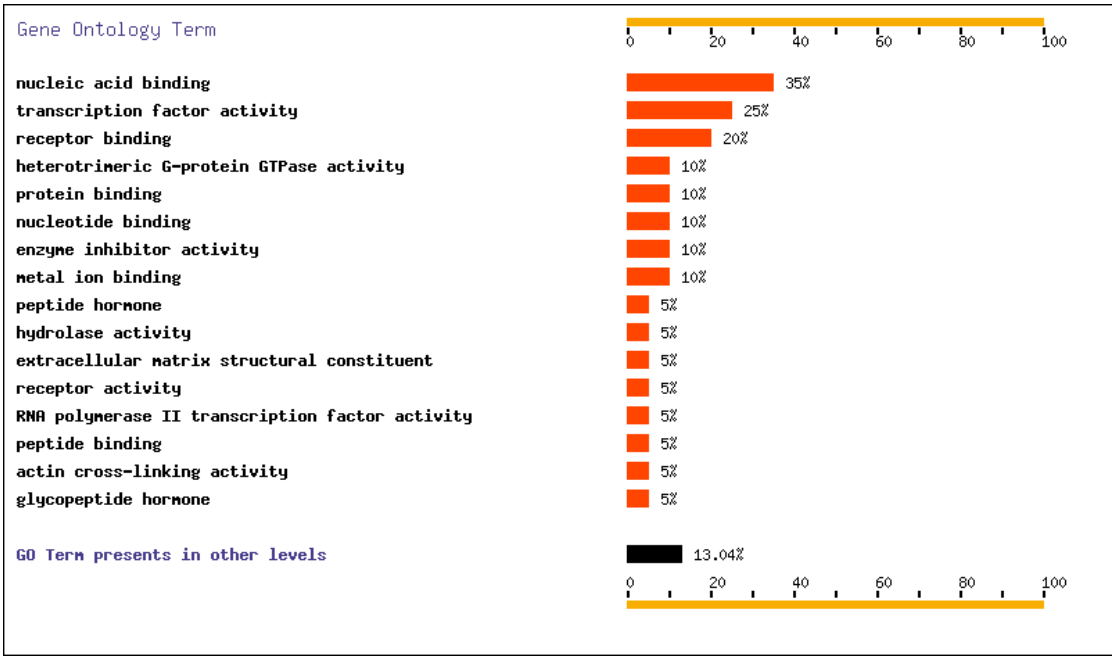


Figure 12. Classification of the 39 genes after analysis with FatiGO. FatiGO: a web tool for finding significant associations of Gene Ontology terms with groups of genes. *Bioinformatics 2004 20: 578-580*

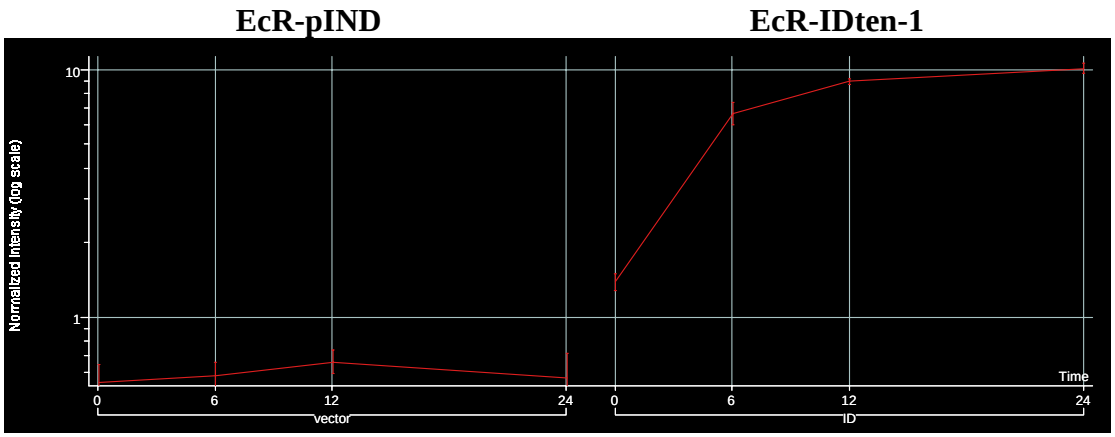


Figure 13. Expression kinetics of the AB03066 gene affected by the expression of IDten-1. The expression of this gene is induced by IDten-1 over a 24-hour time course and remains expressed at a low level in EcR-pIND control cells.

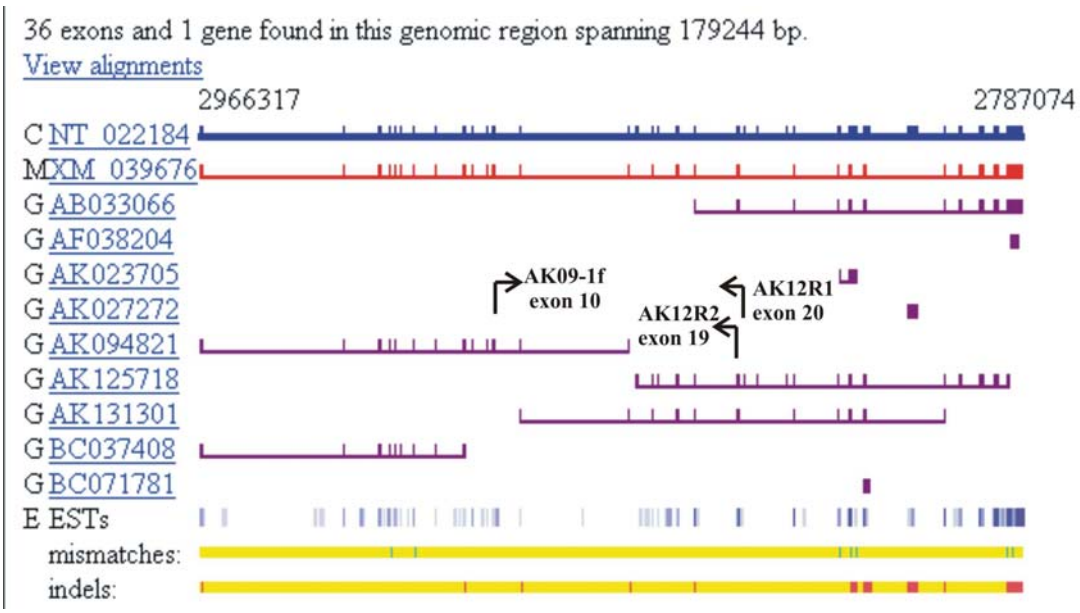


Figure 14. Known ESTs and cDNAs covering the entire coding region of the AB033066 transcript. PubMed predicts that the two consecutive transcripts (AB033066/AK125718 and AK094821) are joined together to form a single longer transcript XM_039676 depicted in red as M. Each spike corresponds to an exon.

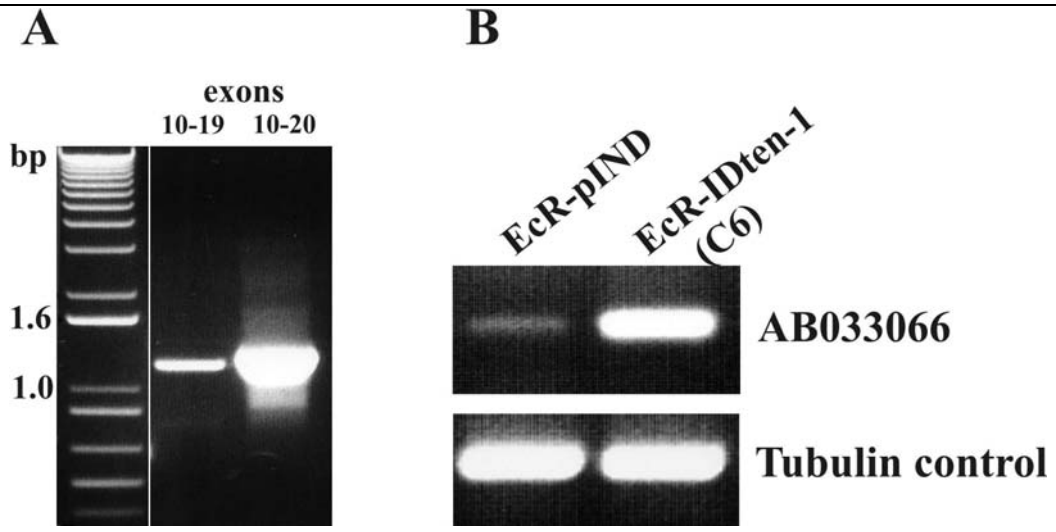


Figure 15. PCR analysis of the XM_039676 gene and of the induction of the transcript induced by IDten-1. (A) PCR reactions were performed with the primers indicated in figure 14 and confirmed that the AB033066 and the AK094821 genes are joined together forming a longer transcript (XM_039676). **(B)** RT-PCR of mRNA isolated from EcR cells induced to express IDten-1 for 24 hours shows the induction of the XM_039676 gene whereas the expression level of the AB033066 gene in vector control cells is very low. Tubulin, which was used as internal control, remains unaltered.

