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Analysis of the interaction of TIP60β and PIN1 with the *ets* family transcription factor ETV6

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Vorgelegt von

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For my Mother and Father

Contents

1. Introduction

Chromosomal rearrangements, especially balanced chromosomal translocations, play a pivotal role in leukemogenesis. The best known example of the causal relationship between a chromosomal translocation and a malignancy is the Philadelphia chromosome and chronic myeloid leukemia (CML). The Philadelphia chromosome, which was identified in the early 1960ies, is the result of a balanced translocation of chromosomes 9 and 22. This translocation results in the production of a fusion protein, the BCR/ABL fusion protein, which is directly responsible for the malignant transformation in CML. Today we know of more than 100 recurring chromosomal rearrangements that lead to the formation of fusion genes and fusion proteins (Mitelman, 1994). Balanced chromosomal translocations do not only lead to the formation of fusion genes but can also result in transcriptional deregulation of genes close to the breakpoints. This is, for example, the case in the t(8;14) translocation found in Burkitt's lymphoma which leads to the overexpression of the MYC oncogene through juxtapposition of the immunoglobulin heavy chain enhancer / promoter. Chromosomal rearrangements affecting band 12p13 have been the focus of investigators since the early 1990ies. 12p rearrangements, both translocation

and deletions, can be found in a wide spectrum of hematopoietic malignancies (acute lymphoblastic leukemia (ALL), chronic myelomonocytic leukemia (CMMoL)) and also in certain solid tumors, like congenital fibrosarcome and secretory breast carcinoma. In 1994, Golub et al. identified the *TEL* gene (later called *ETV6*) on 12p13 as the fusion partner of the *PDGFRβ*(platelet derived growth factor) gene in cases with CMMoL and a t(5;12). Subsequently, *ETV6* has been identified as the fusion partner of at least 17 other genes. While in most of these translocations fusion proteins are formed between ETV6 and the partner genes, deregulation of oncogenes seems also to play a role in a few translocation involving ETV6 (Rawat et al., 2004). Deletions affecting 12p have a smallest region of overlap encompassing ETV6 (Sato et al., 1995). These and other data suggest that ETV6 might also function as a tumor suppressor gene. Because of its prominent function in malignant transformation in the hematopoietic system, studies trying to elucidate the function of ETV6 are the focus of this work.

1.1 ETV6

1.1.1 The *ets* family of transcription factors

ETV6 (*ETS* variant gene 6) was originally called *TEL,* for translocation *ets* leukemia gene. ETV6 is a member of the *ets* (erythroblast transformation specific) family of transcription factors. *ets* proteins all share a conserved 85 amino acid long DNA binding domain called the *ets* domain. Some *ets* proteins also have another conserved domain: the SAM domain (also called *pointed* domain, helix-loop-helix domain). There are more than 40 members of this family, which have been found in species ranging from sponges, nematodes and insects to humans. *ets* domain proteins are involved in many cellular and organismal functions like proliferation, differentiation, development, transformation, and apoptosis. Most members of the *ets* family have been shown to be transcriptional activators except for three *ets* proteins, YAN, ERF and ETV6, which act as transcriptional repressors. There are also some members which function as activators or repressors depending on their interactions with specific cofactors and on the promoter context, such as ELK-1 (Janknecht et al., 1994; Yang et al., 2001), SAP-2/NET (Price et al., 1995), NERF-1/-2 (Cho et al., 2004), and ESE-1/-2/-3. (Table 1.1, (Sharrocks, 2001)).

Table 1.1 The *ets* domain proteins

ETS-domain protein	Pointed domain	Activator (A) or repressor (R)	Biological role(s)
Ets sub-family	Yes		
Ets-1		A	*T-cell survival; natural killer cell differentiation
Ets-2		A	*Extraembryonic tissue generation; extracellular matrix remodelling; hair development.
Pointed‡		A	Multiple developmental roles
TCF sub-family	No		Immediate-early gene regulation; cellular proliferation?
Elk-1		A/R	Neuronal differentiation
SAP-1		A	§T-cell differentiation
SAP-2/Net		R/A	*Lymphatic vessel formation
Lin-1‖		R?	Vulval development
Erg sub-family	Yes		
Fli-1		A	*Megakaryocyte differentiation; vasculogenesis/angiogenesis?
Erg		A	
PEA3 sub-family	No		Neuronal pathfinding
PEA3		A	*Neuronal function? Muscle differentiation
ERM		A	
ER81		A	Directing sensory–motor neuron connections
GABP sub-family	Yes		
GABPα		A	Muscular synapse function
Elf sub-family	No		
Elf-1		A	
NERF-1/-2		A/R	
MEF		A	
Spi sub-family	No		
PU.1		A	*Myeloid and lymphoid differentiation
SpiB		A	*B-cell function
SpiC			A
Yan sub-family	Yes		
TEL		R	*Yolk-sac angiogenesis; adult haematopoiesis.
TEL2		R	
Yan‡		R	Several developmental roles.
Erf sub-family	No		
ERF		R	
PE-1		?	
Others			
ESE-1/-2/-3	Yes	A/R	Epithelium-specific (expression)
PDEF	Yes	A	
ER71	No	?	

*Shown by mouse knockout studies; ‡proteins from *Drosophila*; §R. Treisman, personal communication; ‖proteins from *C. elegans*.

Table 1.1 The *ets* domain protein family. Sharrocks, 2001

The *ets* domain is not only a DNA binding domain but also an interface for protein-protein interaction. This *ets* domain binds a purine rich GGAA/T core DNA (Karim et al., 1990). The structure and biochemical studies show that

the *ets* domain has a winged helix-turn-helix structure (Donaldson et al., 1996). In recent years, structural studies of *ets* domains complexed with DNA have show that also some non-conserved amino acid residues of the *ets* domain contribute to the protein-DNA interaction. These findings explain why different members of the *ets* family that have highly similar *ets* domain recognize different DNA sequences. (Mo et al., 2000; Obika et al., 2003). The *ets* domain has also been shown to function as an activator or repressor of transcription (Virbasius et al., 1993; Maurer et al., 2003; Graves et al., 1996). Protein-protein interactions may modify the DNA binding properties of the *ets* domains, which in turn may change the transcriptional regulation at specific promoters (Baert et al., 2002; Carrere et al., 1998). These interactions either positively or negatively influence DNA binding (Table.1.2) (Galanis et al., 2001).

Table 1.2 Binding partners for ETS-domain transcription factors

Co-regulatory binding partner	ETS-domain protein	Domains involved	Functions affected	References
DNA-binding regulation				
Runt-domain proteins (e.g. AML-1/CBFα2)	Ets-1	ETS-domain and immediate amino- and carboxy-terminal flanking regions	Enhances DNA binding	28,29
Pax proteins (e.g. Pax-5)	Ets-1	ETS-domain	Enhances DNA binding	24
Id proteins	TCFs	ETS-domain	Inhibits DNA binding	34
GABPβ	GABPα	ETS-domain and immediate carboxy-terminal region	Enhances DNA binding	20
SRF	TCFs	B-box	Enhances DNA binding	38,39
Pip	PU.1	PEST-region	Enhances DNA binding	35
Mae	Yan	Pointed domain	Inhibits DNA binding	11
Transcriptional regulation				
bHLH proteins (e.g. USF-1)	Ets-1	ETS-domain	Transcription activation and DNA binding enhanced	107
	PEA3	ETS-domain and immediate amino- and carboxy-terminal flanking regions	DNA binding enhanced	36
hZIP proteins (e.g. MafB)	Ets-1	ETS-domain	Represses Ets-1-mediated transcriptional activation	108
GATA proteins (e.g. GATA-1)	PU.1	ETS-domain	Inhibition of GATA-1/PU.1 transactivation	109–111
Nuclear hormone receptors (e.g. VDR)	Ets-1	ETS-domain	Transcriptional activation of VDR enhanced	112
mSin3A	Elk-1	ETS-domain	Enhances transcriptional repression	41
	TEL	Pointed domain	Enhances transcriptional repression	12
NcoR	TEL	Region between pointed domain and ETS-domain	Enhances transcriptional repression	68
CtBP	Net	CtBP interaction domain	Enhances transcriptional repression	40
CBP/p300	Ets-1	Region between pointed domain and ETS-domain	Enhances transcriptional activation	42
	ER81	ETS-domain	Enhances transcriptional activation	43
TEL	TEL	Pointed domain	Enhances transcriptional repression	10
MAPKs	TCFs	D-domain/FxF motif	Enhances TCF phosphorylation and activation	56,57,59,60
	Ets-1	Pointed domain	Enhances Ets-1 phosphorylation	*

See ref. 15 for additional examples. *J. Seidel and B. Graves, personal communication. TCFs, ternary complex factors; bHLH, basic helix–loop–helix; bZIP, basic leucine zipper; MAPKs, mitogen-activated protein kinases.

Table 1.2 The DNA binding partners of *ets* proteins. Sharrocks, 2001

The *pointed* or SAM (Sterile alpha motif) domain is an evolutionarily conserved protein binding domain with 65 to 70 amino acids found in more

than 250 regulatory proteins including receptor tyrosine kinases and transcription factors (Kim et al., 2001; Kyba and Brock, 1998; Schultz et al., 1997). This domain is involved in the regulation of numerous developmental processes in diverse eukaryotes. The *pointed* domain mediates protein-protein interaction to form homotypic or heterotypic oligomers with other *pointed* (or SAM) domains, which organize protein complexes in the cell (Barrera et al., 2002). Various members of the *ets* family such as *Ets-1/-2*, *Fli-1*, *Erg*, *GABP*, *YAN*, *ETV7* and *ETV6* have this domain (Jansen et al., 2001; McGrath et al., 2001; Sharrocks, 2001).

ets proteins are found in most multicellular organisms. They are involved in mammalian developmental processes at the cellular, tissue and organ level. Various studies show that *ets* proteins play important roles in hematopoiesis and vasculogenesis/angiogenesis, at the earliest stages of embryogenesis and later in tissue development. *ets* genes are expressed in hematopoietic cells, the vasculature, brain and central nervous system, mammary gland, endometrium, ovaries, testes, kidneys and lungs. Different members of the *ets* family have different functions in the regulation of cell proliferation, differentiation, migration, apoptosis and in cellular interactions (reviewed by (Sharrocks, 2001; Sementchenko and Watson, 2000)).

Extracellular signals from ligand-receptor interactions on the cell surface are conveyed through numerous cytoplasmic signaling pathways to the nucleus. The responses of the cell to these signals are temporary or permanent changes in gene expression resulting in cell proliferation, differentiation or survival. For examples, the mitogen-activated protein kinase (MAPK) pathway and calcium dependent kinases pathway play important roles in cellular signal transduction. Many *ets* family proteins have been shown to be the nuclear targets of such signaling pathways acting as downstream effectors, especially in MAPK pathway. *ets* proteins with *pointed* domains like Ets-1 and Ets-2 as well as YAN have a single MAPK phosphorylation site, which localizes near the *pointed* domain. On the other hand, TCF which does not have a *pointed* domain also has a phosphorylation domain and serves as an integration point for different MAPK pathways. Phosphorylation generally enhances the transcriptional activity of *ets* proteins leading to the increased affinity of the proteins to their DNA binding sites, like Ras response elements (RREs) or serum response elements (SREs) which are found in the promoters of many immediate early response genes. The identification of docking domains for MAPKs in *ets* proteins showed that the binding of MAPKs is specific for different substrate *ets* proteins (Fig.1.1, Sharrocks, 2001). As mentioned above, *ets* proteins can be positive or negative regulators of transcription. Most *ets* proteins have been shown to be

transcriptional activators (see Table.1.1). Earlier studies showed that some factors such as ERF, NET, YAN and ETV6 act as transcriptional repressors (Sgouras et al., 1995; Lai et al., 1997; Wood et al., 2003). The identification of the repression domains of NET and ETV6 showed that their transcriptional repression functions depend in part on the recruitment of histone deacetylase. These four proteins are all regulated by MAPKs. Recently, it could be demonstrated that several *ets* family members function both as transcriptional activators and repressors. For examples, NET can change its repressor role to an activator when it is activated by Ras (Giovane et al., 1994). Elk-1 was originally described as an activator protein, but recent studies revealed a repression domain at the N-terminus of Elk-1, which is highly homologous to the CRD1 repression domain of p-300 (Yang et al., 2002; Yang et al., 2003b). Sumoylation of Elk-1 leads to transcriptional repression which depends on the histone deacetylase complex (Yang et al., 2001; Yang et al., 2003a).