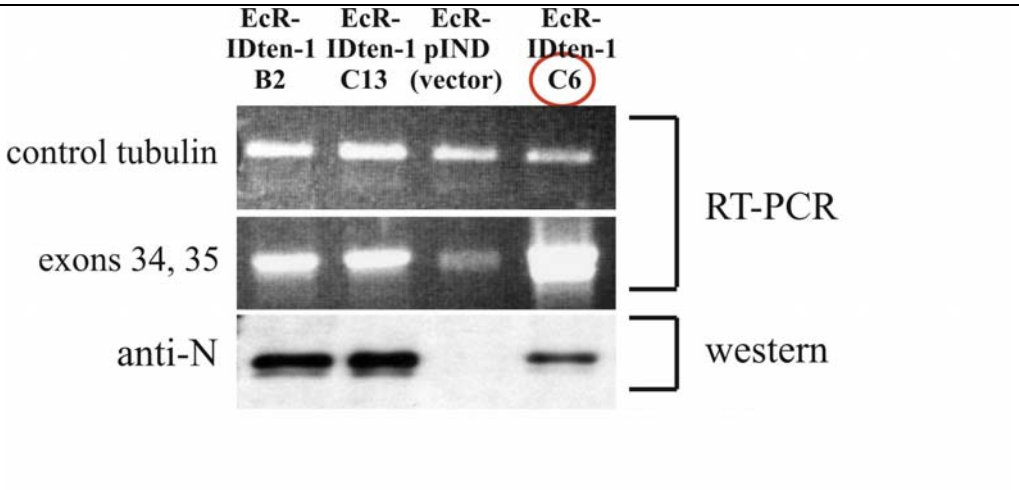


Figure 16. Analysis of the induction of the XM_039676 transcript in other clones induced to express IDten-1. Clones B2, C13 and C6 have been induced to express IDten-1 for 24 hours. IDten-1 can be detected by Western blots of cell extracts, except for the vector control cells (bottom panel). The RT-PCR analysis shows that all clones express similar amounts of tubulin transcripts (top panel) whereas XM_039676 transcripts are highly expressed in clones induced to express IDten-1 as compared to the vector alone (middle panel). The primers used to assess the induction of XM_039676 transcripts amplify the region spanning exons 34-35.

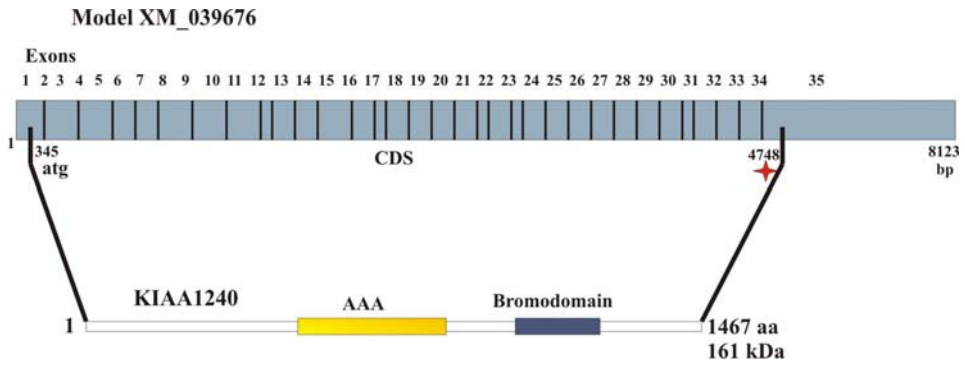


Figure 17. Domain structure of the KIAA1240 protein. The XM_039676 gene contains 35 exons. The predicted molecular weight of the protein encoded by the XM_039676 gene is 161kDa. The protein harbours a central AAA ATPase domain and a Bromodomain.

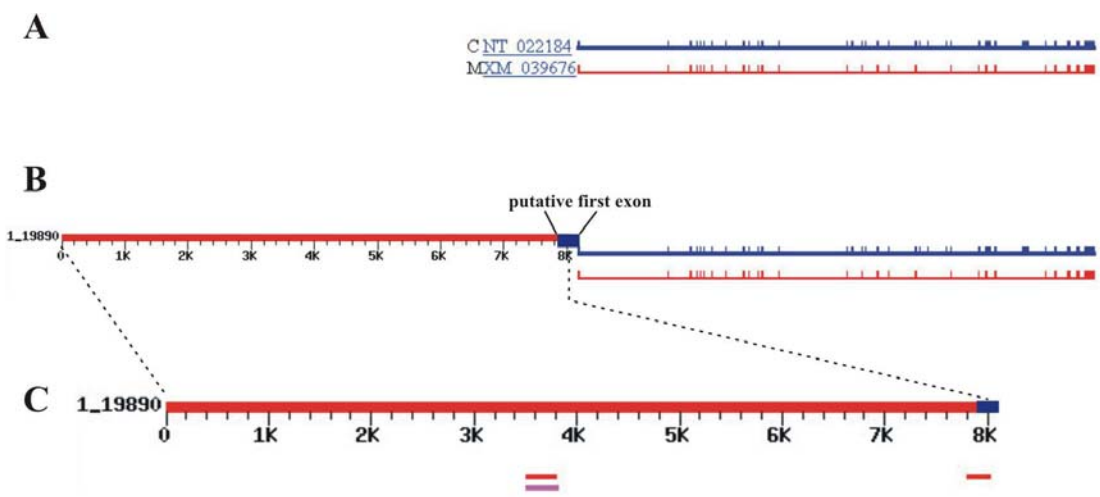


Figure 18. Schematic representation of the putative promoter region of the XM_039676 gene. (A) Gene prediction of XM_039676. **(B)** An 8 kb region upstream and including the first putative exon of the XM_039676 gene was used for the blast search against the mouse genome. **(C)** This search revealed two highly homologous regions of about 200bp each (red bars underlined). The one on the right corresponds to the first exon, whereas the one 4kb upstream may indicate the presence of yet another exon containing 5'UTR sequences of the XM_039676 transcript.

IV. DISCUSSION

Recently, the newly described mechanism defined as Regulated Intramembrane Proteolysis (RIP) has been implicated in the processing of transmembrane proteins. Such a mechanism is also envisaged for the teneurins. It would allow for example, neurons expressing the cell-anchored teneurin, to deliver a signal to the nucleus and counteract the proliferating program of cells and/or promote differentiation. The postulation that teneurin-2 promotes differentiation is based on the fact that in brain its transcripts could not be detected in the cell dense ventricular zone. That suggested that teneurin-2 was not expressed by neuroblasts, but by postmitotic neurons about to establish new connections (Rubin et al., 1999).

Interestingly, cells overexpressing IDten-1 show a reduced rate in proliferation as measured by BrdU incorporation assays. If one considers similar nuclear functions for both teneurin proteins, it can be speculated that this result is due to a modulation of gene expression in order to facilitate a transition from a proliferating state to a differentiating one.

During development, teneurin was found to play a crucial role in the determination of the *Drosophila* segmental body pattern. Genetic analysis has revealed that the *ten-m/odz* gene belongs to the pair-rule class of transcription factors based on the mutant phenotype observed. In addition, *ten-m/odz* was shown to cooperate with *opa* in the same signalling cascade (Baumgartner et al., 1994). *Zic* genes are the vertebrate homologues of *opa*. In a recent study of molecular mechanisms of retinal axon guidance, disruption of gradient expression of Zic3 resulted in abnormal intraretinal axon projection (Zhang et al., 2004). Since in the *Drosophila* work the authors have postulated that *ten-m* may influence the activity of *opa* (Baumgartner et al., 1994) and later on teneurin-2 was shown to repress zic-1-mediated transcription (Bagutti et al., 2003), it is possible that a functional interaction of zic proteins with the teneurin family members exists *in vivo*.

The strategies used to investigate the synthesis and processing of teneurin-1 and the involvement of its intracellular domain in gene regulation

were the following: (1) generation of antibodies to detect endogenous and different forms of overexpressed teneurin-1 in cell culture; (2) yeast two-hybrid screen to identify interacting proteins of IDten-1 and (3) microarray analysis to identify possible target genes of IDten-1. The results of the three approaches described in this thesis will the basis for the following discussion, including an extension of the discussion already provided in the published Part 1 of the Results section (Nunes et al., 2005).

Strategy 1. Generation of antibodies and overexpression of different forms of teneurin-1 in cell culture

Antibodies against teneurin-1 were raised to have a tool for cytological staining and biochemical analyses. The use of specific antibodies was useful to reveal the subcellular localisation of various forms of the protein and their expression pattern in overexpression experiments, including the one of the constructs encoding the intracellular domain of teneurin-1 that mimic cleavage of the endogenous protein.