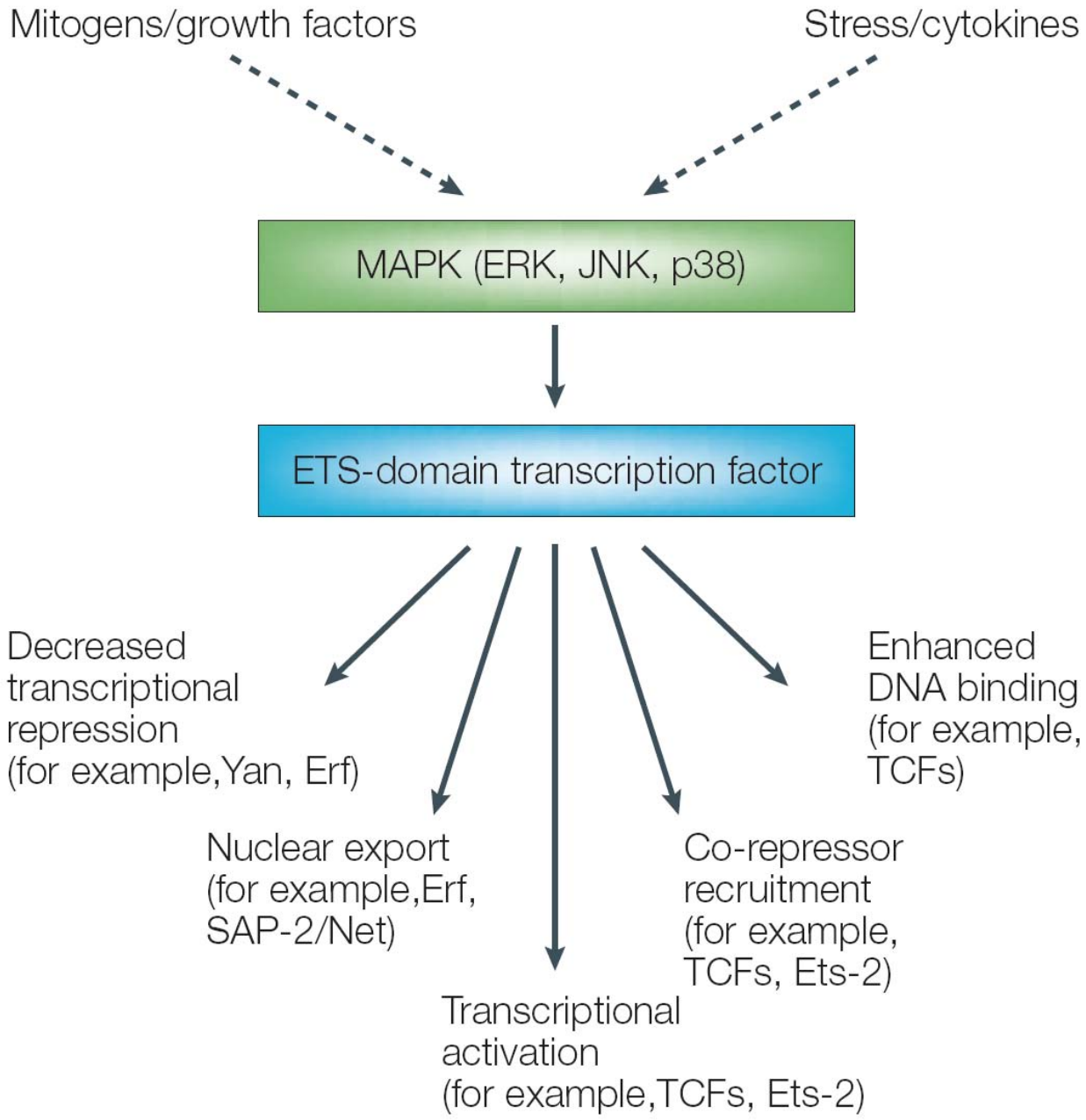


Fig. 1.1 *ets* proteins are regulated by phosphorylation. Sharrocks, 2001

1.1.2 Biological characterization of ETV6

1.1.2.1 The *ETV6* gene and its protein domains

1.1.2.1.1 Cloning and characterization of *ETV6*

ETV6 was cloned as the fusion partner of *PDGFRβ* from the leukemic cells of a patient with CMMoL in 1994 (Golub et al., 1994). The *ETV6-PDGFRβ* fusion gene is the result of a chromosomal translocation between chromosomes 12 and 5, t(5;12)(q33;p13). The *ETV6* gene encodes two nuclear proteins, one of 452 amino acid residues, and the other of 409 amino acid residues which results when an alternative start cordon at position 43 is used. Human *ETV6* is 85% homologous to mouse *ETV6* (NCBI database). In the *ETV6/PDGFRβ* fusion gene, the sequence coding for the N-terminal portion of *ETV6* including the *pointed* domain is fused to the C-terminal portion of *PDGFRβ*, which codes for the tyrosine kinase domain. Subsequently, more than 17 *ETV6* fusion genes resulting from chromosomal translocations have been identified in different types of leukemias and also in some solid tumors. The *ETV6* fusion partners include receptor tyrosine kinase such as *PDGFRβ*, *JAK2*, or transcription factors such as *RUNX1* or *CDX2* (Kim et al., 2001; Bohlander, 2000).

1.1.2.1.2 ETV6 protein domains

Analysis of the ETV6 amino acid sequence revealed two domains, which are characteristic of *ets* proteins: an N-terminal *pointed* domain (SAM, helix-loop-helix (HLH) domain) and a C-terminal *ets* DNA binding domain. The *pointed* domain of ETV6 consists of 65 amino acids. The amino acids in this domain are highly conserved when compared to other *pointed* domains of *ets* proteins. This domain has been shown function as an oligomerization interface and mediates transcriptional repression through recruitment of the co-repressor L(3)MBT, a polycomb group protein. The *ets* domain of ETV6 is highly similar to the *ets* domain of other *ets* family members. The central domain of ETV6, which is the region between the *pointed* and the *ets* domain, has been shown to have repression function through the binding of the co-repressors SMRT, NcoR and mSin3A (Chakrabarti and Nucifora, 1999). The central domain is not conserved (Figure 1.2).

Comparision of ETV6 from *Fugu* and human show that *Fugu* ETV6 is highly related to its human homologue with an overall amino acid identity of about 58% (72% similarity) and that the *pointed* and *ets* domains of different vertebrate species (human, *Fugu*, chicken, mouse) are highly conserved with 81% to 95% amino acid sequence identity (Montpetit and Sinnett, 2001). In

2000, a human gene with great similarities to *ETV6* was described and named *ETV7*. *ETV7* is located on chromosome 6 band p21 and encodes for a protein of 341 amino acids which has a *pointed* and an *ets* domain (Potter et al., 2000).

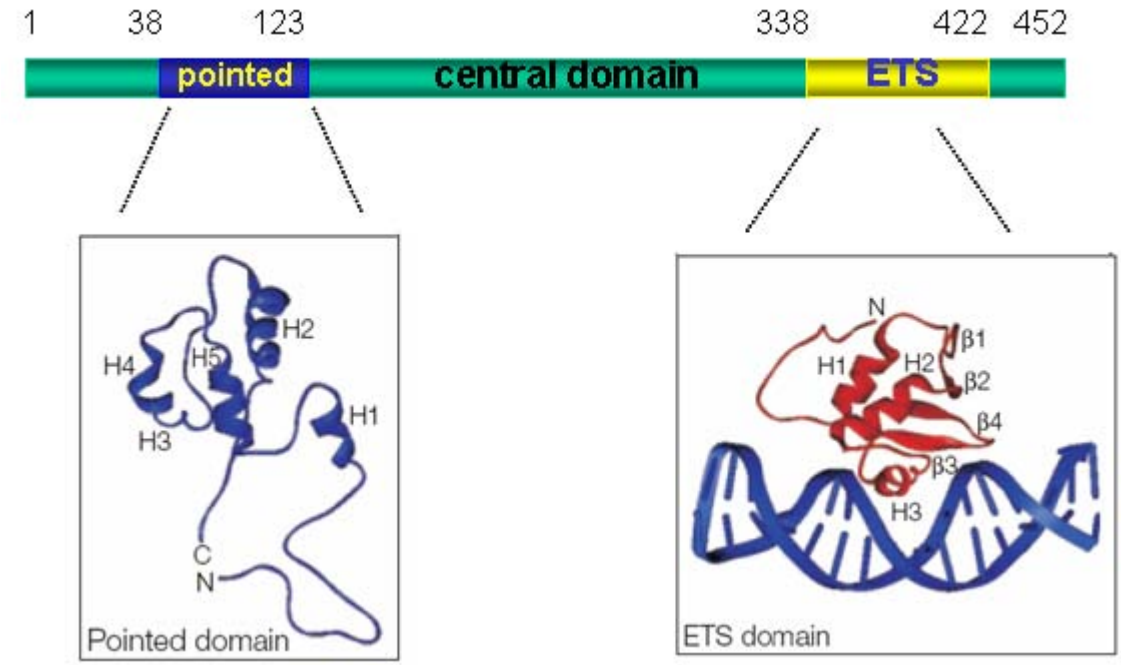


Fig. 1.2 ETV6 structure.

1.1.2.2 ETV6 function

1.1.2.2.1 *ETV6* in mammalian development

The *ETV6* gene is widely expressed in embryonic development and in the adult. Knock-out experiments have shown that ETV6 is not essential for embryonic hematopoiesis (Wang et al., 1998). Analysis of *ETV6* expression

in mouse development shows that embryos express *ETV6* at high levels in multiple organs including the developing lungs, kidneys and liver. High levels of *ETV6* expression are also found in neural tissues, such as the cranial nerve ganglia, the dorsal root ganglia, the midbrain and the ventral region of the caudal neural tube. At mid-gestation, high expression of *ETV6* is found in the fetal lungs, thymus, liver and abundantly in hematopoietic cell lineages. In adult tissues *ETV6* is expressed in the heart, lungs, brain, spleen, liver, kidneys, testes and skeletal muscle (Table 1.3). In *ETV6* knock out mouse embryos, the development of the erythroid and myeloid lineages proceeds normally. These studies showed that *ETV6* is required for the development of the vascular network in the yolk sac and for the survival of selected cell types within the embryo proper. Mice with targeted disruption of *ETV6* die between E10.5-E11.5 with defective yolk sac angiogenesis and intra-embryonic apoptosis of mesenchymal and neural cells (Wang et al., 1997). However, studies using chimeric mice consisting of *ETV6* knock-out and *ETV6* wild type cells demonstrated that *ETV6* is important for the development of T and B cells (Fig. 1.3). These studies also showed that *ETV6* is essential for establishing final hematopoiesis in the bone marrow and is crucial for the maintenance of hematopoietic stem and progenitor cells in the bone marrow compartment (Wang et al., 1998). ETV7, which is closely related to ETV6, is also expressed in hematopoietic tissues and has been shown to interact with

ETV6 (Potter et al., 2000; Poirel et al., 2000).

regions	tissue	E10.5	E11.5	E12.5	E13.5	E14.5	E15.5	E16	E17.5	E18.0	E19.5	D1	D7	D14	D21	adult
head	brain			■	■	■	■	■	■	■	■	■	■	■	■	■
	lung			■	■	■	■	■	■	■	■	■	■	■	■	■
chest	heart															■
	thymus			■	■	■	■	■	■	■	■	■	■	■	■	■
	liver			■	■	■	■	■	■	■	■	■	■	■	■	■
abdomen	spleen															■
	kidney			■	■	■	■	■	■	■	■	■	■	■	■	■
	sex organs	■														

Table 1.3 The expression of *ETV6* in different organs, modified from (Maroulakou and Bowe, 2000)

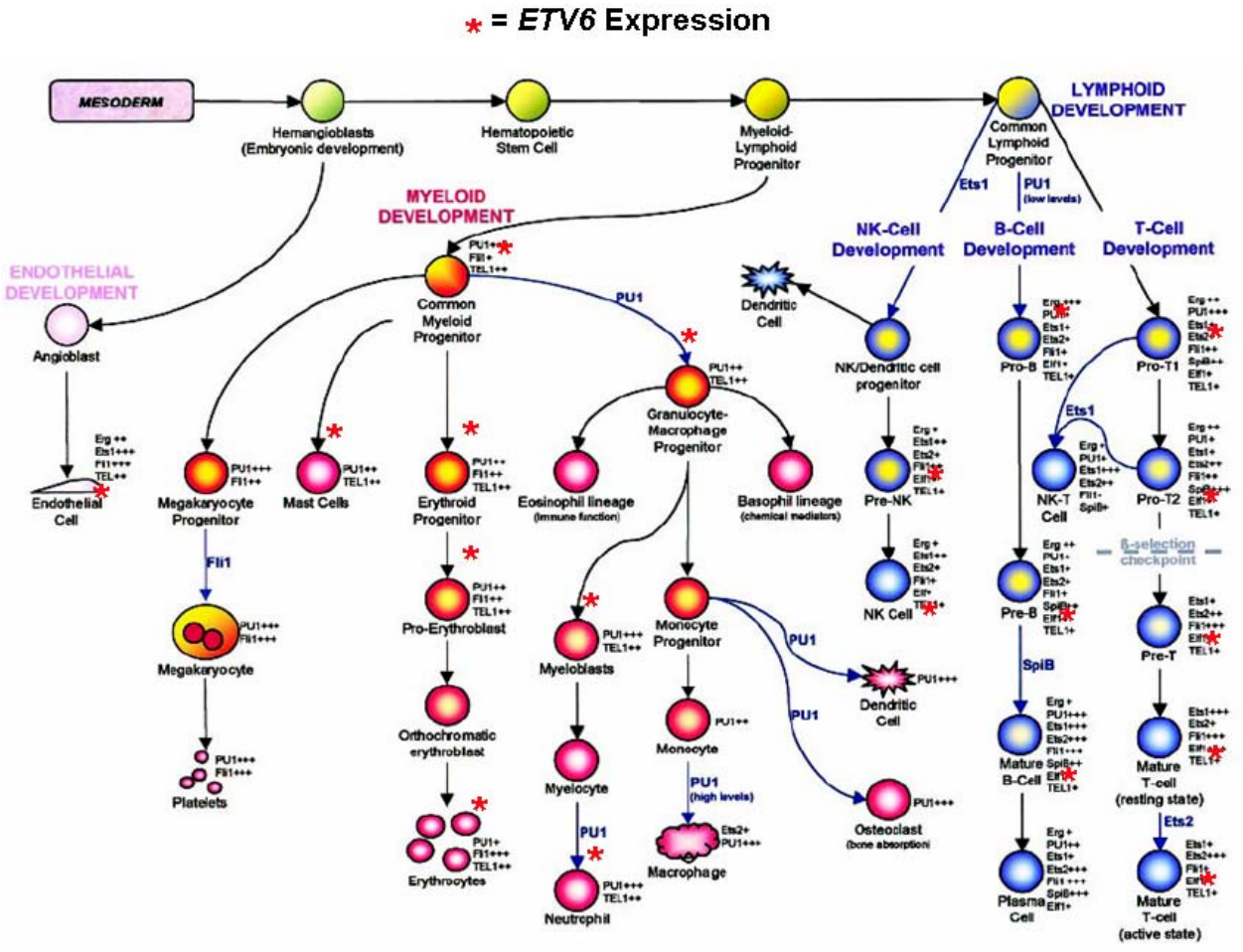


Fig. 1.3 *ETV6* expresses in hematopoietic and endothelial cell development. Modified from Maroulakou and Bowe. (2000)

1.1.2.2.2 *ETV6* fusion genes

ETV6 was initially cloned as the fusion partner of *PDGFRβ* in a balanced chromosomal translocation involving chromosomes 5 and 12 band q33 and p13 (Golub et al., 1994). Subsequently, *ETV6* has been found fused to numerous different genes in different types leukemia and in some solid

tumors. Up to now, some 35 different chromosome bands have been shown to be involved in *ETV6* related translocation, which suggests that *ETV6* is involved in at least 41 different chromosomal translocations (Odero et al., 2001). Almost 20 different fusion partners of *ETV6* have been found from protein tyrosine kinases, such as *ARG, NTRK3/TRKC, PDGFRβ, JAK2, ABL, SYK*, and *FGFR3*, to transcription factors, such as *AML1/RUNX1, EVI1, HLXB9, MN1* or *PAX5,* to genes of various or unknown functions, such as *STL, BTL, ARNT, MDS2, TTL, ACS2*. Most cases of *ETV6* fusion genes are found in patients with leukemia or certain solid tumors.