Interestingly, the pattern of overexpressed IDten-1 protein was highly similar to that of overexpressed IDten-2 in cells. Despite the accumulation of both proteins in subnuclear domains, the teneurin-2 intracellular domain frequently co-stained with PML bodies (Bagutti et al., 2003), while this was only occasionally the case for IDten-1. Overexpressed IDten-1 protein was found to be present in a special subnuclear structure thought to be involved in processes such as DNA replication, transcription and mRNA splicing, the nuclear matrix (Nunes et al., 2005). It would be of interest to investigate whether IDten-2 also cofractionates in the nuclear matrix and whether any correlation can be made between the presence of these proteins in the nuclear matrix and colocalisation with PML bodies. Alternatively, does interaction with different protein partners as shown in (Nunes et al., 2005) play a role in targeting members of the teneurin family to distinct subnuclear structures? Despite the high degree of similarity between teneurin-1 and -2, the two proteins are expressed in a specific subset of neurons of different but

interconnected sites of the developing chicken brain (Rubin et al., 2002). Therefore, it could well be that despite the nuclear localisation of both proteins the interaction with particular subnuclear structures (e.g. nuclear matrix) is mutually exclusive, and in turn responsible for mediating independent functions.

In order to gain a better understanding of IDten-1 signalling, it could be investigated if an association with the nuclear matrix occurs as a consequence of specific signals as described for PCNA. PCNA translocates to the nuclear matrix in mid-G1 and (from the nuclear matrix) to chromatin when DNA replication starts (Naryzhny and Lee, 2004). This could be achieved by staining of synchronized IDten-1 expressing cells at different stages of the cell cycle.

In this context, it would be compelling to check whether the reduced rate in proliferation of cells overexpressing IDten-1 is linked to association of IDten-1 with the nuclear matrix as opposed to direct effects on the activities of specific signalling pathways. For example, it would be possible to measure the proliferation rate of cells expressing IDten-1 mutated at sites containing putative nuclear matrix association signal(s)/or nuclear localisation signal(s).

The possibility that both mechanisms of action might be integrated would be even more exciting. For example, triggering of a specific signalling pathway by IDten-1 promotes its shuttling to the nuclear matrix and as a result of this event cell cycle progression is delayed. Some interesting candidate pathways that could be affected by IDten-1 such as MAPK, PKB and p53 activation were investigated. However, no changes based on the presence of IDten-1 could be detected. In the case of p53, one limitation encountered was the fact that its activation was checked in an indirect way. Instead of a reporter assay, in the future one could analyse the expression of p53 target genes previously shown to be involved in slowing cell cycle progression e.g. p21.

The experiments done with the anti-teneurin-1 antibodies provided some evidence of an extracellular and intramembrane cleavage of teneurin-1 and its translocation to the nucleus.

(A) By Western blotting, detection of the induced form of the full-length teneurin-1 protein was accompanied by additional smaller fragments when anti-N terminus antibody and not anti-C antibody was used. The same smaller fragments could be detected with the antibody against the Flag-tag of the protein, corroborating the hypothesis that these fragments are cleaved forms containing the N-terminus of teneurin-1. In sub-fractionation experiments, both the soluble fraction and the insoluble nuclear matrix of cells were positive for IDten-1 expression. However, only the nuclear matrix fraction of cells induced to express the full-length teneurin-1 and not the one of un-induced cells revealed the presence of an exclusive band, which ran at the same size as the putative intracellular domain (Nunes et al., 2005).

(B) Based on the pattern of expression of the overexpressed protein, we attempted to stain endogenous teneurin-1 by immunofluorescence of chicken embryo fibroblasts. In agreement with the overexpression studies, IDten-1 not only localised to the nucleus (Nunes et al., 2005), but its staining was also positive in the nuclear matrix of these cells.

(C) Using two different polyclonal antibodies against the same N-terminal region of the protein, immunoprecipitation experiments followed by Western blotting revealed the presence of a 75kDa band in lysates of chicken brain. No immunoprecipitated band could be detected in liver, in agreement with the absence of the teneurin-1 mRNA in this organ (Minet et al., 1999). Although speculative, a few explanations are possible.

First, the immunoprecipitated band could correspond to a cleaved form of the protein, which would still be membrane bound. This is supported by the absence of a signal when an anti "C" antibody against the C-terminus of the protein was used, indicating that this part of the protein was not precipitated. Although teneurin-1 does not seem to possess a perfect furin consensus site like teneurin-2 (Rubin et al., 1999) and Notch (Logeat et al., 1998) the possibility remains that related proteases are involved in similar cleavage mechanisms of teneurin-1.

Interestingly, a S1P site (RXX/L/K) (Espenshade et al., 1999) present in the teneurin-1 sequence (at amino acid position 746 - RCTL, (highlighted in red in **figure 19**) could generate a fragment with a molecular weight of approximately 80kDa. In this case, however, in contradiction to what has been shown to be a prerequisite for a secondary intramembrane cleavage, the extracytosolic portion remaining would be much longer than the 30 amino acids described in a previous report (Brown et al., 2000). On the other hand, considering that teneurins might be cleaved in a constitutive manner similar to Notch, the fragment of 75kDa might represent a constitutively cleaved chain that participates in forming the intact molecule. Dibasic sites have been observed frequently as recognition sites for a family of mammalian proteases with similarity to bacterial subtisilin (Barr et al., 1991). All mouse ten-m proteins contain several dibasic sites close to the membrane-spanning region and one of them is conserved in all sequences and in the *Drosophila* ten-m/odz and ten-a. Dibasic sites can also be found in the chicken teneurin-1 sequence (**figure 19**). Another indication for extracellular cleavage of teneurins comes from the following publication. Schaefer et al. have detected a soluble form of γ-heregulin in the supernatant of the breast cancer cell line MDA-MB-175, suggesting proteolytic processing of this protein (Schaefer et al., 1997). In this cell line, the γ-heregulin gene has been found rearranged and fused with the DOC4 (rat teneurin-4) gene (Wang et al., 1999). The resulting ten-m4 γ-heregulin fusion protein includes the dibasic site sequence juxtaposed to the transmembrane domain. Therefore, the potential for shedding of the extracellular domain may be a characteristic of all ten-m family members (Oohashi et al., 1999). In other words, besides teneurin-2 (which has been shown to be processed at a furin site) the activity of other teneurins and related proteins might also be subject to proteolytic cleavage (Rubin et al., 1999).

The second explanation is based on the *in-vitro* sub-fractionation experiment of transfected U2OS and EcR-IDten-1 expressing cells (discussed in (A)), where IDten-1 was found associated with the insoluble nuclear matrix fraction of these cells. Assuming that the same occurs *in vivo*, it is conceivable

that the nuclear matrix associated part of the protein cannot be immunoprecipitated from brain extracts since it cannot be solubilised in the first place. This may explain why we failed to detect a smaller form of teneurin-1 (e.g. the 55kDa form) by immunoprecipitation. In fact, many proteins are known to shuttle between different compartments e.g. (nuclear/cytoplasmic soluble or nuclear matrix associated/ insoluble form).

Yet another speculative explanation for the 75kDa fragment is that this size of protein corresponds to a post-translationally modified intracellular form of IDten-1, which, in a similar manner as described above, shuttles between different compartments within the cell. Modifications such as ubiquitination or sumoylation will cause a protein to migrate slower in SDS-PAGE as compared to its unmodified form. Ubiquitination and sumoylation are known to target proteins to nuclear substructures and play a role in the regulation of gene expression (Gill, 2004). Thus, immunoprecipitated teneurin-1 could be analysed by immunoblotting with anti-ubiquitin and anti- sumo antibodies for the presence of these modifications.

Intriguingly, on Western blots of nuclear matrices of chicken brain extracts and CEF cells, bands of 75kDa and 55kDa were detected with anti-N antibody that were absent with control pre-immune serum (**cf. supplementary figure 2**). This provides support for the possibility that the 75kDa band could be a modified nuclear form of the teneurin-1 intracellular domain. More experiments will be necessary to confirm the specificity of the signals detected in the Western blots. One approach could be RNA-interference in CEF cells to confirm the specificity of the bands observed.

Strategy 2. Yeast two-hybrid screen to identify interacting proteins of the IDten-1

In collaboration with Jacqueline Ferralli, who performed the yeast two-hybrid screen, it was possible to identify two highly interesting proteins interacting with IDten-1; CAP/ponsin and MBD1. Hopefully they will contribute to a better understanding of teneurin-1 function. The experiments

describing the interacting proteins of teneurin-1; CAP/ponsin and MBD1 are discussed in detail in (Nunes et al., 2005). Since CAP/ponsin is found in a multi-protein complex at cell-cell and cell-ECM sites and teneurin-1 is present as a transmembrane protein, we envisaged a scenario where both proteins would co-function at these sites. Indeed, in overexpression experiments, both proteins showed a colocalisation at cell-cell interaction sites. Interestingly CAP/ponsin, which by itself showed specific cytoskeletal staining, upon co-transfection with IDten-1 was translocated to the nucleus, the main site where IDten-1 accumulated. Previously, a nuclear function for CAP/ponsin has been reported. The protein was shown to interact with the mouse homologue of the human zinc-finger transcription factor INSM1 (IA-1) expressed during embryo development (Xie et al., 2002). IA-1 had a restricted distribution in the embryonic nervous system and tumours of neuroendocrine origins (Breslin et al., 2003). In addition, CAP/ponsin was found to be present in the nucleus of the mouse mammary tumour cell line MTD-1A but the significance of this nuclear staining was unclear (Mandai et al., 1999). All these findings provide additional indications that CAP/ponsin and IDten-1 may function in the nucleus.

The other interacting protein of the IDten-1 found in the two-hybrid screen was the transcription repressor MBD1. This was the first direct link of IDten-1 to gene regulation since certain domains present in MBD1 are responsible for binding to DNA and for remodelling of chromatin (Fujita et al., 2003). However, despite the identification of these interacting proteins and the knowledge of some of their functions, many questions remain. For instance, what is the relevance of these interactions and their functional consequence? When are they taking place in a cell and to which signalling pathway do they belong to? Ultimately, what is the contribution of these interactions to gene regulation? In the case of MBD1, it was shown that the protein could repress transcription specifically (Fujita et al., 1999). Therefore, it could be tested whether interaction with IDten-1 increases or relieves this repression.

Strategy 3. Microarray analysis

Microarray experiments were performed using the EcR-IDten-1 cell line, where we can mimic the induction of the putative intracellular teneurin fragment that translocates to the nucleus. Expression patterns of genes responding to the absence or presence of IDten-1 were analysed. Interestingly, the expression of the XM_039676 gene was found to be dramatically induced after stimulation of IDten-1 protein expression and it was therefore chosen for further analyses. The protein encoded by this gene (KIAA1240) is predicted to harbour a bromodomain, which is often present in proteins possessing chromatin-remodelling properties. Proteins that contain this motif are implicated both in transcriptional activation and repression (Denis, 2001). However, the domain is also found in several proteins not directly participating in chromatin modification - the *Drosophila fsh* gene and the related *RING3* gene encode nuclear protein kinases (Denis et al., 2000), and several of the non-enzymatic components of the ATP-dependent remodelers (e.g. RSC1/2/3 and Polybromo) also contain essential bromodomains (Cairns et al., 1999). Overall, bromodomain-containing proteins have been implicated in functions as diverse as cell-cycle control (CCG1 and RING3) (Denis et al., 2000), cellular differentiation (the *Drosophila* genes *fsh* and *brm*) (Tamkun, 1995), transcriptional regulation (SWI/SNF and RSC) and mitotic chromatin dynamics (Dey et al., 2000). Moreover, the relationship between acetyllysine and bromodomains is reminiscent of the specific recognition of phosphorylated residues by phospho-specific binding molecules such as SH2 domains and 14-3-3 proteins. Bromodomains of Gcn5, PCAF, TAF1 and CBP are able to recognize acetyllysine residues in histones, HIV Tat, p53, c-Myb or MyoD (Yang, 2004).

In addition to the bromodomain an AAA domain (ATPase Associated to a variety of cellular Activities) was found in the protein encoded by the XM_039676 gene. The key feature of this domain is a highly conserved module of 230 amino acids present as one or two copies per protein (Confalonieri and Duguet, 1995). Very recent reports implicate a highly

conserved AAA-ATPase in spindle disassembly at the end of mitosis (Cheeseman and Desai, 2004). The proteasome and related energy-dependent proteases, as well as NSF, p97, and Hsp104, all employ AAA+ ATPase domains for reactions that involve protein remodelling, unfolding, or disaggregation. ATPases can also function independently of peptidases as disassembly chaperones to dismantle protein complexes and to solubilise aggregates (Sauer et al., 2004).

Interestingly, the *Drosophila* brahma (*brm*) gene contains both bromo and ATPase domains (Tamkun et al., 1992). The brahma complex of *Drosophila* is a SWI/SNF-related chromatin remodelling complex required to correctly maintain proper states of gene expression through ATP-dependent modification of chromatin structure (Marenda et al., 2004). Further studies will need to be performed to determine the function of our newly identified KIAA1240 protein harbouring these domains.

To exclude the possibility that induction of XM_039676 by IDten-1 was an artefact of the particular clone used for the gene array, the activation of the XM_039676 gene was tested in other clones overexpressing IDten-1. We could confirm that this induction was reproducible in several other clones but not all clones induced expression of the transcript to the same extent. This could be interpreted as follows: (1) there are cell clone-dependent differences in the way this gene is regulated that are independent of IDten-1, (2) each clone behaves somewhat different depending on the insertion site of the transgene into the host genome and in some clones the transgene may even have been rearranged when it inserted into the host cell genome, thus expressing a potentially non-functional IDten-1, (3) each clone displays different kinetics and possibly varied levels of expression of IDten-1 and (4) there is the possibility of a negative feedback loop to repress XM_039676 expression after it has been induced. If one assumes that IDten-1 must form stoichiometric complexes with co-factors to regulate gene expression it is conceivable that too high levels of IDten-1 protein eventually results in the formation of non-functional

complexes, mimicking a dominant negative effect. These hypotheses are highly speculative, but merit further investigations in the future.

Clearly, the following points need to be addressed: (i) is the IDten-1 involved directly in the activation of the XM_039676 gene and (ii) does it function as a co-factor in association with other proteins in a yet-to-be defined signalling complex?

One possibility is that IDten-1/MBD1/Ponsin complexes are involved in regulating the methylation status of the XM_039676 promoter region. Thus, the gene could be induced by removal of inhibitory DNA-methylation. In this context, it could be checked if the promoter region of the XM_039676 is methylated or not. Furthermore, depletion of MBD1 and/or Ponsin (the former may be required to recognize methylated DNA regions) by siRNA could be useful to determine if they are required for the induction of the XM_039676 gene in cell clones expressing IDten-1.

If the promoter region of this newly identified gene induced by IDten-1 is a direct target of IDten-1-containing DNA regulatory complexes, it will be possible to use the induction of XM_039676 expression as a read out of teneurin-1 signalling. For example, one could compare different stimuli and measure the levels of activation of the XM_039676 gene as a consequence of cleavage and translocation of IDten-1 to the nucleus. The promoter sequence could be cloned in front of a luciferase reporter to monitor its activation. This would allow pinpointing the IDten-1 dependent gene-regulatory region by introducing deletions. Using this approach, it could also be possible to identify regions containing modulators that might up- or downregulate the expression of the target gene.

In a more indirect way, cell clones stably expressing full-length teneurin-1 could be transfected with various protease-encoding constructs and screened for induction of the XM_039676 gene (e.g. by RT-PCR) or of a cotransfected luciferase reporter construct in an attempt to identify the physiologically relevant candidate that promotes teneurin-1 cleavage. An alternative way to find teneurin-1 cleaving proteases would be the adaptation of

a widely used strategy in protease(s) screenings. Steiner et al. have reported an *in vivo* assay for the identification of target proteases, which cleave membrane-associated substrates (Steiner et al., 1999). For instance, by measuring the activity of a reporter gene, one could test if the transmembrane sequence of teneurin-1, containing the cleavage sites for potential target proteases cloned in frame with GAL 4, would be able to induce the growth of a yeast strain harbouring a *GAL1-His* operon in the absence of histidine.

Apart from the XM_039676 gene, some other interesting genes still remain to be analysed. Of interest are the ones present in the analysis that can be classified in a group with the XM_039676 gene based on their expression profile (**table 4**). In addition, candidate genes that could lead to reduced proliferation and genes potentially associated with increased differentiation were analysed. Some interesting candidates from the 1-Way ANOVA list are shown in the next table (**table 5**). However, such correlations may become more evident only when performing microarray analysis after longer IDten-1 induction.

Table 4: Group of genes with similar expression kinetics as compared to the XM_039676 gene

C9orf7 (chromosome 9 open reading frame 7)
KCND1 (potassium voltage-gated channel, Shal-related subfamily member 1)
CLDN3 (claudin 3)
MGC3038 (hypothetical protein similar to actin related protein 2/3 complex subunit 5)
PBXIP1 (pre-B-cell leukemia transcription factor interacting protein 1)
EGLN2 (egl nine homolog C. elegans)
JPH2 (junctophilin 2)
IGKC (immunoglobulin kappa constant)

Table 5: Genes that are potentially involved in cell proliferation and cell differentiation

YWHAH (evidence of involvement in regulation of neuron differentiation)
GADD45B (evidence of involvement in cell differentiation)
IFITM1 (negative regulation of cell proliferation)
RBM9 (regulation of cell proliferation)

Finally, chromatin immunoprecipitation could be an appropriate extension of the DNA-microarray analysis. With the use of the antibodies generated to immunoprecipitate IDten-1 it would be possible to investigate whether IDten-1 and/or MBD1 are part of a protein complex binding to DNA that contains the XM_039676 gene detected by PCR.