The molecular mechanisms by which the various ETV6 fusion proteins cause leukemia are quite well understood in the case of the ETV6-tyrosine kinase (PTK) fusions and less well understood in the case of ETV6 transcription factor (TF) fusions or fusion with genes of unknown function. It could be shown that the *pointed* domain mediates dimerization or oligomerization of the ETV6-PTK fusion proteins which leads to constitutive activation of the PTK and aberrant signaling (Golub et al., 1996; Argani et al., 2000; Gloc et al., 2002; Atfi et al., 1999). Deletion of the *pointed* domain in the ETV6-PTK fusion abrogates the transforming properties of the fusion protein. In the two cases of ETV6-TF fusions that are well studied the pathogenic mechanism seems to be the formation of an aberrant transcription factor. This

mechanism is described in the next paragraph.

1.1.2.2.3 ETV6 in transcriptional regulation

Unlike most members of the *ets* family of transcription factors, which are activators of transcription and induce cellular transformation when over-expressed, ETV6 is a transcriptional repressor.

Functional studies of ETV6 fusion genes show that these chimeric proteins behave as transcriptional activators or repressors depending on their fusion partner and on which domains of ETV6 are retained in the fusion protein. For example, the t(12; 22)(p13;q11) found in two patients (one with myeloid leukemia and one with myelodysplastic syndrome (MDS)), results in the formation of the *MN1-ETV6* and *ETV6-MN1* fusion genes. The MN1-ETV6 fusion protein consists of the N-terminal portion of MN1 which is rich in prolines and has two polyglutamine stretches, and the C-terminus of ETV6 with the *ets* DNA binding domain (Buijs et al., 1995). This chimeric protein, unlike ETV6 or MN1, transforms murine fibroblast NIH3T3 cells. The transformation potential depends on both the N-terminal MN1 portion and a functional ETV6 DBD, which is also essential for the transcriptional activity of the MN1-ETV6 fusion protein (Buijs et al., 2000).

The *ETV6-AML1* fusion gene caused by the t(12;21)(p13;q22) originally described in two cases of childhood pre-B ALL encodes for a fusion protein which contains the *pointed* domain of ETV6 and the DNA binding and transactivation domain of AML1 (Golub et al., 1995). This fusion gene was later shown to be the most common fusion gene in childhood ALL (Shurtleff et al., 1995; Fears et al., 1996). The ETV6-AML1 fusion protein interacts with the Core-Binding Factor Beta (CBFβ), an AML1 binding protein. The ETV6-AML1 fusion protein represses transcription of a reporter gene under the control of the T-cell receptor beta enhancer, which is normally activated by AML1. The repression mediated by ETV6-AML1 is dependent on the *pointed* domain of ETV6 (Hiebert et al., 1996). This observation led to the identification of an association between ETV6 and the co-repressors mSin3A and SMRT. The *pointed* domain and the central region of ETV6 play a role in transcriptional repression using two different mechanisms. The central domain requires interaction with SMRT and mSin3A for its repressional activity. SMRT and mSin3A recruit histone deacetylase enzymes (HDAC) (Chakrabarti and Nucifora, 1999). Recently, it could be shown that the transcriptional repression mediated by the *pointed* domain of ETV6 requires interaction with the polycomb group gene L(3)MBT (Boccuni et al., 2003).

Frequent unbalanced chromosomal translocations and deletions affecting

the the short arm of chromosome 12 in different hematopoietic malignancies suggest the presence of a tumor suppressor gene in this region. It could be shown that the minmal region of overlap of these deletions contains ETV6 and the cyclin dependent kinase inhibitor KIP1 (Wang et al., 1997; Sato et al., 1995; Hatta et al., 1997). In more than 70% of childhood acute lymphoblastic leukemias with an ETV6/RUNX1 fusion gene a deletion of the non-rearranged ETV6 allele is observed (Romana et al., 1996). These findings suggest that ETV6 is a tumor suppressor gene. However, direct evidence supporting this hypothesis is still lacking.

Since ETV6 plays an important role in chromosomal translocations in different types of human leukemia and solid tumors and also has a prominent role mammalian hematopoietic development, a yeast two-hybrid screen was performed using ETV6 as bait. A HeLa cDNA library was used in this screen. The aim of this screen was to find more protein interaction partners of ETV6, which would provide more information about the function of ETV6. The screen was carried out by Sigrun Bartels, a technician in Prof. Bohlanders laboratory at the Human Genetics Institute in Göttingen in 1996. The following interaction partners were identified TIP60β (human HIV Tat interaction protein, Mw 60 KDa), PIN1 (protein interaction with NIMA1), and UBC9 (ubiquitin-conjugating enzyme 9) (Putnik, Ph.D. thesis, 2001). Several

of the experiments confirming the physical interaction between ETV6 and TIP60 or PIN1 were performed by Jasmina Putnik and Michael Kickstein (Putnik, Ph.D. thesis, 2001). The focus of the thesis work presented here is the detailed analysis of the functional consequences of the interaction of ETV6 with TIP60β and of ETV6 with PIN1.

In the following paragraphs some background information on TIP60 and PIN1 is given.

1.2 TIP60

TIP60 was identified in a yeast two-hybrid screen in a human B-lymphoblastoid cDNA library as a HIV-1 Tat protein interacting partner. The human HIV Tat protein is important in activating HIV gene expression. TIP60 directly interacts with the HIV-1 Tat activation domain and acts as a Tat transcriptional co-activator on the HIV-1 promoter (Kamine et al., 1996). TIP60 is a member of the MYST family of histone acetyltransferases (HAT).

1.2.1 Histone acetyltransferases

Eukaryotic transcription is a highly regulated process in which numerous protein factors participate. Acetylation plays an important role in this process for modifying the structure of chromatin through the acetylation of histone tails and through the direct acetylation of some transcription factors. Histone acetyltransferases (HAT) transfer acetyl groups from acetyl Co-enzyme A to the epsilon-amino groups of an amino-terminal lysine residue in the N-terminal tail of the core histone proteins (H2A, H2B, H3 and H4). Several non-histone transcription factors have also been shown to be modified by HATs *in vitro* and *in vivo*. All these observations show that HATs play a major role in transcriptional regulation (Sterner and Berger, 2000).

HATs can be separated into at least two classes. Type A HATs are localized in nucleus and acetylate histones and some transcription factors to regulate gene expression. Type B HATs can be purified from cytoplasmic fraction and are involved in acetylating newly synthesized histones. Recent studies also show that type B protein HATs can also be found in the nucleus to regulate gene silencing (Sterner and Berger, 2000).

HATs usually function as transcriptional co-activators. The exact nature of

their transcriptional activation function is dependent on their interaction with different transcription factors. However, some HATs can act both as transcriptional co-activators and as transcriptional co-repressors (Sterner and Berger, 2000; Yang and Sharrocks, 2004; Yang, 2004).

1.2.2 MYST domain proteins

The MYST domain is an about 200 amino acid long highly conserved protein domain. The name MYST is an acronym derived from the names of the four founding proteins with this conserved domain: human MOZ (monocytic leukemia zinc finger protein) (Borrow et al., 1996), yeast Ybf2 (renamed Sas3, something about silencing 3), yeast Sas2 and human TIP60 (HIV Tat-interacting protein, 60 kDa) (Kamine et al., 1996). There are at least five MYST domain proteins in the human genome: TIP60, MOF, HBO1, MOZ and MORF. The MYST domain contains a C2HC zinc finger and a histone acetyl Co-enzyme A binding site which is responsible for the histone acetyltransferase catalytic activity of these proteins (Fig. 1.4 modified from (Yang, 2004)).

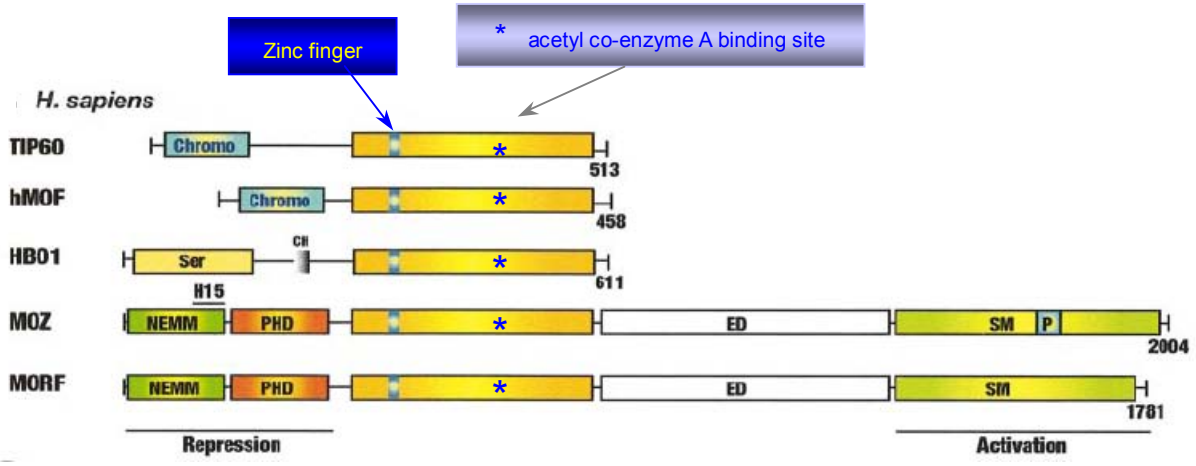


Fig. 1.4 Structure of human MYST domain proteins (modified from X-J. Yang, 2004)

MYST domain proteins can be found in various species from yeast, to *Drosophila* to mammals. Usually, the MYST proteins are found in large multiprotein complexes *in vivo*. For example, the complex in which the yeast Esa1 protein is found, NuA4, consists of at least 12 subunits. The NuA4 HAT complex is highly conserved in eukaryotes. It plays a role in regulation transcription, cellular response to DNA damage, and cell cycle progression (Doyon et al., 2004). MYST domain HATs have diverse regulatory functions in biological processes. This depends among other things on the other proteins they are associated with. Esa1 plays an important role in DNA repair and cell cycle progression (Clarke et al., 1999). The NuA4 protein complex

has also been shown to link spindle stress to transcription control (Doyon et al., 2004). MOZ and MORF contain both transcriptional repression and activation domains and might function both as co-activators and as co-repressors of transcription (Champagne et al., 1999; Champagne et al., 2001).

1.2.3 The structure and function of TIP60

There are three protein isoforms of TIP60 resulting from alternative splicing: TIP60alpha, TIP60beta (Sheridan et al., 2001; Bohlander et al., 2001; Ran and Pereira-Smith, 2000) and a larger form of TIP60 (Legube and Trouche, 2003). TIP60alpha is 513 amino acids long with a MYST domain at the C-terminus, a proline rich domain and a chromo domain at the N-terminus. TIP60beta is 461 amino acids in length and lacks 52 amino acids at the N-terminus. The larger form of TIP60 has 33 amino acids more than TIP60alpha at the N-terminus. A diagram of the three TIP60 isoforms is presented in Fig. 1.5.

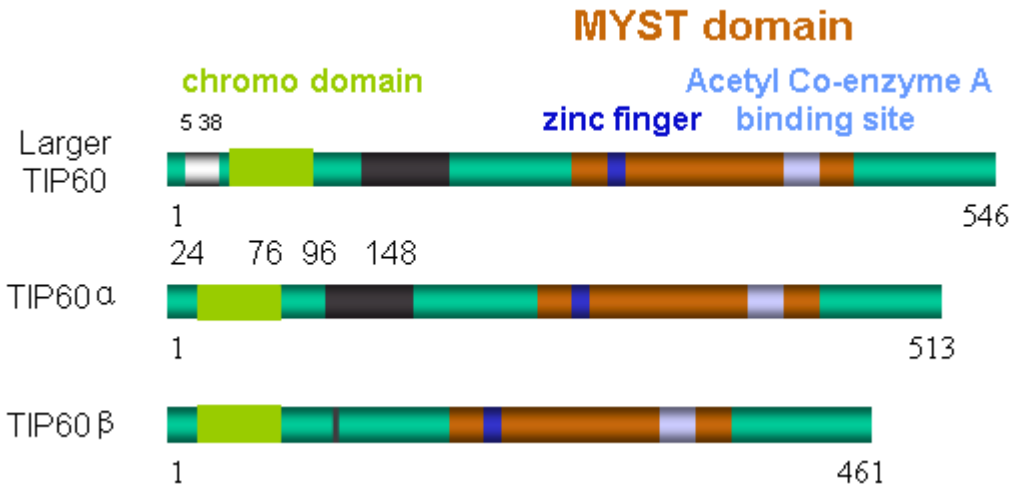


Fig. 1.5 TIP60 isoforms

Various studies have shown that the histone acetyltransferase TIP60 has a role in DNA repair, apoptosis and signal transduction. These studies were mainly performed with the TIP60α isoform.

Initially, TIP60 was isolated as a protein that interacted with the essential 31 amino acid residues at the N-terminus of the HIV1 Tat protein. This interaction causes a 4-fold increase of Tat transactivation of the HIV1 promoter without increasing the basal activity of the HIV promoter or activating the heterologous RSV promoter (Kamine et al., 1996).

The TIP60α isoform has been shown to be a part of a large protein complex in mammalian cells. This protein complex has histone acetylase activity on chromatin and is involved in ATPase, DNA helicase and structural DNA

binding activities (Ikura et al., 2000). A recent study showed that both TIP60α and TIP60β are both subunits of the human NuA4 protein complex, which has a similar HAT activity like the yeast NuA4 complex. There are 11 subunits conserved between the human and the yeast NuA4 complex. TIP60, EPC1 and ING3 form the core of the NuA4 protein complex, which has a strong HAT activity on nucleosomal templates. Extrapolating from the role of the NuA4 complex in yeast, mammalian TIP60 in the NuA4 complex probably plays a key role in the regulation of gene expression and cell cycle progression (Ceol and Horvitz, 2004).

Although subcellular localization data show that TIP60 is mainly a nuclear protein, there is also some data that suggests that TIP60 might have a role in signal transduction in the cytoplasm. TIP60 forms a protein complex in the cytoplasm with the intracellular tail fragment of the amyloid-beta precursor protein (APP) and the nuclear adaptor protein Fe65. This protein complex potentially activates transcription (Cao and Sudhof, 2001). Additional studies of this protein complex show that the membrane part of the APP intracellular domain (AICD) recruits Fe65 and mediates the activation of bound Fe65 that is then released by proteolytic cleavage and undergoes nuclear translocation together with the AICD (Cao and Sudhof, 2004). TIP60 has also been reported to interact with the cytoplasmic tail of the alpha chain of the human

interleukin 9 receptor (IL-9 Rα) (Sliva et al., 1999). TIP60 enhances Bcl-3-p50 dependent transcription through NF-kappa B binding sites (Baek et al., 2002) and is a co-activator of nuclear hormone receptors (Brady et al., 1999; Halkidou et al., 2003).