To finalize I would like to propose a possible model that would integrate some of the findings discussed above (**figure 20**). Teneurin-1, upon yet-to-be identified stimuli, is cleaved at the cell membrane and its intracellular domain translocates to the nucleus together with CAP/ponsin. In the nucleus, most likely as a response to specific signals, the IDten-1 shuttles to the insoluble nuclear matrix. In complexes that may involve MBD1/Ponsin or not, IDten-1 appears to be involved in regulating XM_039676 gene expression. The KIAA1240 protein may itself be involved in chromatin remodelling and may trigger changes in histone acetylation by recruiting HDACs to those genes that are ultimately responsible for the observed reduction in the rate of cell proliferation of cells expressing IDten-1. Indeed, changes in chromatin topology e.g. induced by HDAC inhibitors can block cell proliferation, induce apoptosis or differentiation (Atadja et al., 2004; Nimmanapalli et al., 2003).

MEQMDCKPYQPLSKVKHEVDLTYTSSSDESEDGRKQRQSYDSRETLNEYSQELRLNYNSQSR
KRKNTDQSTQDMEFCETPHILCSGYQTDLHGVSEHSYPLEVGSDVDTETEGGASPDHALRMW
MRGMKSEHSSCLSSRANSALSLTDTDHERKSDGENDMPGSPHNQFTFRPLPPPPPPPHACTC
TRKPPPAADSLQRRSMTTRSQPSPAAPTPPTSTQDSVHLHNSWVLNSNIPLETRHFLFKHGS
GSSAIFSAASQNYPLTSNTVYSPPPRPLPRNTFSRPAFTFSKPYRCCNWKCTALSATAITVT
LALLLAYVIAVHLFGLTWQLQPVEGQLYENGVSKGNKGAESTDTTYSPIGGKVSDKTEKKVF
QKGRAIDTGEVEIGAQVMQTIPPGLFWRFQITIHHPVYLKFNISLAKDSLLGIYGRRNIPPT
HTQFDFVKLMDGKQLIKQEPKNSEEPQQAPRNLILTSLQETGFIEYMDQGAWHMAFYNDGKK
VEQVFVLTTAIEVLDDCSTNCNGNGECISGHCHCFPGFLGPDCAKDSCPVLCSGNGEYEKGH
CVCRNGWKGPECDVPEEQCIDPTCFGHGTCIMGVCICVPGYKGEICEEEDCLDPMCSGHGVC
VQGECHCSAGWGGVNCETSLPICQEHCSGHGTFLLDVGLCSCEPQWTGSDCSTELCTLDCGS
HGVCSRGICQCEEGWVGPTCEERTCHSHCAEHGQCKDGKCECSPGWEGDHCTIDGCPGLCYG
NGRCTLDQNGWHCVCQVGWSGSGCNVVMEMACGDNLDNDGDGLTDCVDPDCCQQNNCYASPL
CQGSPDPLDLIQHSQPPFSQHPPRLFYDRIRFLIGKESTHVIPGDISFESRRASVIRGQVVA
IDGTPLVGVNVSFLHHDEYGYTISRQDGSFDLVAVGGISVTLVFDRSPFISEKRTLWLSWNR
FVIVDKVVMQRAESDIPSCDVSSFISPNPIVLPSPLTAFGGSCPERGTVIPELQVVQEEIPI
PSSFVKLSYLSSRTPGYKTLLRVILTHTTIPSGMTKVHLIIAVEGRLLQKWFPAAANLVYTF
AWNKTDIYGQKVSGLAEAMVSVGYEYETCPDFILWEKRTVILQGFEMDASNLGGWSINKHHV
LNPQSGIVHKGNGENMFISQQPPVISTMMGNGHQRSVSCSNCNGLALNSKLFAPVALTSGPD
GSVYIGDFNFVRRIFPSGNSIGILELRNRDTRHSTSPAHKYYLAVDPVSESLYLSDTNTRRV
YKAKSLIETKDLAKNVDVVAGTGDQCLPFDQSHCGDGGKASEASLNSPRGITIDKHGFIYFV
DGTMIRKIDENGMITTIIGSNGLTSTQPLSCDSGMDITQVRLEWPTDLTVNPLDNSLYVLDN
NIVLQISESRRVRIIAGRPIHCQVPGIDHFIVSKVAIHSTLESARAIAVSHSGIPYIRETDE
RKINRIQQVTTNGEISIIAGAPSDCDCKIDPNCDCFSGDGGYAKDAKLKAPSSLAVSPDDTL
YVADLGNIRIRAVSRNKAHLSDTNMYEIASPADQELYQFTINGTHLHTLNLITRDYIYNFTY
SGEGDVATITSSNGNSVHIRRDTSGLPLWVVVPGGQVYWLTISSNGVLKRVYAQGYNLALMT
YPGNTGLLATKSDENGWTTVYEYDSDGHLTNATFPTGEVSSFHSDVEKLTRVELDTSNRENM
VTATNFSATSTIYTLKQDNTQNIYRVSPDGSLRVTFASGMEITLNTEPHILAGVVSPTLGKC
NISLPGEHNSNLIEWRQRREQTKGNISTFERRLRAHNRNLLSIDFDHVTRTGKIYDDHRKFT
LRIMYDQTGRPVLWSPISKYNEVNITYSHSGLVTYIQRGTWTEKMEYDPSGNIISRTWADGK
IWSYTYLEKSVMLLLHSQRRYIFEYDQSDYLLSVTMPSMVRHALQTMLSVGYYRNIYTPPDS
GAAFIQDVTRDGRLLQTLYPGTGRRVLYKYSKQSRLSEILYDTTQVTFTYEESSGVIKTIHL
MHDGFICTIRYRQTGPLIGRQIFRFSEEGLVNARFDYSYNNFRVTSMQAMINETPLPIDLYR
YVDVSGRTEQFGKFSVINYDLNQVITTTVMKHTKIFSANGQVIEVQYEILKSIAYWMTIQYD
NMGRMVICDIRVGVDANITRYFYEYDRDGQLQTVSVNDKTQWRYSYDLNGNINLLSHGNSAR
LTPLRYDLRDRITRLGEIQYKMDEDGFLRQRGNEIFEYNSNGLLNKAYNKVSGWTVQYCYDG
LGRRVASKSSLGQHLQFFYADLSNPIRVTHLYNHSSSEITSLYYDLQGHLIAMELSSGEEYY
VACDNTGTPLAVFSSRGQVIKEILYTPYGEIYQDTNPDFQVVIGFHGGLYDSLTKLVHLGQR
DYDVIAGRWTTPNHHIWKHLNAVPQPFNLYSFENNYPVGRIQDVAKYTTDIGSWLELFGFQL
HNVLPGFPKPEIEALETTYELLQLQTKTQEWDPGKTILGIQCELQKQLRNFISLDQLPMTPR
YSDGKCYEGVKQPRFAAIPSVFGKGIKFAIKDGIVTADIIGVANEDSRRIAAILNNAHYLEN
LHFTIEGRDTHYFIKLGSLEEDLSLIGNTGGRRILENGVNVTVSQMTSVINGRTRRFADIQL
QHGALCFNVRYGTTVEEEKNHVLEVARQRAVAQAWTKEQRRLQEGEEGIRAWTDGEKQQLLN
TGRVQGYDGYFVLSVEQYLELSDSANNIHFMRQSEIGRR

Figure 19. Amino acid sequence of teneurin-1 protein. Putative S1P cleavage sites present in the protein are highlighted in blue. The particular S1P cleavage site of interest that could generate a fragment of approximately 80kDa is depicted in red. The teneurin-1 transmembrane domain is highlighted in yellow. Dibasic arginine and lysine residues in the vicinity of the transmembrane domain that have the potential to serve as recognition sites for proteases are shown underlined.