TIP60 has not only been shown to be a transcriptional co-activator, but has also been shown to be a transcriptional co-repressor depending on the proteins that it interacts with. When TIP60 is expressed as a GAL4DNA binding domain fusion (GAL4DBD) it represses the expression of a luciferase reporter gene, which is under the control of a thymidine kinase promoter and Gal4 DNA binding site. This repression can be enhanced by coexpression of HDAC7, which interacts with the zinc finger of the TIP60 MYST domain (Xiao et al., 2003). TIP60 interacts with STAT3 and represses STAT3-mediated gene expression. This repression can be further enhanced by co-transfection of HDAC7 (Xiao et al., 2003). Other HATs such as CBP/p300 and NcoA/SRC1a also play essential roles in STAT3-mediated transcriptional activation (Giraud et al., 2002).

The interaction between ETV6 and TIP60 has been shown to result in co-repression in a reporter gene system using SEAP (secreted alkaline phosphatase) as a reporter. The SEAP reporter gene was under the control

of the beta-globin promoter, three *ets* binding sites, Gal4 binding sites and an ERE (oestrogen response element) (Nordentoft and Jorgensen, 2003). Our own data show similar results: a TIP60β-YFP fusion (YFP: yellow fluorescent protein) represses the transcription of a luciferase reporter gene which is under the control of a portion of the stromelysin-1 promoter containing an *ets* binding site and an ETV6 binding site (Zhang and Bohlander, 2003).

The histone acetyltransferase activity of TIP60 is modulated *in vivo* by phosphorylation of TIP60 at two main phosphorylation sites (Ser86 and Ser90). S90 is specifically phosphorylated *in vitro* by the cyclin B/Cdc2 complex. Phosphorylation of TIP60 was enhanced after drug-induced arrest of cells at G(2)/M (Lemercier et al., 2003).

1.3 PIN1

The other interacting partner of ETV6 found in the Hela cDNA library screen was PIN1 (protein interacting with NIMA1 (never in mitosis A)), a member of the parvulin family peptidyl-prolyl cis/trans isomerases. PIN1 was first identified as an interacting partner of NIMA1 in a yeast two-hybrid screen (Lu et al., 1996).

1.3.1 Description of PIN1

The *PIN1* gene is located at chromosome 19 band p13 (Campbell et al., 1997). It is highly conserved in evolution and thus found in numerous species from primitive chordates to mammals. PIN1 forms a new class of peptidyl-prolyl cis/trans isomerases together with other genes including the *E. coli parvulin,* yeast *ESS1*, and *Drosophila melanogaster dodo* gene. All these suggest that *PIN1* plays an important role in basic cellular processes.

Human *PIN1* encodes a 163 amino acid long protein with two functional domains: the WW domain and the PPIase domain. Mouse PIN1 consists of 165 amino acid residues, which have a very high homology to human PIN1 especially in the two domains (NCBI database).

The WW domain was first described in 1994 (Bork and Sudol, 1994). Since then, several studies have shown that it is an important functional domain for protein-protein interaction. Usually, WW domains consist of around 40 amino acids with two signature tryptophan (W) residues spaced at 20 to 22 amino acids, which have important roles in the structure and function of the WW domain. This domain can identify and bind to proline-rich motifs in other proteins. The WW domain in PIN1 is specialized on interacting with

phosphorylated Ser/Thr-Pro motifs. The N-terminus of the WW domain provides the binding site for the interaction with the substrate. Three-dimensional models of the PIN1 WW domain derived from x-ray crystallography and NMR studies provide evidence for these interactions (Bayer et al., 2003; Ranganathan et al., 1997). Furthermore, it could be shown that phosphorylation of Ser16 in the WW domain of PIN1 is essential for the interaction of PIN1 with its subtrates and its function in the cell cycle (Lu et al., 2002). The isolated WW domain of PIN1 has been shown to have a similar ability of binding the substrate like full length PIN1 (Landrieu et al., 2001; Bayer et al., 2003). For the interaction between PIN1 and Cdc25C, the threonine phosphorylation at Thr48-Pro and Thr67-Pro is essential (Zhou et al., 2000). In p53, phosphorylation of three Threonine/Serine- Proline motifs (Thr23-Pro, Thr81-Pro and Ser315-Pro) are important for the interaction with PIN1 (Zacchi et al., 2002).

The other functional domain on PIN1 is the PPIase domain. It spans 113 amino acids from aa 50 to aa 163. The C-terminal portion of this domain contains the catalytic function for the peoptidyl-prolyl isomerization reaction of the substrate at the pSer/pThr-Pro peptide bonds. This conformational change in the substrate depends on the binding of substrate through the WW domain, which brings the substrate into close proximity to the PPIase

catalytic site. The isolated PPIase domain shows moderate affinity or no affinity towards to the substrates (Verdecia et al., 2000; Shen et al., 1998). The conformational changes of Cdc25C induced by PIN1 peptidly-prolyl cis-trans isomerization makes Cdc25 sensitive to cleavage by proteases or allows binding of MPM-2 (phosphospecific mitosis marker antibody) (Shen et al., 1998) (Fig. 1.6).

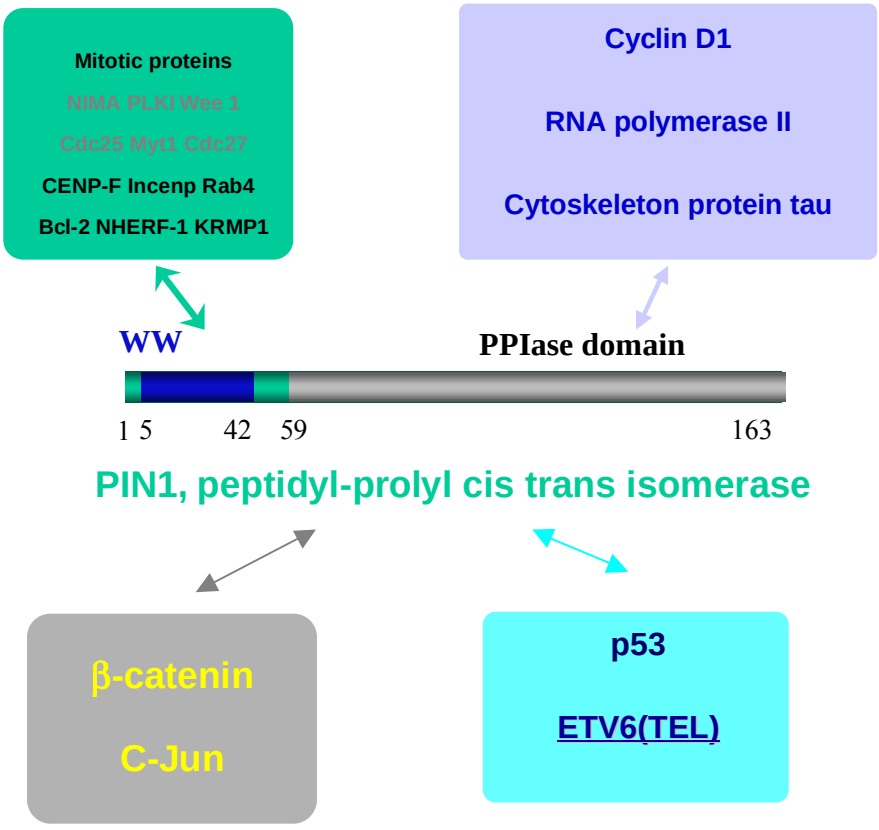


Fig.1.6 The primary structure of PIN1 and some of its protein interacting partners

The X-ray and NMR structures of PIN1 show that these two functional domains share a common interface. The WW domain and PPIase domain

are connected by a glycine and serine rich polypeptide chain (Kowalski et al., 2002; Wintjens et al., 2001). Additional NMR studies of PIN1 and its substrates in solution demonstrate that the linker between these two domains contributes to substrate binding (Bayer et al., 2003).

1.3.2 PIN1 function

More than 20 interacting partners of PIN1 have been identified to date. These proteins belong to mitotic proteins, cytoskeleton proteins, transcription factors or cell cycle regulators. The interaction between PIN1 and its substrates causes a conformational change in the substrates, which affects their properties and functions such as phophorylation status, their interactions with other proteins, their subcellular localization or stability. For example, the conformational change of Cdc25 caused by PIN1 causes Cdc25 to become dephosphorylated and become subject to proteolytic cleavage (Shen et al., 1998; Stukenberg and Kirschner, 2001; Zhou et al., 2000).

PIN1 has been shown to be involved in the regulation of cell cycle progression, proliferation and differentiation. It is also involved in the transcriptional regulation of some genes in response to DNA damage

response through the regulation of TP53 function (Wulf et al., 2002). On a pathological level, PIN1 has been found to play a role in processes related to Alzheimer's disease and is over-expressed in some solid tumors (Ayala et al., 2003; Hamdane et al., 2002; Joseph et al., 2003; Shaw, 2002; Uchida et al., 2003; Wulf et al., 2003).

PIN1 has been shown to be important for the regulation of several cell cycle transitions including the G1/S and G2/M transitions. It also plays a role in the DNA replication checkpoint (Winkler et al., 2000; Wulf et al., 2002). Depletion of PIN1 causes mitotic arrest and apoptosis in yeast and HeLa cells (Lu et al., 1996). The interaction between cell cycle regulator Cyclin D1 and PIN1 has been studied *in vitro* and *in vivo*. Cyclin D1 is overexpressed in one half of breast cancer and contributes to cellular transformation. In the Cyclin D1 overexpressing tumors such as breast cancer, PIN1 is also expressed two times as high as in CyclinD1 negative tumors (Tsuchida et al., 2003).

PIN1 overexpression has been described not only in breast cancer but also in other solid tumors like lung, prostate cancers, oral squamous cell carcinoma and thyroid tumor (Nakashima et al., 2004; Miyashita et al., 2003). The expression level of PIN1 is positively correlated to tumor grade in breast cancer (Wulf et al., 2001).

TP53, a known tumor suppressor, plays a pivotal role in cell cycle checkpoints, genomic stability and apoptosis (Chang, 2002). DNA damage causes the stabilization and accumulation of TP53, which is dependent on TP53 phosphorylation at multiple Ser/Thr residues. The role of PIN1 in DNA damage response is mediated through regulation of TP53. DNA damage enhances the specific interaction between PIN1 and TP53 through binding of the PIN1 WW domain to phosphorylated Ser-Pro or Thr-Pro motifs in TP53. The conformational change of TP53 catalyzed by PIN1 through peptidyl-prolyl cis-trans isomerization leads to a reduced affinity of TP53 for its ubiquitin ligase, MDM2. This in turn increases TP53 half life by reducing proteasomal degradation of TP53 (Yang et al., 2004).

The phosphorylation of proteins at Ser/Thr-Pro residues is an important signaling mechanism in many cellular processes. It is regulated by a large superfamily of serine-threonine kinases such as cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs) and glycogen synthase kinase 3 beta. The discovery that PIN1 changes the conformation of phosphorylated Ser/Thr-Pro bonds in target proteins revealed a new post-phosphorylation regulatory mechanism of cellular signaling (Ryo et al., 2003).

1.4 Aim of this study

It is the aim of this work to analyze the interactions of ETV6 with TIP60β and of ETV6 with PIN1. In particular, how the interaction with these two proteins, a MYST domain histone acetyltransferase and a peptidyl-prolyl cis trans isomerase, changes the function of ETV6 as a transcriptional repressor.

2. Materials

2.1 Chemicals, enzymes

2.1.1 Chemicals

100bp DNA Ladder	BibcoBRL Life technologies, USA
1Kb DNA Ladder	BibcoBRL Life technologies, USA
3-amino-1, 2, 4-triazole (3-AT)	Sigma, Germany
Acetic acid	Roth, Germany
Acrylamide	Roth, Germany
Agar	Roth, Germany
Agarose	Roth, Germany
Albumin bovine fraction V	Sigma, Germany
Ampicillin	Roth, Germany
Bacto-peptone	Difco, USA
Bacto-yeast extracts	Difco, USA
BioRad protein assay	BioRad, USA
Bis-acrylamide	Roth, Germany
Boric acid	Roth, Germany

Bromophenol blue	Roth, Germany
BSA (bovine serum albumin)	Roth, Germany
Calcium chloride dihydrate	Roth, Germany
Coomassie Blue. G250 Brilliant Blue	Roth, Germany
D-Galactose	Sigma, Germany
Diethyl-1, 3, 6, 8-tetrahydro-1, 3, 6, 8-tetraoxobenzo[lmn][3,8] phenanthroline-2,7-diacetate (DTTPD)	Calchem, UK
Dithiothreitol (DTT)	Roth, Germany
Dulbecco's modified eagle medium (DMEM)	PAN, Germany
Dimethylformamaide (DMF)	Merck, Germany
Dimethyl Sulfoxid (DMSO)	Sigma, Germany
DPBS	PAN, Germany
ECL Hyperfilm	Amersham-Pharmacia, UK
Ethylenediaminetetraacetic acid (EDTA)	Roth, Germany
Ethanol	Merck, Germany
Ethidium Bromide	Roth, Germany
Fetal calf serum (FCS)	PAN, Germany; Gibco, USA
Fish sperm DNA	Sigma, Germany
Formalddehyde 37%	Roth, Germany

Glycerin (Glycerol)	Roth, Germany
Glucose	Merck, Germany
Glutathione-Sepharose 4B	Amersham-Pharmacia, UK
HCl	Roth, Germany
Hoechst 33342	Sigma, Germany
Hybond-ECL (Nitrocellulose)	Amersham-Pharmacia, UK
IPTG (Isopropyl-1-thio-β-D-galactopyranoside)	Roth, Germany
Isopropanol	Roth, Germany
K_2HPO_4	Roth, Germany
Kanamycin	Roth, Germany
L-amino acids 20 types	Sigma, Germany
L-Glutamine	Sigma, Germany
Lithium acetate (LiAc)	Sigma, Germany
2- Mercaptoethanol (2-ME)	Sigma, Germany
Methanol	Merck, Germany
Milk powder	Roth, Germany
Magnesium chloride hexahydrate	Roth, Germany
Magnesium sulphate heptahydrate	Roth Germany
NP-40	Sigma, Germany
PBS (phosphate-buffered saline)	PAN, Germany

PEG (polyethylene glycol) 4000	Sigma, Germany
Penicillin/Streptomycin	PAN, Germany
PMSF (Phenylmethylsulfonyl fluoride)	Sigma, Germany
Poly-Lysine	Sigma, Germany
Propidium iodide (PI)	Sigma, Germany
Protease inhibitors	Sigma, Germany
Protein A agarose	Santa Cruz, USA
Protein A/G PLUS-Agarose	Santa Cruz, USA
Protein G agarose	Santa Cruz, USA
Roti-Fect	Roth, Germany
SeeBlue protein standards	BD, Germany
Sepherose 4B beads	Amersham-Pharmacia, UK
SDS (sodium dodecyl sulphate)	Roth, Germany
Sodium acetate	Sigma, Germany
Sodium azide	Roth, Germany
Sodium chloride	Roth, Germany
Sodium deoxycholade	Sigma, Germany
Sodium di-hydrogen phosphate-monohydrate	Roth, Germany
Sodium fluoride	Sigma, Germany
Sodium orthovanadate	Aldrich, Germany

TEMED	Roth, Germany
Trichostatin A	Sigma, Germany
Tris base	Roth, Germany
Triton X-100	Roth, Germany
Trypsin/EDTA	PAN, Germany
Tryptone	Roth, Germany
Tween 20	Roth, Germany
X-gal (5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside)	Roth, Germany
Yeast extracts	Difco, USA
Yeast nitrogen base	Difco, USA
$ZnSO_4 \cdot 7H_2O$	Merck, Germany

2.1.2 Enzymes for DNA manipulation

Klenow fragment	MBI-Fermentas, USA
DNA restriction endouncleases	MBI-Fermentas, USA
RNAse A	Qiagen, Germany
T4 DNA Ligase	Gibco, USA
T4 DNA Polymerase	MBI-Fermentas, USA
rTaq® DNA Polymerase	PAN, Germany

2.2 Commercial kits

Dual-Luciferase® Reporter Assay System	Promega, USA
ECL Westernblotting Detection Reagents Kit	Amersham-Pharmacia, UK
GeneAmp PCR Reagent Kit	Perkin Elmer USA
Qiagen Plasmid Mini Kit	Qiagen GmbH, Germany
Qiagen Plasmid Maxi Endo-free Kit	Qiagen GmbH, Germany
Qiaquick Gel-Extract Kit	Qiagen GmbH, Germany
QuikChange® Multi Site-Directed Mutagenesis Kit	Stratagene, USA

2.3 Labware and Equipment

FACS (Moflo (Modular Flow Cytometer))	Cytomation, USA
Sterile filter 0.22 µm	Roth, Germany
Micro-pestle 100-1000 µl	Roth, Germany
Fluorescent Microscope	Karl Zeiss, Germany
TD-20/20 Luminometer	Turner Designg, Germany

Whatmann 3MM paper	Whatmann, USA

2.4 Bacterial and yeast strains

2.4.1 Bacterial strains

E.coli strains XL1-Blue	Stratagene, USA
E.coli strains XL10-Gold	Stratagene, USA
E.coli strains BL21	Amersham-Pharmacia, UK

2.4.2 Yeast strains

CG-1945 (Clontech, USA) was used in yeast two-hybrid screening and protein-protein interaction model in yeast with a highly sensitive HIS3 reporter gene and a lacZ reporter gene. It has the genotype: *MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3,*

112, gal4-542, gal80-538, cyh^{r}2, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URA3:: GAL4$_{17\text{-}mers(x3)}$-CYC1$_{TATA}$-lacZ.

2.5 Mammalian cell lines

HEK 293T (Human embryonal kidney adherent fibroblastoid cells growing as monolayer), from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

NIH 3T3 (Swiss mouse embryo fibroblast adherent monolayer cells), from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

2.6 Plasmids and oligonucleotides

2.6.1 Cloning vectors

Plasmid name	Used for	Company
pCMV-3B	Expressing a myc tag fusion protein in mammalian cells	Stratagene, USA
pCMV-2A	Expressing a flag tag fusion protein in mammalian cells	Stratagene, USA
pGAD-424	Expressing a GAL4 DNA activation domain fusion protein in yeast	Clontech, USA
pGBT9	Expressing a GAL4 DNA binding domain	Clontech, USA

	fusion protein in yeast	
pECFP-C1	Expressing a cyan fluorescence fusion protein in mammalian cells	Clontech, USA
pEYFP-N1	Expressing a yellow fluorescence fusion protein in mammalian cells	Clontech, USA
pGEX4T2	Expressing a GST fusion protein in bacteria	Amersham, UK
pM1	Expressing a GAL4 DNA binding domain fusion protein in mammalian cells	Promega, USA

2.6.2 Oligonucleotides used

2.6.2.1 DNA amplification primers:

ETV6(964-1032) fragment amplification primers:

Tel 964 Eco-T:

5’-GTGACGTCGACTGCTATTCTCCCAAT-3'

ETV6 1032B SAL1:

5’- CTCTGAATTCTACATG AACCACATCATGGTC -3'

ETV6(964-1047) fragment amplification primers:

Tel 964 Eco-T:

5’-GTGACGTCGACTCAAAGCAGTCTACAGT-3'

ETV6 1047B SAL1:

5’- CTCTGAATTCTACATG AACCACATCATGGTC -3'

For the amplification of full length PIN1 gene:

PIN1ATGXhol:

5’-CACCTCGAGGGAAGATGGCGGACGAGGAGAAGCTGCCGCCC-3’

PIN1B498EcoRI:

5’-ACAGAATTCTCTCAGTGCGGAGGATG- 3’

For the amplification of full length TIP60 gene:

TIP60ATGXhol:

5’-CATCTCGAGAAGATGGCGGAGGTGGGGGAGATAATCGAGGGC-3’

TIP60B1765EcoRI:

5’-GTAGAATTCGCCACTTCCCCCTCTTG-3’

2.6.2.2 Sequencing primers:

- EGFP-C1(1240-1258): 5’- AAA GAC CCC AAC GAG AAG C-3’
- DsRed-C 1(1214-1231): 5’-ACA TCA CCT CCC ACA ACG-3’
- PEYFPN-1(513-534): 5'-CATTGACGCAAATGGGCGGTAG-3'
- Y2H1: 5’-TCATCGGGAAGAGAGT-3’
- Y2H2: 5’-TACCACTACAATGGATG-3’
- TIP-MYST 1270T: 5’-TCT CGA ATT CTA TGA CTG TAA GGG CTT CAA C-3’
- TIP505B: 5’ CAC CTG TCG ACC ACC TCT CAC TCT GGA GAG CC-3’

2.6.2.3 Site-directed mutagenesis primers:

ETV6 mutagenesis primers:

ETV6(S106A):

ETV6(S106A) T:

5 ' -ACTTTCGCTATCGAGCCCCTCATTCAGGT-3 '

ETV6(S106A) B:

5 ' -ACCTGAATGAGGGGCTCGATAGCGAAAGT-3 '

ETV6(S257A):

ETV6(S257A) T:

5’-ACCCGAAGCCATCCGCACCCCGGCAGGAG-3’

ETV6(S257A) B:

5’-CTCCTGCCGGGGTGCGGATGGCTTCGGGT-3’

ETV6(S323A):

ETV6(S323A)-T:

5’-TCATGGTCTCT GTC GCC CCG CCT GAA GAG -3’

ETV6(S323A)-B:

5’- CTC TTC AGG CGG GGC GAC AGA GAC CATGA-3’

TIP60β mutagenesis primers:

TIP60-DRS-T:

5'-CCTACCAGCGCCGGGACTACCGGAAGCTGAGCATCGAGTTCAG C 3'

TIP60-DRS-B:

5'-GCTGAACTCGATGCTCAGCTTCCGGTAGTCCCGGCGCTGGTAG G-3'

2.6.3 Constructs used in experiments

No.	Plasmids	Antibiotics resistant	Insert	Obtained from
1	pCMV-3B ETV6	Ampicillin	ETV6	Michael Kickstein, 2000
2	pGBT9 ETV6	Ampicillin	ETV6	Sigrun Bartels, 1997
3	pGAD424 ETV6(1-59)	Ampicillin	ETV6(1-59)	Michael Kickstein, 2000
4	pGAD424 ETV6(1-126)	Ampicillin	ETV6(1-126)	Michael Kickstein, 2000
5	pGAD424 ETV6(118-345)	Ampicillin	ETV6(118-345)	Michael Kickstein, 2000
6	pGAD424 ETV6(118-218)	Ampicillin	ETV6(118-218)	Michael Kickstein, 2000
7	pGAD424 ETV6(219-287)	Ampicillin	ETV6(219-287)	Michael Kickstein, 2000
8	pGAD424 ETV6(282-345)	Ampicillin	ETV6(282-345)	Michael Kickstein, 2000
9	pGAD424 ETV6(314-336)	Ampicillin	ETV6(314-336)	Chang-Dong Zhang, 2002

10	pGAD424 ETV6(314-341)	Ampicillin	ETV6(314-341)	Chang-Dong Zhang & Michael Kickstein, 2000, Chang-Dong Zhang, 2002
11	pGAD424 ETV6(340-420)	Ampicillin	ETV6(340-420)	Michael Kickstein, 2000
12	pECFP C1	Kanamycin		Clontech
13	pECFP C1 ETV6(S106A)	Kanamycin	ETV6(S106A)	Chang-Dong Zhang, 2004
14	pECFP C1 ETV6(S257A)	Kanamycin	ETV6(S257A)	Chang-Dong Zhang, 2004
15	pECFP C1 ETV6(S323A)	Kanamycin	ETV6(S323A)	Chang-Dong Zhang, 2003
16	pECFP C1 ETV6	Kanamycin	ETV6	Chang-Dong Zhang & Britta Kaltwasser, 2001
17	pM1 ETV6	Ampicillin	ETV6	Sigrun Bartels, 1996
18	pM1 ETV6(282-345)	Ampicillin	ETV6(282-345)	Sigrun Bartels, 1996
19	pM1 ETV6(S323A)	Ampicillin	ETV6(S323A)	Chang-Dong Zhang, 2002
20	pCMV 2A TIP60β	Kanamycin	TIP60β	Michael Kickstein, 2000
21	pEYFP C1 TIP60β	Kanamycin	TIP60β	Chang-Dong Zhang, 2001
22	pEYFP N1 TIP60β	Kanamycin	TIP60β	Chang-Dong Zhang, 2002
23	pDasRed C1	Kanamycin		Clontech
24	pDasRed C1 TIP60β	Kanamycin	TIP60β	Chang-Dong Zhang, 2001
25	pGEX4T2 TIP60β	Ampicillin	TIP60β	Jasmina Putnik, 1998
26	pGAD GH TIP60β	Ampicillin	TIP60β	Hela cDNA library, Clontech
27	pGAD GH TIP60β DRS	Ampicillin	TIP60β DRS	Chang-Dong Zhang, 2001
28	pEYFP N1 PIN1	Kanamycin	PIN1	Chang-Dong Zhang, 2002

29	pGAD GH PIN1	Ampicillin	PIN1	Hela cDNA library, Clontech
30	pGAD424 PIN1 WWD	Ampicillin	PIN1 WWD	Michael Kickstein, 2000
31	pGAD424 PIN1 PPIase	Ampicillin	PIN1 PPIase	Michael Kickstein, 2000
32	pGAL4-TK Luc	Ampicillin	GAL4 DB, TK promoter, firefly luciferase	Jasmina Putnik, 1998
33	pTR 89	Ampicillin	luciferase	Hiebert S.W., 2000
34	pTR 334	Ampicillin	stromelysin-1 promoter, luciferase	Hiebert S.W., 2000
35	phRL-null	Ampicillin	Renilla luciferase	Clontech

2.7 Media and cell culture

2.7.1 Medium for *E.coli*

LB (Luria Bertani) medium contains 10 g/l tryptone, 5 g/L yeast extract and 5 g/L NaCl, pH 7.2. LB/Ampicillin medium was used for selecting the transformed colonies of Ampicillin resistant plasmids with a final concentration of 50 µg/ml Ampicillin.

LB/ Kanamycin medium was used for selecting the transformed colonies of Kanamycin resistant plasmids with a final concentration of 25 µg/ml Kanamycin.

NZY+ medium consisted of 10 g/l NZ amine (casein hydrolysate), 5 g/l yeast extract, 5 g/l NaCl, pH 7.5, to 1 liter of meduim prior to use the following supplements were added: 12.5 ml of 1 M $MgCl_2$ to a final concentration of 12.5 mM, 12.5 ml of 1 M $MgSO_4$ to a final concentration of 12.5 mM, 20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose) to a final concentration of 20 mM. NZY+/Ampicillin and NZY+/Kanamycin medium was used for selecting site directed mutagenesis clones. For plates, 18g/l agar was added to the medium.

2.7.2 Yeast Medium

YPD medium was used for culturing CG1945 yeast strain to make competent cells for plasmid DNA transformation. YPD medium: 20 g/L peptone, 10 g/l yeast extract, pH5.8. After autoclaving, glucose was added to a final concentration of 2% at 55°C. For plates, 20 g/l agar was added to the medium.

2.7.3 SD (synthetic drop-out) medium

Synthetic drop-out medium was used to test for yeast autotrophy after transformation and for the expression of reporter genes that would indicate

protein-protein interaction. SD medium: 6.7 g/l yeast nitrogen base, 20 g/L agar, 100 ml of appropriate 10x dropout stock solution, pH5.8. After autoclaving, glucose was added to a final concentration of 2% (w/v) and 3-AT was added to a final concentration of 0.1 mM, 0.5 mM, 1 mM, 2.5 mM or 5 mM at 55 °C. Plates containing 3-AT were stored at 4°C for up to 2 months.

2.7.4 Medium for mammalian cell culture

Dulbecco's Modified Eagle Medium (DMEM) with 4.5 mg/ml Glucose, 10% FCS, 2 mM Glutamine was used for HEK 293T and NIH 3T3 cells. For some experiments, Penicillin/Streptomycin was added in the medium at the final concentration of 1% (10.000 U/ml Penicillin and 10 mg/ml Streptomycin).

2.8 Stock solutions and buffers

The stock solutions listed below were all prepared with deionized H_2O. "x" refers to the fold concentration of the stock solution.