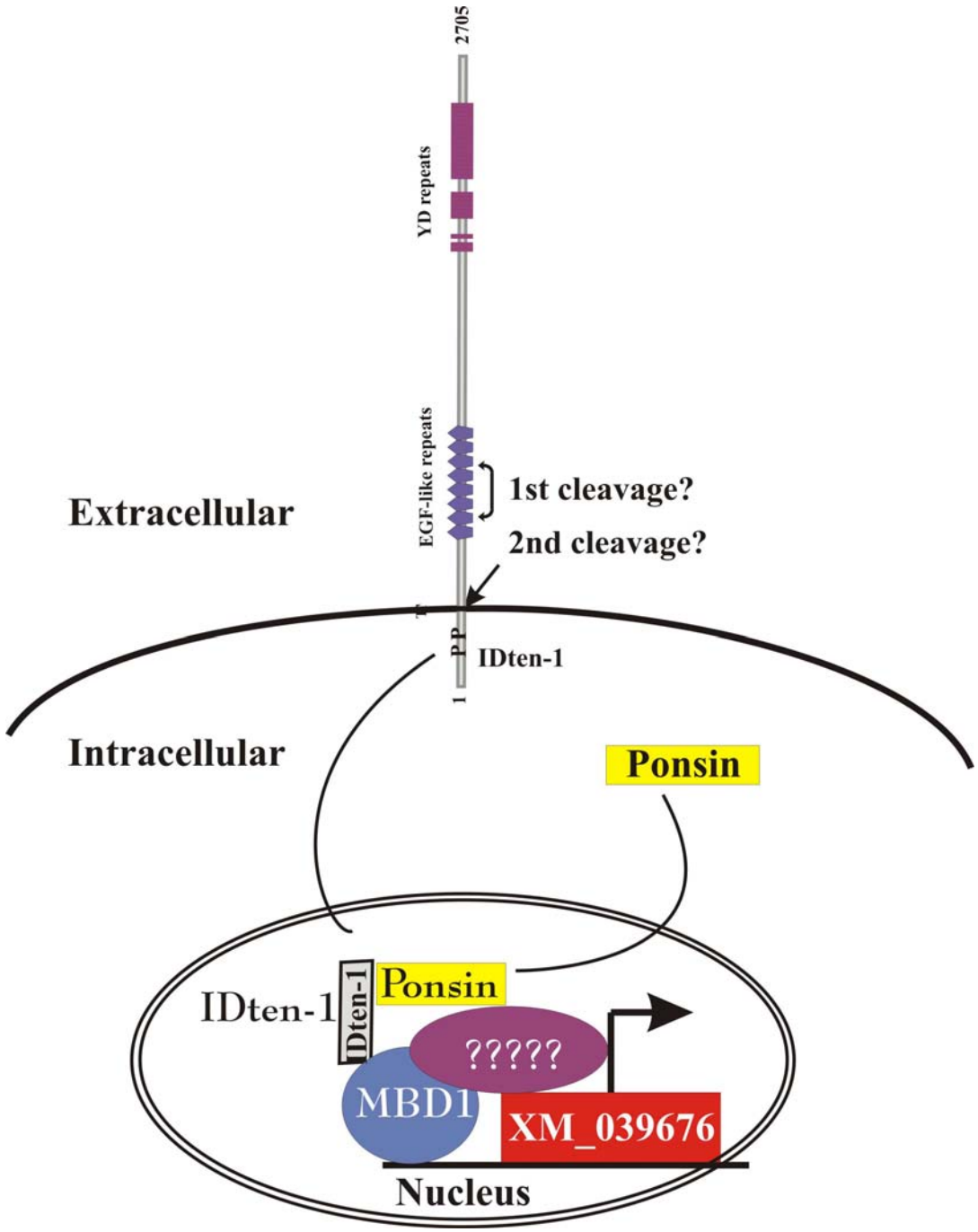


Figure 20. Proposed model for teneurin-1 signalling. Teneurin-1, upon cleavage at the cell membrane by unknown proteases, releases its intracellular domain. The IDten-1 then translocates to the nucleus together with CAP/ponsin where, in association with MBD1, it may be involved in the regulation of target genes like for example the XM_039676 gene, which was identified in the microarray analysis.

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VI. APPENDIX

List I - 1 Way ANOVA

Affy Annotation	Description	Abbreviation
221168_at	PR domain containing 13	PRDM13
213135_at	T-cell lymphoma invasion and metastasis 1	TIAM1
210276_s_at	Tara-like protein	HRIHFB2122
208663_s_at	tetratricopeptide repeat domain 3	TTC3
213721_at	SRY (sex determining region Y)-box 2	SOX2
220209_at	peptide YY, 2 (seminalplasmin)	PYY2
213641_at	KIAA0557 protein	KIAA0557
201739_at	serum/glucocorticoid regulated kinase	SGK
201300_s_at	prion protein (p27-30) (Creutzfeld-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia)	PRNP
214464_at	CDC42 binding protein kinase alpha (DMPK-like)	CDC42BPA
202477_s_at	tubulin, gamma complex associated protein 2	TUBGCP2
217403_s_at	zinc finger protein 227	ZNF227
204720_s_at	DnaJ (Hsp40) homolog, subfamily C, member 6	DNAJC6
218294_s_at	nucleoporin 50kDa	NUP50
217775_s_at	retinol dehydrogenase 11 (all-trans and 9-cis)	RDH11
211952_at	karyopherin (importin) beta 3	KPNB3
210793_s_at	nucleoporin 98kDa	NUP98
204004_at	PRKC, apoptosis, WT1, regulator	PAWR
201708_s_at	nipsnap homolog 1 (C. elegans)	NIPSNAP1
200676_s_at	ubiquitin-conjugating enzyme E2L 3	UBE2L3
207753_at	zinc finger protein 304	ZNF304
201611_s_at	isoprenylcysteine carboxyl methyltransferase	ICMT
217986_s_at	bromodomain adjacent to zinc finger domain, 1A	BAZ1A
204047_s_at	chromosome 6 open reading frame 56	C6orf56
207081_s_at	phosphatidylinositol 4-kinase, catalytic, alpha polypeptide	PIK4CA
221646_s_at	zinc finger, DHHC domain containing 11	ZDHHC11
218726_at	hypothetical protein DKFZp762E1312	DKFZp762E1312
208802_at	signal recognition particle 72kDa	SRP72
208228_s_at	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)	FGFR2
218092_s_at	HIV-1 Rev binding protein	HRB
213376_at	zinc finger and BTB domain containing 1	ZBTB1
204799_at	zinc finger, BED domain containing 4	ZBED4
208934_s_at	lectin, galactoside-binding, soluble, 8 (galectin 8)	LGALS8
212361_s_at	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	ATP2A2
203570_at	lysyl oxidase-like 1	LOXL1
220707_s_at	hypothetical protein FLJ23322	FLJ23322

207574_s_at	growth arrest and DNA-damage-inducible, beta	**GADD45B**
207826_s_at	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	**ID3**
217975_at	pp21 homolog	**LOC51186**
213598_at	putative dimethyladenosine transferase	**HSA9761**
212693_at	Homo sapiens transcribed sequences	**---**
202703_at	dual specificity phosphatase 11 (RNA/RNP complex 1-interacting)	**DUSP11**
202943_s_at	N-acetylgalactosaminidase, alpha-	**NAGA**
212248_at	LYRIC/3D3	**LYRIC**
212206_s_at	histone H2A.F/Z variant	**H2AV**
210735_s_at	carbonic anhydrase XII	**CA12**
201819_at	scavenger receptor class B, member 1	**SCARB1**
221987_s_at	hypothetical protein FLJ10534	**FLJ10534**
203058_s_at	3'-phosphoadenosine 5'-phosphosulfate synthase 2	**PAPSS2**
208933_s_at	hypothetical protein FLJ10359	**FLJ10359**
212113_at	Homo sapiens mRNA; cDNA DKFZp313P052 (from clone DKFZp313P052)	**---**
216215_s_at	RNA binding motif protein 9	**RBM9**
206061_s_at	Dicer1, Dcr-1 homolog (Drosophila)	**DICER1**
213035_at	KIAA0379 protein	**KIAA0379**
218358_at	hypothetical protein MGC11256	**MGC11256**
218626_at	eukaryotic translation initiation factor 4E nuclear import factor 1	**EIF4ENIF1**
201900_s_at	aldo-keto reductase family 1, member A1 (aldehyde reductase)	**AKR1A1**
205501_at	Homo sapiens cDNA FLJ25677 fis, clone TST04054	**---**
201150_s_at	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	**TIMP3**
207781_s_at	zinc finger protein 6 (CMPX1)	**ZNF6**
221516_s_at	hypothetical protein FLJ20232	**FLJ20232**
212271_at	mitogen-activated protein kinase 1	**MAPK1**
204637_at	glycoprotein hormones, alpha polypeptide	**CGA**
214659_x_at	ZAP3 protein	**ZAP3**
210416_s_at	CHK2 checkpoint homolog (S. pombe)	**CHEK2**
212157_at	syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan)	**SDC2**
207060_at	engrailed homolog 2	**EN2**
213682_at	nucleoporin 50kDa	**NUP50**
219437_s_at	nasopharyngeal carcinoma susceptibility protein	**LZ16**
203031_s_at	uroporphyrinogen III synthase (congenital erythropoietic porphyria)	**UROS**
203089_s_at	protease, serine, 25	**PRSS25**
206245_s_at	influenza virus NS1A binding protein	**IVNS1ABP**
202944_at	N-acetylgalactosaminidase, alpha-	**NAGA**
201569_s_at	CGI-51 protein	**CGI-51**