3-AT

1 M 3-AT was prepared in deionized H_2O, filter sterilized and stored at 4 °C.

Dropout Solution (10X) for SD medium

10x –Leu/–Trp DO Solution (lacking leucine and tryptrophan):

200 mg adenine hemisulfate

200 mg arginine HCl

200 mg histidine HCl monohydrate

300 mg isoleucine

300 mg lysine HCl

200 mg methionine

500 mg phenylalanine

2000 mg threonine

300 mg tyrosine

200 mg uracil

1500 mg valine

Components were dissolved in 1 L deionized H_2O and autoclaved.

10x –Leu/–Trp/-His DO Solution (lacking leucine, tryptrophan and histidine)

200 mg adenine hemisulfate

200 mg arginine HCl

300 mg isoleucine

300 mg lysine HCl

200 mg methionine

500 mg phenylalanine

2000 mg threonine

300 mg tyrosine

200 mg uracil

1500 mg valine

Components were dissolved in 1 L deionized H_2O and autoclaved.

IPTG (10mM)

24 mg of isopropyl-1-thio-β-Dgalactopyranoside (IPTG) were dissolved in 10 ml of sterile dH_2O and stored at –20 °C.

Laemmli-buffer (2 x)

187.5 mM	Tris-Cl pH 6.8
6 %	SDS
30 %	Glycerol
0.01 %	Bromophenol Blue

5 %	2-ME

LiAc (10 x)

1 M	LiAc-Hac

pH7.5, autoclaved

P1 buffer (resuspension buffer)

50 mM	Tris·Cl
10 mM	EDTA
100 µg/ml	RNase A

pH 8.0, stored at 2–8°C

P2 buffer (lysis buffer)

200 mM	NaOH
1%	SDS (w/v)

P3 buffer (neutralization buffer)

3.0 M	potassium acetate
pH 5.5	

PBS (10 x)

1.4 M	NaCl
27 mM	KCl
100 mM	Na_2HPO_4
18 mM	KH_2PO_4
pH7.3	

PEG 4000 (50%)

50 g PEG4000 was dissolved in 100 ml H_2O and autoclaved

RIPA buffer

1% (v/v)	NP40
0.5%(w/v)	Deoxycholate
0.1%(w/v)	SDS
0.15 M	NaCl

5mM	EDTA
50 mM	Tris
pH 8.0	

TBE (5 x)

0.45 M	Tris-Borate
0.01 M	EDTA
pH8.3, autoclaved	

TBS (10 x)

0.2 M	Tris base
1.5 M	NaCl
pH7.4, autoclaved	

TE (10 x)

0.1 M	Tris-HCl
0.01 M	EDTA
pH 7.5, autoclaved	

TFB I

10 mM	MES
45 mM	$MnCl_2\,4H_2O$
10 mM	$CaCl_2\,2H_2O$
100 mM	KCl
3 mM	Hexamminecobalt chloride

filter sterilized and stored at 4 °C

TFB II

10 mM	KAc
45 Mm	$MnCl_2\,4H_2O$
10 mM	$CaCl_2\,2H_2O$
10 mM	KCl
100 mM	Hexamminecobalt chloride
10%(v/v)	Glycerol

pH 6.4. filtered and stored at 4 °C.

TSA (Trichostatin A)

100 mM	TSA

Stored at -20 °C

X-Gal (2%)

0.2 g of 5-bromo-4-chloro-3-inodlyl-β-Dgalactopyranoside (X-Gal) was dissolved in 10 ml of dimethylformamide (DMF), stored at –20 °C

2.9 Antibodies

The following primary antibodies were used either in immunoblot analysis or as primary antibodies in immunoprecipitation.

2.9.1 Primary antibodies:

GFP antibody (A11122, Molecular Probes, Germany):

Rabbit polyclonal IgG with a stock concentration of 2 mg/ml. This antibody was used for testing the expression of CFP fusion ETV6, YFP fusion TIP60β and YFP fusion PIN1 in transient transfection experiments.

Primary antibodies from Santa Cruz, USA:

TEL/ETV6 (N-19): sc-8546

Goat polyclonal IgG at a stock concentration of 200 µg/ml. This antibody was used to test the ETV6 protein expression in transiently transfected mammalian cells lysate.

TIP60 (N-17): sc-5725

Goat polyclonal IgG at a stock concentration of 200 µg/ml. This antibody was used to test the TIP60β expression in transiently transfected mammalian cells.

PIN1 (H-123): sc-15340

Rabbit polyclonal IgG with a stock concentration of 200 µg/ml. This antibody was used to test PIN1 expression in mammalian cells.

2.9.2 Secondary antibodies:

The following HRP-conjugated secondary antibodies from Santa Cruz were used in conjunction with the ECL Kit (Amershan Pharmacia, UK) to visualize

proteins in immunoblot (Western blot) analysis:

Donkey anti-goat IgG HRP: sc-2056

Donkey anti-rabbit IgG-HRP: sc-2305

2.10 Softeware used

Adobe photoshop

This image manipulation software was mainly used to change the size and improve the contrast of experimental images.

MacVector

This software was used analyze the secondary structure (hairpin etc.) and annealing temperature of PCR primers. It was also used for comparing the homology between the target DNA and sequenced DNA. Some protein characteristics such as isoelectric point (PI), molecular weight (MW) were analyzed by this software.

Openlab

This image analysis software was used to capture images (bright field and fluorescent) from the CCD attached to the Zeiss inverted microscope. It was also used to acquire Z- stacks of cells and to deconvolve theses images.

WinMDI2.8

It was used to analyze FACS data.

3. Methods

3.1 Molecular Biology

3.1.1 Preparation and purification of plasmid DNA from bacteria

The procedures were based on the alkaline lysis method with some modifications. Mini preparation was performed by following the modified protocol of QIAprep Spin Miniprep Kit without QIAprep Spin Column. The mini preparation plasmids were used as templates for DNA sequencing. Plasmid Midi and Max preparations were carried out with commercial kits from Qiagen. The process followed the manuals provided with the kits. The Plasmid Midi Kit was used to purify up to 100 µg of plasmid DNA. The Endo-free plasmid Max preparation Kit was used to obtain endotoxin-free plasmid DNA for transfection into mammalian cell. The following is a sample process of preparation and purification of a plasmid DNA minipreparation.

Procedure:

1. A single fresh colony was cultured in 4 ml LB medium containing the appropriate selective antibiotic at 37°C overnight with vigorous shaking.

2. The cells were harvested by centrifugation and resuspended with 300 µl buffer P1 in a 1.5 ml centrifugation tube.

3. 300 µl buffer P2 was added and mixed gently.

4. 300 µl buffer P3 was added and mixed gently.

5. The tube was centrifuged at 12000 g for 10 minutes.

6. The clear supernatant was moved to a new 1.5 ml centrifugation tube. 630 µl isopropanol was added and mixed.

7. The tube was centrifuged again at 12000 g for 10 minutes. The supernatant was discarded.

8. 1 ml 70 % ethanol was added to wash the plasmid DNA pellet. The tube was centrifuged at 12000 g for 10 minutes. The supernatant was carefully removed.

9. The tube was air-dried for 10 minutes. 10 µl TE buffer was added to dissolve the plasmid DNA pellet.

10. The tube with plasmid DNA sample can be stored at 4 °C for one month or –20 °C for several years.

3.1.2 Digestion of DNA with restriction endonucleases

The restriction digestions were performed by the protocol provided by the supplier companies. For example, 1 µg plasmid DNA was digested with 5

units of a given enzyme and 1x digestion buffer in 20 µl total volume for an incubation of one hour at 37°C.

3.1.3 Blunt ending 3' or 5' overhangs

It was occasionally necessary to fill in the recessed 3' ends of DNA molecules left after the digestion with restriction enzyme in some cloning experiments by using Klenow fragment in the reaction. For example, a single digestion was performed at first then the reaction mixture was incubated at the appropriate temperature to inactivate the first enzyme inactive. 5 units of Klenow and dNTP were mixed with reaction products and incubated at room temperature for 15 minutes or longer. Then the reaction was incubated at 65 °C for 10 minutes to inactivate the Klenow enzyme. The blunted DNA product could be purified by QIAquickGelExtractionkit (Qiagen, USA).

3.1.4 Polymerase chain reaction (PCR)

3.1.4.1 Amplification of DNA fragments by PCR

DNA amplification in the polymerase chain reaction (PCR) is based on multi-cycles of the denaturation of double stranded template (plasmid),

primer annealing and synthesis of new DNA sequence with a heat stable DNA polymerase. The following table is an example of standard reaction conditions for PCR, the total reaction volume is 20 µl.

Tris HCl pH 8.3	Mg^{2+}	KCl	dNTP	Primers	DNA polymerase	Template DNA
10 mM	1.5 mM	50 mM	200 µM	1 µM	1-5 units	1 pg to 1 µg

The denaturation, annealing and polymerization times and temperature are shown in the table below.

Loop	1st	2nd			3rd	
Temperature	94 °C	94 °C	58 °C	72 °C	72 °C	4 °C
Time	2 min	30 sec	30 sec	1 min	10 min	∞
Cycle	x1	x25			x1	

3.1.4.2 Sequencing PCR reaction

Cycle sequencing was performed using the dye dideoxy chain termination method based on the original dideoxy chain termination method developed by Sanger. In this work, it was performed by using pre-prepared solutions from a kit (Perkin Elmer (PE) Company) as listed below.

BigDye	Primer	Template	H_2O	Total Volume
4 µl	1 µM	1 µg	4 µl	10 µl

The denaturation, annealing and polymerization times and temperature are shown in below table.

Loop	1st	2nd			3rd
Temperature	96 °C	96 °C	50 °C	60 °C	4 °C
Time	1 min	30 sec	15 sec	4 min	∞
Cycle	x1	x25			x1

3.1.5 Site-directed mutagenesis of DNA

Site-directed mutagenesis technique is an important tool for studying gene and protein function in vivo and vitro. The method is used to specifically mutate a nucleotide in a DNA sequence. In this work, the quick-change site directed mutagenesis method was used to obtain mutant plasmids. It was based on a three step (mutagenesis primer design, Dpn I digestion and mutant ss-DNA repair in vivo) protocol from *Stratagene*.

3.1.5.1 Primer design for site directed mutagenesis

The mutagenic oligonucleotide primers were designed to incorporate the desired point mutations and bind to adjacent sequences of the template plasmids. The length of primer should be between 25 and 45 bases with the melting temperature at least 75 degree. The GC percent of the whole primer should be more than 40% and at least one C or G at 3' terminal.

3.1.5.2 Process of PCR

PCR with the primers carrying the mutation was performed according to the manufacturer's protocol with some modification as listed below.

Reaction components, total volume were 20 µl:

10 x reaction buffer	H_2O	Qucik solution	Plasmid DNA template	Mutagenesis primers	dNTP	*PfuTurbo* DNA polymerase
2 µl	14 µl	0.5 µl	1µl (100 ng/µl)	1 µl (125 ng/µl)	1 µl	0.5 µl

Cycling parameters:

Loop	1st	2nd			3rd
Temperature	95 °C	95 °C	55 °C	65 °C	4 °C
Time	1 min	1 min	1 min	2 min/1kb of plasmid	2 min
Cycle	X1	X25			X1

3.1.5.3 *Dpn* I digestion of the amplification products

The *Dpn* I endonuclease (target sequence: 5´-Gm6ATC-3´) is specific for methylated and hemimethylated DNA. DNA isolated from almost all *Escherichia coli* strains is dam methylated. So it was used to specifically digest the parental (wild type) DNA template. After PCR, 1 µl diluted *Dpn* I (1 µg/µl, 1:10) was added into the reaction tube and incubated at 37 °C for 1 hour.

3.1.5.4 Transformation of mutated plasmids into XL-10 ultra competent cell

In this step, the *Dpn* I treated reaction mixture was transformed into XL10-Gold® ultra competent cells. The nicked mutated plasmids would be repaired in vivo. The transformation was performed according to the manufacturer's instructions.

3.1.6 DNA gel electrophoresis

DNA gel electrophoresis was used to check the results of restriction enzyme digestions, PCR reactions and DNA purification. Fragments of between 400 bp and 12 Kbp were separated by using 0.5% agarose gels. The gels were stained with ethidium bromide (EB) at a concentration of 0.5 µg/ml and the DNA was visualized under UV light (365 nm). DNA fragments less than 400 bp were separated on 1% or 1.5% agarose gels.

3.1.7 Purification of DNA fragments from agarose

In order to obtain single DNA fragments from digested plasmids, gel electrophoresis was performed at first. After that, the QIAquick Gel Extraction

kit was used to extract the DNA from the gel. The band of interest was immediately excised from the gel and the weight of the gel slice was determined. Three volumes solubilisation buffer (components not given in Kit) from the kit were added into the sample tube. The tube was incubated and vortexed at 50 °C for 10 minutes or longer until the agarose was completely dissolved. The solution was loaded onto the ion–exchange column by centrifugation at 12000 g for one minute. 500 µl solubilisation buffer was added to wash the column that was centrifuged at 12000g for one minute again. Three volumes high salt ethanol wash buffer were used for washing the column. Elution of the DNA from the column was carried out by adding 50 µl of 10 mM Tris-HCl, pH8.5 or H_2O on the column, which was centrifuged at 12000g for one minute. To increase the DNA concentration in the eluate, a centrifugation step under vacuum was performed. This procedure was also used for the purification of PCR products.

3.1.8 Precipitation of DNA

When the DNA fragment was used for sequencing or some enzymatic reaction, that was sensitive to residual salt, it was necessary to precipitate the DNA. The precipitation of DNA was achieved by adding 0.1 Vol 3 M KAc and 2.5 Vol 100% ethanol and incubated at –20 °C for 10 minutes. After

removal of the supernatant the pellet was washed with 70 % ethanol and air-dried.

3.1.9 Cloning of DNA

3.1.9.1 DNA ligation

This procedure was performed to construct new plasmid by ligating the target DNA fragment with vector plasmid. The DNA ligation reaction was performed following the protocol for T4 DNA Ligase (Gibco, USA). 4 µl 5x Ligase Reaction Buffer (250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% PEG8000) from kit, 1 µl T4 DNA Ligase (1U/µl) and 3:1 (molar ratio) insert DNA: vector DNA were mixed together in a microcentrifuge tube. The tube was kept at room temperature for 2 to 4 hours or for 16 hours at 16°C.

3.1.9.2 Preparation of competent *E.coli* cell

Highly competent *E.coli* are important for transforming ligation products. The preparation of competent cells was carried out according to the method published by Hanahan in1983. 1 µl stock *E.coli.* XL-1 Blue was incubated in

10 ml LB liquid medium with 125 µg/µl Tetacyclin overnight at 37 °C. 2 ml of the overnight culture were added into 200 ml LB liquid medium and incubated for 2 to 3 hours at 37 °C until the O.D. (595) was between 0.5 and 0.7. The cells were placed on ice for 10 minutes. Then the cells were pelleted at 3000 rpm for 5 minutes at 4 °C. The pellet was resuspended in 30 ml ice-cold TFB I solution and kept on the ice for 10 minutes. The cells were centrifuged again and resuspended in 4 ml TFB II. 100 µl aliquots of the competent cells were stored at –80 °C.

3.1.9.3 Transformation of plasmid DNA into competent *E.coli*. cells

10 to 100 ng supercoiled DNA or 4 µl of ligation reaction product were mixed with 100 µl competent *E.coli.* cells. This mixture was incubated on ice for 30 minutes then heat-shocked by rapidly transferring it to a water bath preheated to 42 °C for 90 seconds and immediately placing it back on ice for two minutes. 900 µl LB medium was added to the mixture and incubated at 37 °C for 40 minutes. The cells were pelleted and resuspended in 100 µl LB medium. The resuspended cells were spread on LB plates with the appropriate antibiotic selection. The plates were incubated for 16 to 20 hours at 37 °C after which individual colonies became visible.

3.1.10 DNA sequencing

DNA sequencing was performed by the automated sequencing way using DNA molecular labeled with fluorescent dyes developed by Smith et al., 1986. After running the sequencing PCR reaction, the product was precipitated and dissolved in 20 µl H_2O. 10 µl of the product was analyzed on the sequencing machine (ABI PRISM 310 Genetic Analyzer, PE). The DNA sequences were analyzed using the MacVector software or web-based tools (www.ncbi.nlm.nih.gov).

3.2 Protein chemistry and biochemistry

3.2.1 Preparation of mammalian cell lysates

In order to analyze target protein expression in mammalian cells, cells were lyzed in RIPA buffer. Transfected cells were washed with PBS two times at room temperature, then Trypsin/EDTA was added and incubated with at 37 °C until the adherent cells were detached. DMEM medium with fetal calf serum was added to inhibit the activity of Trypsin. The cells were transferred to a fresh 15 ml tube and centrifuged at 750 rpm for 5 minutes. Ice-cold PBS was added to wash the cell pellet two times. 0.6 ml of RIPA buffer was added

to resuspend the cell pellet. The resuspended cells were transferred to a fresh microcentrifuge tube and incubated on ice for 30 minutes. The sample was votexed for 30 seconds every 10 minutes. Then the solution was centrifuged at 10,000 g for 10 minutes at 4 °C. The supernatant fluid was the total cell lysate. The supernatant was transferred to a new microcentrifuge tube.

3.2.2 Electrophoretic separation of proteins by SDS-PAGE

Pre-cast one-dimensional denaturing NuPAGE Novex 4-12% Bis-Tris gels were obtained from Life Technologies. Protein samples were solubilised by boiling in the presence of Laemmli buffer (2x). An aliquot of the sample was loaded on the gel. Electrophoresis was performed at constant 200 V in a cold room. The gel front was allowed to migrate out of the gel before electrophoresis was stopped. Commercial protein standards enabled the estimation of apparent molecular weights.

3.2.3 Protein blotting

Protein transfer from Bis-Tris gel to Hybond-ECL Nitro-Cellulose membrane was carried out by electroblotting in a semi-dry electroblotter as described by

the manufacturer (Invitrogen, USA). The protein transfer process lasted 1 to 2 hours at a constant current of 120 mA/64 cm^2. After transfer, the protein standards should be clearly visible on the membrane.

3.2.4 Immunodetection (Western blotting)

The procedure was performed according to the protocol from Santa Cruz Biotechnology. Briefly, the membrane was blocked against non-specific binding by incubating the membrane in freshly prepared 0.5% TBS/Milk for 1 hour at room temperature or overnight at 4 °C. The membrane was washed in TBS two times and incubated with primary antibody diluted at 1:1000 in 0.5 % TBS/Milk for 1 hour at room temperature. The membrane was washed in TBS and incubated with the 2^{nd} antibody-HRP (horseradish peroxidase) conjugate at room temperature for one hour. After this, the membrane was washed three times for 15 minutes at room temperature to remove excess 2^{nd} antibody conjugate. The specific protein bands were detected using chemiluminescence (ECL kit, Amersham-Pharmacia, UK) staining for 1 minute. Membranes with cheminluminescence reagent were sealed in plastic and exposed to X-ray films for 1 to 30 minutes.

3.3 Detection of protein-protein interaction in the Yeast two-hybrid system

The yeast two hybrid system used in this work was a GAL4 based two-hybrid system (Clontech, USA) that provided a transcriptional assay for detecting protein-protein interaction in vivo in yeast. The principle of this method is shown in the diagram below (Fig. 3.1). A bait protein (protein X) was expressed as a fusion to the GAL4 DNA-binding domain (GAL4 BD) and the putative interacting protein (protein Y) was expressed as a fusion to the GAL4 activation domain (GAL4 AD). When bait and its interacting protein were binding, the DNA-BD and AD were brought into proximity, thus activating transcription of reporter genes. This method can be used to identify novel protein interactions, confirm suspected interactions, and define interacting domains.

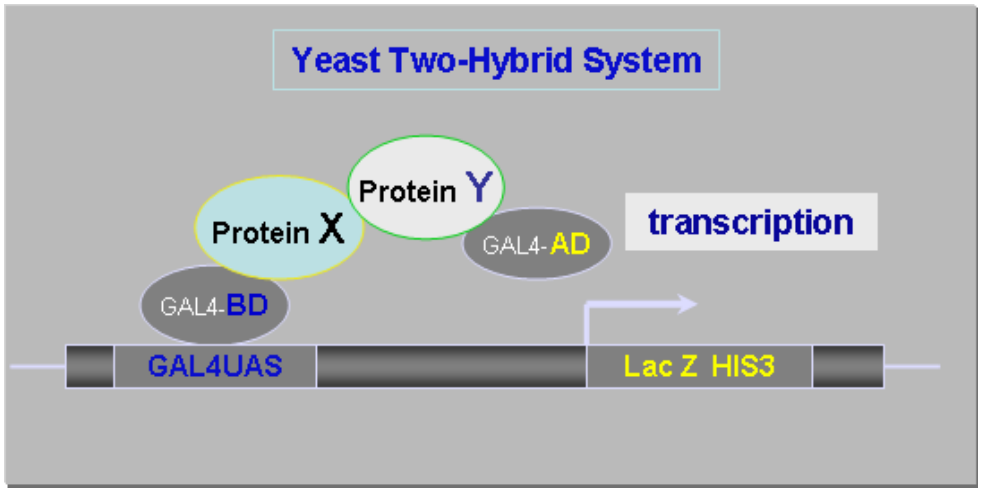


Fig. 3.1 Yeast two-hybrid protein-protein interaction model

3.3.1 Preparation of competent yeast cell

Competent yeast cells were used for the transformation of two plasmid DNA encoding the two interaction proteins. The process of making competent cells was mainly carried out of Yeast Handbook from Clontech Laboratories.Inc. Several fresh *CG1945* colonies (one to three weeks old, 2–3 mm in diameter) were suspended in 1 ml of YPD or SD medium. The resuspended cells were incubated in 50 ml of YPD or the appropriate SD medium at 30 °C overnight for 16–18 hours with shaking at 250 rpm until stationary phase was reached (OD_{600}>1.5). This overnight culture was transferred to a flask containing 300 ml of YPD and incubated at 30 °C for about 3 hours with shaking (230 rpm) until the OD_{600} reached 0.4–0.6. The cells were collected and washed with H_2O. 5 ml sterile 1X TE/1X LiAc buffer were used to suspend the cells. 100 µl cells were aliquotted into 1.5 ml microcentrifugation tubes and stored at –80 °C.

3.3.2 Transformation of plasmids into competent yeast cell

0.1 µg of plasmid DNA and 100 µg of herring testes carrier DNA were mixed and added to a tube with 100 µl of yeast competent cells. Then 0.6 ml of sterile PEG/LiAc solution was added and vortexed at high speed for 10 sec to

completely mix all components. The tubes were incubated at 30 °C for 30 min with shaking at 200 rpm. 70 µl of DMSO was added and mixed well by gentle inversion. A heat shock was performed at 42 °C water bath for 15 minutes after which the tubes were placed on ice for 1 to 2 minutes. Cell pellets were obtained by a centrifugation at room temperature. The pellet was resuspended in 100 µl of sterile 1X TE buffer. These cells were spread on SD agar plates and the plates were incubated at 30 °C until colonies appeared (generally, 2 to 4 days).

3.3.3 Detection of protein-protein interaction in yeast

To test for interaction between two proteins, the bait and prey protein expressing plasmids (see 3.3) were co-transformed into competent yeast cells and the cells were plated on SD plates lacking the transformation markers for each of the two plasmids (usually tryptophan for the bait and leucine for the prey plasmid). Then the double transformants were streaked out on plates lacking histidine, tryptophan and leucine (-H,-L,-W) and incubated at 30 °C for 2 to 4 days. Colony growth on -H,-L,-W plates indicated activation of the HIS 3 reporter gene, which is a consequence of the physical interaction between bait and prey protein.

3.4 Cell biology

3.4.1 General cell culture techniques

All mammalian cell lines (NIH3T3 and HEK 293T) were cultured in a humidified incubator (Heraeus CO2-AUTO-ZEKO) at 37 °C with 5% CO_2. Cell culture work was done in an airflow hood (BDK Luft-and Reinraumtechnik GMBH). All materials used in the laminar-flow hood were sterilized by treating the surface with 70% ethanol or UV light for 15 minutes. Cells were kept in DMEM medium with 5% FCS and 1% Penicillin/ Streptomycin (10.000 U/ml Penicillin and 10 mg/ml Streptomycin). Cell line stocks were frozen in liquid nitrogen in freezing medium (10% DMSO and 90% FCS).

3.4.2 Transient transfection of mammalian cells with plasmids

Two different methods of introducing plasmid DNA into mammalian cells (calcium phosphate precipitation or liposome mediated transfection) were used in this work. The calcium phosphate method was carried out as described by Jordan et al. (1996). But since the transfection efficiency was not high with in this method, the liposomal transfection reagent Roti-fect

(Roth, Germany) was used in the experiments. The procedure was carried out according to the manufacturer's description with some minor modifications. The ratio of plasmid DNA (weight in microgram) and liposome reagent (volume in micro liter) was between 1 to 1 or 1 to 2 depending on the particular plasmid and cell type. The tissue culture plates (1 million cells per 35 mm plate) were prepared one day before the transfection. The incubation time of plasmid DNA/liposome mixture with the cells was about 6 hours at 37 °C with 5% CO_2 after which time fresh medium was added to the plates. Two days after transfection, the cells were harvested by different ways depending on the exact experiments. The transfection efficiency was assessed by using a parallel transfection with the pECFP C1 or pEYFP N1 plasmid, which usually showed around 90% transfection efficiency. But the transfection efficiencies of pECFP C1 ETV6, pEYFP N1 TIP60β and pEYFP N1 PIN1 were much lower than the vector control about 40%, 10% and 40%, respectively.

3.4.3 Protein-protein co-localization in mammalian cells

To perform in vivo and in situ protein (co) localization, fluorescent protein tagged proteins were expressed in NIH3T3 mouse fibroblast cells using transient transfection. For colocalization studies, cyan and yellow fluorescent

proteins were used as tags because there is minimal spectral overlap between the emission and excitation spectra of these two proteins at the excitation and emission wavelength used in our microscope set-up. The cells were plated on glass cover slips. The method of transfecting plasmid DNA into mammalian cells was carried out as described above. Two days after transfection, cells were washed two times with PBS. Then the cells were fixed with freshly prepared 3.7% formalin/PBS for 15 minutes at room temperature. The cells were washed with PBS two times. Then 1% Triton-X100 was added for 15 minutes. Then the cells were washed again two times with PBS. Cover slips were put on the microscope slides and sealed at the edges by rubber cement. Protein co-localization was observed with a fluorescence microscope (Karl Zeiss, Germany) with filter combinations for cyan (475 nm emission maximum) and yellow fluorescent protein (527 nm emission maximum). The Openlab software package was used to control the CCD camera, and take digital photos of observed cells as well as to perform volume deconvolution on image stacks. Cells transfected with only one plasmid (CFP or YFP alone) were used as controls.

3.4.4 Cell cycle analysis

It is known that PIN1 plays an important role in cell cycle regulation. We

therefore decided to investigate whether the expression of ETV6, PIN1 or TIP60 as well as the coexpression of ETV6 with PIN1 and ETV6 with TIP60 would have an effect on the cell cycle of HEK293T cells. The transfection procedure was carried out as described in Methods (chapter 3.4.2). As negative control, mock transfected cells were used. These cells were plated at the same density and treated the same way as the experimental cells except that no DNA was added in the transfection step. The cells were harvested 48 hours after transfection and stained with 5 µg/ml Hoechst 334 dye and 50 µg/ml propidium iodide. The cells were analyzed by FACS. The DNA contents of the cells were measured using the Hoechst stain. The PI stain was used to exclude dead cells (PI positive cells). To analyze the DNA contents of the transfected cells, cells were gated for GFP, YFP or GFP/YFP positivity.

3.5 Reporter gene assays

3.5.1 Reporter plasmids

Two different reporter systems were used in this work. One for analyzing the transcriptional properties of proteins and protein domains were fused to a GAL4 DNA binding domain. Here the reporter plasmid pGAL4 TK Luc which

contains 5 GAL4 DBD binding sites and a TK (Herpes simplex virus thymidine kinase) minimal promoter followed by the firefly luciferase reporter gene, was used. This plasmid has a medium to high expression level of firefly luciferase and was used to study transcriptional repression. The other reporter gene system was based on the ETV6 responsive stromelysin-1 promoter. Two reporter plasmid, were used in this system: pTR334 and pTR89. pTR334 had a 334 bp fragment of the rat stromelysin-1 promoter, which contains an ETV6 (EBS) and two *ets* potential binding sites (TBS), upstream of the firefly luciferase gene. pTR 89 was used as a control plasmid. It has only 89 bp of the rat stromelysin-1 promoter without EBS and TBS. Both pTR 334 and pTR 89 were obtained from Prof. Hiebert, Nashville, Ten. To control for transfection efficiency the phRL-null plasmid, which encodes the Renilla luciferase under the control of a minimal promoter, was used.