205980_s_at	Rho GTPase activating protein 8	ARHGAP8
64474_g_at	hypothetical protein FLJ22127	FLJ22127
219180_s_at	peroxisome biogenesis factor 26	PEX26
202478_at	tribbles homolog 2	TRB2
217445_s_at	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	GART
200749_at	RAN, member RAS oncogene family	RAN
204593_s_at	hypothetical protein FLJ20232	FLJ20232
208712_at	cyclin D1 (PRAD1: parathyroid adenomatosis 1)	CCND1
203639_s_at	fibroblast growth factor receptor 2 (bacteria expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)	FGFR2
201020_at	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	YWHAH
202225_at	Homo sapiens cDNA FLJ38130 fis, clone D6OST2000464.	---
202211_at	ADP-ribosylation factor GTPase activating protein 3	ARFGAP3
212596_s_at	high-mobility group protein 2-like 1	HMG2L1
203401_at	phosphoribosyl pyrophosphate synthetase 2	PRPS2
202990_at	phosphorylase, glycogen; liver (Hers disease, glycogen storage disease type VI)	PYGL
208890_s_at	plexin B2	PLXNB2
207830_s_at	protein phosphatase 1, regulatory (inhibitor) subunit 8	PPP1R8
213626_at	hypothetical protein FLJ14431	FLJ14431
205206_at	Kallmann syndrome 1 sequence	KAL1
201641_at	bone marrow stromal cell antigen 2	BST2
211725_s_at	BH3 interacting domain death agonist	BID
209994_s_at	ATP-binding cassette, sub-family B (MDR/TAP), member 1	ABCB1
220940_at	KIAA1641 protein	KIAA1641
213577_at	squalene epoxidase	SQLE
216251_s_at	KIAA0153 protein	KIAA0153
201601_x_at	interferon induced transmembrane protein 1 (9-27)	IFITM1
208002_s_at	brain acyl-CoA hydrolase	BACH
55081_at	molecule interacting with Rab13	MIRAB13
205335_s_at	signal recognition particle 19kDa	SRP19
209598_at	paraneoplastic antigen MA2	PNMA2
205350_at	cellular retinoic acid binding protein 1	CRABP1
201928_at	plakophilin 4	PKP4
201929_s_at	plakophilin 4	PKP4
203164_at	solute carrier family 33 (acetyl-CoA transporter), member 1	SLC33A1
206874_s_at	Ste20-related serine/threonine kinase	SLK
213880_at	G protein-coupled receptor 49	GPR49
208817_at	catechol-O-methyltransferase	COMT

202816_s_at	synovial sarcoma translocation, chromosome 18	SS18
218421_at	ceramide kinase	CERK
217870_s_at	UMP-CMP kinase	UMP-CMPK
217738_at	pre-B-cell colony-enhancing factor	PBEF
206106_at	mitogen-activated protein kinase 12	MAPK12
206833_s_at	acylphosphatase 2, muscle type	ACYP2
212993_at	hypothetical protein MGC23427	MGC23427
210732_s_at	lectin, galactoside-binding, soluble, 8 (galectin 8)	LGALS8
201650_at	keratin 19	KRT19
208833_s_at	like mouse brain protein E46	E46L
209305_s_at	growth arrest and DNA-damage-inducible, beta	GADD45B
211754_s_at	solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34kDa), member 17	SLC25A17
204620_s_at	chondroitin sulfate proteoglycan 2 (versican)	CSPG2
214794_at	proliferation-associated 2G4, 38kDa	PA2G4
209407_s_at	deformed epidermal autoregulatory factor 1 (Drosophila)	DEAF1
203196_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	ABCC4
214022_s_at	interferon induced transmembrane protein 1 (9-27)	IFITM1
201627_s_at	insulin induced gene 1	INSIG1
210010_s_at	solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1	SLC25A1
200683_s_at	ubiquitin-conjugating enzyme E2L 3	UBE2L3
200732_s_at	protein tyrosine phosphatase type IVA, member 1	PTP4A1
205881_at	zinc finger protein 74 (Cos52)	ZNF74
208351_s_at	mitogen-activated protein kinase 1	MAPK1
37117_at	Rho GTPase activating protein 8	ARHGAP8
219959_at	molybdenum cofactor sulfurase	HMCS
204594_s_at	hypothetical protein FLJ20232	FLJ20232
205037_at	RAB, member of RAS oncogene family-like 4	RABL4
208284_x_at	gamma-glutamyltransferase 1	GGT1
200684_s_at	ubiquitin-conjugating enzyme E2L 3	UBE2L3
209656_s_at	transmembrane 4 superfamily member 10	TM4SF10
205822_s_at	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	HMGCS1
200597_at	eukaryotic translation initiation factor 3, subunit 10 theta, 150/170kDa	EIF3S10
204126_s_at	CDC45 cell division cycle 45-like (S. cerevisiae)	CDC45L
204693_at	CDC42 effector protein (Rho GTPase binding) 1	CDC42EP1
209590_at	bone morphogenetic protein 7 (osteogenic protein 1)	BMP7
212190_at	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	SERPINE2
214752_x_at	filamin A, alpha (actin binding protein 280)	FLNA
208174_x_at	U2(RNU2) small nuclear RNA auxiliary factor 1-like 2	U2AF1L2
209200_at	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)	MEF2C

201362_at	influenza virus NS1A binding protein	**IVNS1ABP**
208711_s_at	cyclin D1 (PRAD1: parathyroid adenomatosis 1)	**CCND1**
219629_at	hypothetical protein FLJ20635	**FLJ20635**
213405_at	Homo sapiens transcribed sequences	**---**
206002_at	G protein-coupled receptor 64	**GPR64**
206356_s_at	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type	**GNAL**
201570_at	CGI-51 protein	**CGI-51**
215977_x_at	glycerol kinase	**GK**
208667_s_at	suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)	**ST13**
204684_at	neuronal pentraxin I	**NPTX1**
215867_x_at	adaptor-related protein complex 1, gamma 1 subunit	**AP1G1**
209504_s_at	pleckstrin homology domain containing, family B (evectins) member 1	**PLEKHB1**
221787_at	PHD finger protein 10	**PHF10**
209218_at	squalene epoxidase	**SQLE**
215460_x_at	bromodomain containing 1	**BRD1**
214639_s_at	homeo box A1	**HOXA1**
203450_at	chromosome 22 open reading frame 2	**C22orf2**
200632_s_at	N-myc downstream regulated gene 1	**NDRG1**
212124_at	retinoic acid induced 17	**RAI17**
210027_s_at	APEX nuclease (multifunctional DNA repair enzyme) 1	**APEX1**
200859_x_at	filamin A, alpha (actin binding protein 280)	**FLNA**
212480_at	KIAA0376 protein	**KIAA0376**
44783_s_at	hairy/enhancer-of-split related with YRPW motif 1	**HEY1**
209761_s_at	SP110 nuclear body protein	**SP110**
206103_at	ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	**RAC3**
209220_at	glypican 3	**GPC3**
205555_s_at	msh homeo box homolog 2 (Drosophila)	**MSX2**
203963_at	carbonic anhydrase XII	**CA12**
201651_s_at	protein kinase C and casein kinase substrate in neurons 2	**PACSIN2**
211571_s_at	chondroitin sulfate proteoglycan 2 (versican)	**CSPG2**
203638_s_at	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacialdysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)	**FGFR2**
206853_s_at	mitogen-activated protein kinase kinase kinase 7	**MAP3K7**
208504_x_at	protocadherin beta 11	**PCDHB11**
204678_s_at	potassium channel, subfamily K, member 1	**KCNK1**
208666_s_at	suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)	**ST13**
201965_s_at	KIAA0625 protein	**KIAA0625**
218592_s_at	cat eye syndrome chromosome region, candidate 5	**CECR5**
209210_s_at	pleckstrin homology domain containing, family C	**PLEKHC1**

	(with FERM domain) member 1	
212352_s_at	transmembrane trafficking protein	TMP21
33323_r_at	stratifin	SFN
215603_x_at	Homo sapiens similar to Gamma-glutamyltranspeptidase 1 precursor (Gamma-glutamyltransferase 1) (CD224 antigen) (LOC348598), mRNA	---
221805_at	neurofilament, light polypeptide 68kDa	NEFL
201142_at	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	EIF2S1
212646_at	raft-linking protein	RAFTLIN
203438_at	stanniocalcin 2	STC2
201147_s_at	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	TIMP3
202144_s_at	adenylosuccinate lyase	ADSL
209919_x_at	gamma-glutamyltransferase 1	GGT1
218423_x_at	vacuolar protein sorting 54 (yeast)	VPS54
210319_x_at	msh homeo box homolog 2 (Drosophila)	MSX2
208818_s_at	catechol-O-methyltransferase	COMT
212528_at	Homo sapiens, clone IMAGE:3605655, mRNA	---
206291_at	neurotensin	NTS
205241_at	SCO cytochrome oxidase deficient homolog 2 (yeast)	SCO2
207001_x_at	delta sleep inducing peptide, immunoreactor	DSIPI
208370_s_at	Down syndrome critical region gene 1	DSCR1
212597_s_at	high-mobility group protein 2-like 1	HMG2L1
212167_s_at	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	SMARCB1
213746_s_at	filamin A, alpha (actin binding protein 280)	FLNA
207387_s_at	glycerol kinase	GK
210250_x_at	adenylosuccinate lyase	ADSL
215646_s_at	chondroitin sulfate proteoglycan 2 (versican)	CSPG2
213604_at	transcription elongation factor B (SIII), polypeptide 3 (110kDa, elongin A)	TCEB3
203085_s_at	transforming growth factor, beta 1 (Camurati-Engelmann disease)	TGFB1
201363_s_at	influenza virus NS1A binding protein	IVNS1ABP
207131_x_at	gamma-glutamyltransferase 1	GGT1
203417_at	microfibrillar-associated protein 2	MFAP2
217960_s_at	translocase of outer mitochondrial membrane 22 homolog (yeast)	TOMM22
206355_at	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type	GNAL
201755_at	MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)	MCM5
218543_s_at	hypothetical protein FLJ22693	FLJ22693
213508_at	chromosome 14 open reading frame 147	C14orf147

201995_at	exostoses (multiple) 1	**EXT1**
222240_s_at	myo-inositol 1-phosphate synthase A1	**ISYNA1**
217719_at	eukaryotic translation initiation factor 3, subunit 6 interacting protein	**EIF3S6IP**
214265_at	integrin, alpha 8	**ITGA8**
216237_s_at	MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)	**MCM5**
208949_s_at	lectin, galactoside-binding, soluble, 3 (galectin 3)	**LGALS3**
201076_at	NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae)	**NHP2L1**
221731_x_at	chondroitin sulfate proteoglycan 2 (versican)	**CSPG2**
208763_s_at	delta sleep inducing peptide, immunoreactor	**DSIPI**
202391_at	brain abundant, membrane attached signal protein 1	**BASP1**
205110_s_at	fibroblast growth factor 13	**FGF13**
200005_at	eukaryotic translation initiation factor 3, subunit 7 zeta, 66/67kDa	**EIF3S7**
221410_x_at	protocadherin beta 3	**PCDHB3**
218839_at	hairy/enhancer-of-split related with YRPW motif 1	**HEY1**
211919_s_at	chemokine (C-X-C motif) receptor 4	**CXCR4**
205289_at	bone morphogenetic protein 2	**BMP2**
212527_at	DNA segment, Chr 15, Wayne State University 75, expressed	**D15Wsu75e**
211417_x_at	gamma-glutamyltransferase 1	**GGT1**
211998_at	H3 histone, family 3B (H3.3B)	**H3F3B**
213492_at	collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)	**COL2A1**
204735_at	phosphodiesterase 4A, cAMP-specific (phosphodiesterase E2 dunce homolog, Drosophila)	**PDE4A**
205290_s_at	bone morphogenetic protein 2	**BMP2**
212741_at	monoamine oxidase A	**MAOA**
206376_at	homolog of rat orphan transporter v7-3	**NTT73**
204359_at	fibronectin leucine rich transmembrane protein 2	**FLRT2**
209457_at	dual specificity phosphatase 5	**DUSP5**
208937_s_at	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	**ID1**
202431_s_at	v-myc myelocytomatosis viral oncogene homolog (avian)	**MYC**
200792_at	thyroid autoantigen 70kDa (Ku antigen)	**G22P1**
200670_at	X-box binding protein 1	**XBP1**
209278_s_at	tissue factor pathway inhibitor 2	**TFPI2**
218251_at	hypothetical protein STRAIT11499	**STRAIT11499**
213387_at	**KIAA1240 protein**	**KIAA1240**

List II - 2 Way ANOVA

Affy Annotation	Description	Abreviation
212124_at	retinoic acid induced 17	RAI17
221787_at	PHD finger protein 10	PHF10
209278_s_at	tissue factor pathway inhibitor 2	TFPI2
208370_s_at	Down syndrome critical region gene 1	DSCR1
204693_at	CDC42 effector protein (Rho GTPase binding) 1	CDC42EP1
209656_s_at	transmembrane 4 superfamily member 10	TM4SF10
206356_s_at	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type	GNAL
201965_s_at	KIAA0625 protein	KIAA0625
203438_at	stanniocalcin 2	STC2
200684_s_at	ubiquitin-conjugating enzyme E2L 3	UBE2L3
215867_x_at	adaptor-related protein complex 1, gamma 1 subunit	AP1G1
208711_s_at	cyclin D1 (PRAD1: parathyroid adenomatosis 1)	CCND1
206853_s_at	mitogen-activated protein kinase kinase kinase 7	MAP3K7
201147_s_at	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	TIMP3
218423_x_at	vacuolar protein sorting 54 (yeast)	VPS54
218543_s_at	hypothetical protein FLJ22693	FLJ22693
214639_s_at	homeo box A1	HOXA1
201142_at	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	EIF2S1
206355_at	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type	GNAL
221805_at	neurofilament, light polypeptide 68kDa	NEFL
213604_at	transcription elongation factor B (SIII), polypeptide 3 (110kDa, elongin A)	TCEB3
207001_x_at	delta sleep inducing peptide, immunoreactor	DSIPI
205241_at	SCO cytochrome oxidase deficient homolog 2 (yeast)	SCO2
202431_s_at	v-myc myelocytomatosis viral oncogene homolog (avian)	MYC
213746_s_at	filamin A, alpha (actin binding protein 280)	FLNA
203085_s_at	transforming growth factor, beta 1 (Camurati-Engelmann disease)	TGFB1
206376_at	homolog of rat orphan transporter v7-3	NTT73
208763_s_at	delta sleep inducing peptide, immunoreactor	DSIPI
201995_at	exostoses (multiple) 1	EXT1
211998_at	H3 histone, family 3B (H3.3B)	H3F3B
200670_at	X-box binding protein 1	XBP1
205289_at	bone morphogenetic protein 2	BMP2
211919_s_at	chemokine (C-X-C motif) receptor 4	CXCR4
212527_at	DNA segment, Chr 15, Wayne State University 75, expressed	D15Wsu75e

205290_s_at	bone morphogenetic protein 2	**BMP2**
209457_at	dual specificity phosphatase 5	**DUSP5**
208937_s_at	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	**ID1**
218251_at	hypothetical protein STRAIT11499	**STRAIT11499**
213387_at	KIAA1240 protein	**KIAA1240**

CURRICULUM VITAE

PERSONAL INFORMATION

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DEGREES

February 2005: PhD degree in Cell Biology: University of Basel.

December 1998: University degree in Pharmacy: University of São Paulo (USP) Pharmaceutical Sciences College of Ribeirão Preto.

EDUCATION

August 2000 – February 2005: PhD thesis on the molecular mechanisms of cell-extracellular matrix interactions with a focus on the teneurin family of proteins (Friedrich Miescher Institute - FMI, **Basel**, supervisor: Dr. R. Chiquet).

June 1999 - June 2000: International Association for the Exchange of Students for Technical Experience (IAESTE) traineeship; studies on the MEA protein (FMI, **Basel**, supervisor: Prof. U. Grossniklaus).

July 1998 - October 1998: Diploma work; studies on the PKC isoforms and the APC (*Adenomatous polyposis coli*) gene product in colon cancers. (Deutsches Institut für Ernährungsforschung, **Potsdam**, supervisor: Dr. K. Schmehl).

August 1996 - July 1997: Fellowship; analysis of nephrotoxic effects of combined administration of cisplatin and sodium selenite in rats. (Department of Bromatology and Nutrition - USP, supervisor: Dr. ML Bianchi).

January 1996 - July 1996: Traineeship; physical-chemical stability analysis of oil/water (O/W) emulsions (Cosmetic Technology Laboratory - USP, supervisor: Dr. PA Rocha Filho).

January 1994 – December 1998: Undergraduate studies at the University of São Paulo (USP) - Pharmaceutical Sciences College of **Ribeirão Preto**.

EXTRACURRICULAR ACTIVITIES AND COURSES

2005: Intensive German language course, NSH Bildungszentrum, Basel.

2003: Strategies and trends in pharmaceutical development and production course with project work, Pharmazentrum, Basel.

2003: WIN (Women Into Industry) Mentoring Program, University of Basel and Novartis, Basel.

1997: Courses at the Faculty of Economy and Management, University of São Paulo (USP), Ribeirão Preto:

Human Resources Management
Marketing Control and Planning
Basic Marketing
Organization Behaviour

PERSONAL SKILLS

Mother language: Portuguese
Other languages: English (strong command)
German (good)
Spanish (basic)

PUBLICATIONS

Nunes SM, Ferralli J, Choi K, Brown-Luedi M, Minet AD and Chiquet-Ehrismann R. 2005. The intracellular domain of teneurin-1 interacts with MBD1 and CAP/ponsin resulting in subcellular codistribution and translocation to the nuclear matrix. *Experimental Cell Research* **305:** 122-132.

Spillane C, MacDougall C, Stock C, Kohler C, Vielle-Calzada JP, Nunes SM, Grossniklaus U, Goodrich J. 2000. Interaction of the Arabidopsis polycomb group proteins FIE and MEA mediates their common phenotypes. *Current Biology* **10**: 1535-8.

Ferrari M, Maruno M, Goncalves, Nunes S, Rocha Filho PA. 1997. Allantoin Liberation from Liquid Dispersed Systems (O/W Emulsions and O/W/O Multiple Emulsions). *Bollettino Chimico Farmaceutico* **136**: 119.

UNIVERSITY LECTURES ATTENDED

2000- Molecular Medicine (part I); Molecular Mechanisms of Development; Extracellular Matrix from Bone to Brain
2001- Molecular Medicine (part II)
2003- New Literature in Extracellular Matrix; Molecular Aspects of Neurologic Diseases