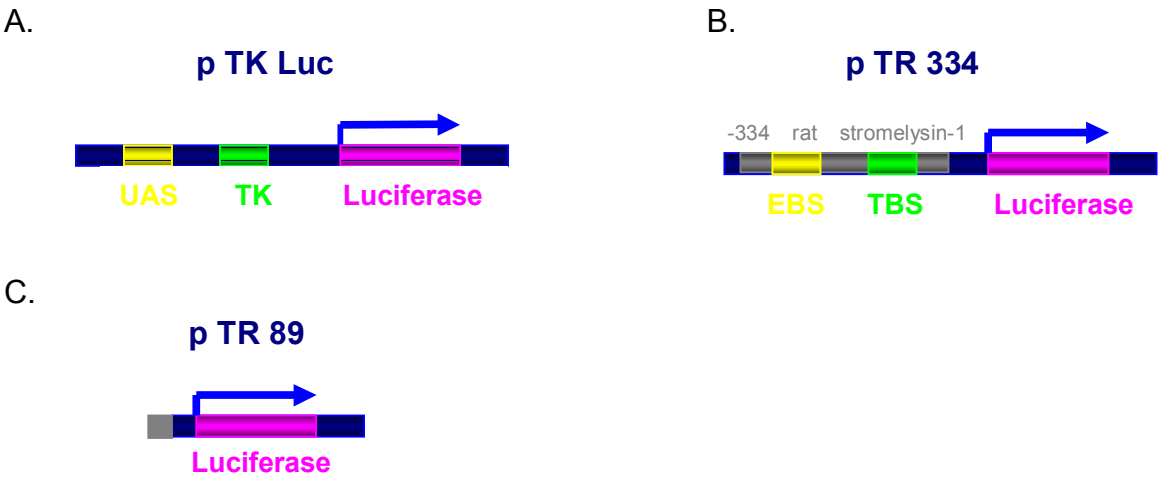


Fig. 3.2 Reporter plasmids used in reporter gene assays

In order to analyze the significance of protein-protein interactions on the

transcriptional activity of ETV6, reportergene assays were performed in which ETV6 or its mutants were cotransfected with TIP60 or PIN1 in HEK 293 T cells. About 2.1 µg of total plasmid DNA was used for $5x10^5$ cells in a single well of a 24 well tissue culture dish. The transfection was carried out as described above. In some experiments, the histone deacetyltransferase (HDAC) inhibitor TSA was added 24 hours after transfection at a concentration of 75 nM. After 48 hours, the transfected cells were washed two times with PBS. 100 µl of passive lysis buffer (PLB) (Dual-Luciferase® Reporter Kit, Promega) was added into each well to lyse the cells. The total cell lysate was centrifuged for 1 minute at 4 °C at 12000 g. 20 µl of supernatant was mixed with 100 µl LAR II substrate for 3 seconds and the firefly luciferase activity was measured for 10 seconds in a luminometer (TURNER DESIGN, Germany). Then 100 µl of stop & Glo reagent (this solution stops the firefly luciferase reaction and initiates the Renilla luciferase reaction) was mixed with the solution and the Renilla luciferase activity was measured for 10 seconds.

4. Results

4.1 Plasmid constructs

4.1.1 Cloning of TIP60β and PIN1 cDNA

The cDNA of TIP60β identified as prey of ETV6 in the yeast two-hybrid screen of a HeLa cDNA library lacked the first 5 codons. In addition, it contained several hundred base pairs of 3' UTR (untranslated region). Using this original TIP60β cDNA cloned into the pEGFP-C1 and pDS-Red-C1 vectors, we did not observe a fluorescence signal in transiently transfected 293T and NIH3T3 cells. We hypothesized that this could be due to the long 3' UTR sequence. The situation for the original PIN1 prey clone was similar: It did not contain the codons for the first 6 amino acids and had a long 3'UTR as well. The pEGFP-C1-PIN1 contstruct showed poor expression in transient transfection experiments as well. The complete cDNA of TIP60β and PIN1 were generated by PCR amplification using linker-adaptor primers (TIP60ATGXhol and TIP60B1765EcoRI; PIN1ATGXhol and PIN1B498 EcoRI) from the original Y2H clone (pGAD-GH vector). The primers also contained the sequences coding for the first 5 amino acids of TIP60β and the first 6 amino acids of PIN1 and a Kozak consensus site. The PCR fragments were

cloned into the EcoRI (5' site) and Sal1 (3' site) of the pEYFP-N1 vector. The 3' primers were designed so that the YFP coding sequence of pEYFP-N1 would be in frame with TIP60β and PIN1. The expression of the fusion proteins TIP60β and PIN1 with YFP were observed using fluorescence microscopy after transient transfection experiments (Fig. 4.1). The proteins were also detected in Western blot experiments. (Fig. 4.2).

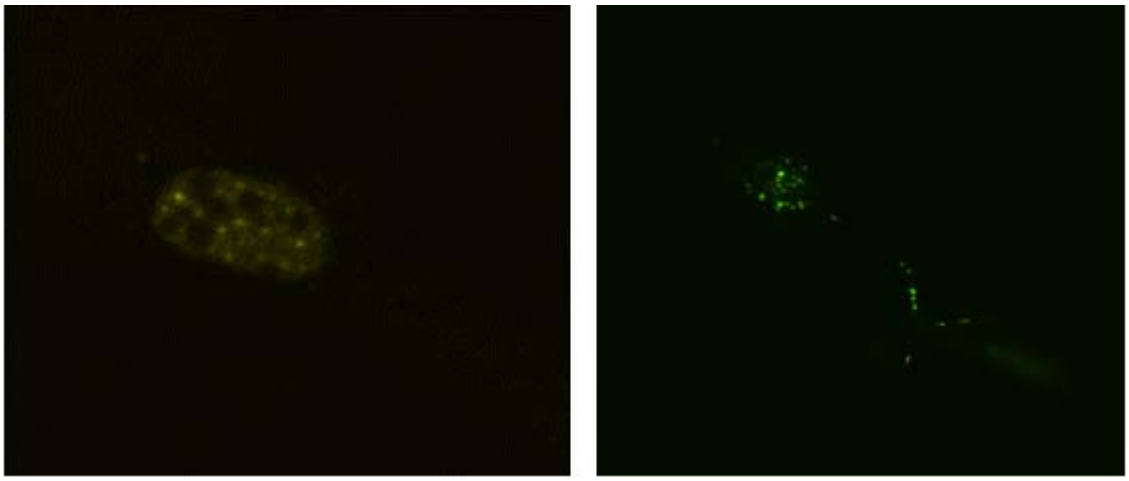
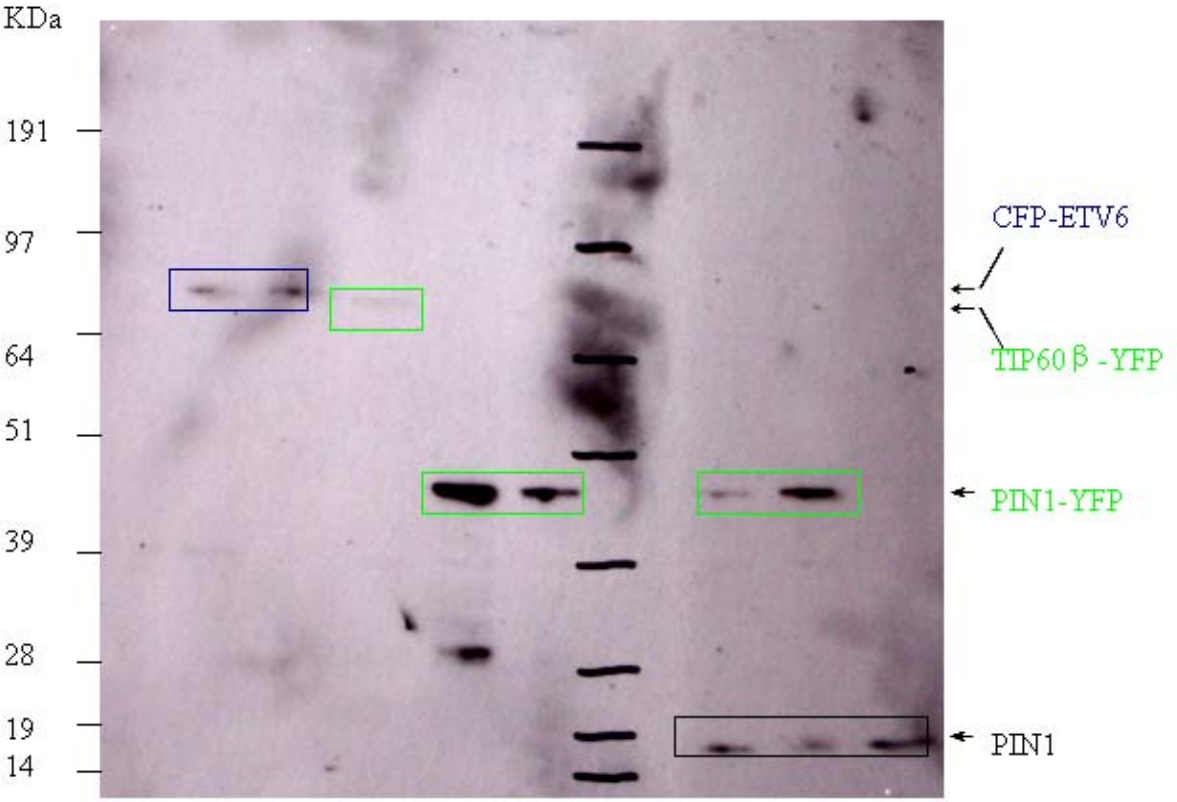


Fig. 4.1 Expression TIP60β-YFP and PIN1-YFP fusion proteins in mammalian cells. 2 µg of pEYFP N1 TIP60β or pEYFP N1 PIN1 plasmid DNA was transfected into 2 x 10^6 mouse NIH3T3 cells. The transfected cells were fixed after 48 hours and observed with a fluorescence microscope. The picture was taken with an excitation and emission filter set, which is specific for YFP (yellow fluorescent protein). The pictures were taken by a CCD camera controlled by Openlab software. The left picture shows the TIP60β-YFP protein in the nucleus of a transfected cell. The right picture shows that the PIN1-YFP protein is localized to both nucleus and cytoplasm. The pictures were acquired as grey scale images by the CCD camera and pseudo-colored in OpenLab to reflect the color of the two fluorescences as seen through the microscope.



1	2	3	4	5	6	7	8	9	10
293T	CFP-ETV6	CFP-ETV6	TIP60β-YFP	PIN1-YFP	PIN1-YFP	Protein standrad	PIN1-YFP	PIN1-YFP	293T
Anti-GFP antibody							Anti-PIN1 antibody		

Fig. 4.2 TIP60β and PIN1 protein expression in mammalian cell. 2 µg of pEYFP N1 TIP60β or pEYFP N1 PIN1 were transfected into 2 x 10^6 human 293T cells. The cells were lysated with RIPA buffer. 10% of the cell lysate was loaded on a 10% PAGE gel and transferred to a nitrocellular membrane after electrophoresis. Rabbit Anti-GFP or anti-PIN1 antibodies were used as primary antibodies. The secondary AB was anti-rabbit-HRP. Plasmids used for transfection are shown below the lanes. The expression CFP-ETV6 was used as a positive control for the anti-GFP antibody, lane 4 shows a weak TIP60β-YFP band. Lane 5 and lane 6 show the PIN1-YFP bands, which serve as the positive control for the anti-PIN1 antibody. Lane 8 and 9 show the PIN1-YFP (upper) and native PIN1 (lower) bands.

4.1.2 Deletion mutants of ETV6

To map the PIN1 and TIP60β interaction domains of ETV6 two deletion mutants of ETV6 were constructed in the yeast expression vector pGBT9. From this vector, proteins are expressed as fusions with the GAL4-DNA binding domain. The deletion mutant ETV6(314-342) interacted with both TIP60β and PIN1. However, ETV6(314-342) was found to have a one base pair deletion at aa 337 which caused a frame shift in the remaining 5 amino acids. To clarify whether PIN1 and TIP60β interact with the sequence between aa 314 and 336 of ETV6 or the frame shifted sequence from aa 337 to 342, two new constructs were made: one expressing ETV6 aa 314 to 336 and the other expressing ETV6 aa 314 to 342 (without frame shift). So the DNA sequences encoding ETV6 amino acids 314-336 and 314-342 were amplified by PCR with linker adaptor primers containing an EcoRI restiction enzyme site in the forward primer and a SalI restriction enzyme site in the backward primered by following a stop codon (Tel 964 Eco-T, ETV6 1032B SAL1, ETV6 1047B SAL1). The PCR products were cloned into the EcoRI and SalI site of pGBT9. The sequence of these constructs was verified by sequencing across the cloning sites.

4.1.3 Site-directed mutagenesis of ETV6 and TIP60β

The conserved amino acids Gly (aa 380) and Leu (aa 385) in the acetyl co-enzyme A binding site of TIP60β were changed into aspartic acid and serine, respectively, by site-directed mutagenesis (A of Table 4.1). The resulting TIP60β-DRS mutant has no acetyltransferase activity (HAT dead mutant) (Gavaravarapu and Kamine, 2000).

It was shown that ETV6 is phosphorylated by the p38 kinase at Ser257 (Arai et al., 2002). We found that ETV6 has two potential PIN1 interaction domains: one in the *pointed* domain, the other in the central domain of ETV6. It is known that PIN1 interacts specifically with Ser-Pro or Thr-Pro sites which are phosphorylated at the serine or threonine. In B of table 4.1, those S-P or T-P motifs in ETV6 that are potential PIN1 binding motifs are underlined. We chose to mutate the serines at positions 106, 257 and 332, which were the most likely targets for PIN1 binding, into alanines. Alanines can not be phosphorylated. The introduced mutations were confirmed by sequencing. The mutated constructs are summarized in table 4.1.

Table 4.1 Mutated constructs of ETV6 and TIP60β

A.

Point mutations in the conserved acetyltransferase motif of the TIP60

	Acetyl Co-enzyme A binding site
TIP60α	YN**VACILTLPPYQRRGYRKLLIEF**
TIP60β DRS	YN**VACILTLPPYQRRDYRKLSIEF**

B.

Amino Acid sequence of ETV6

MSETPAQCSIKQERISYTPPESPVPSYASSTPLHVPVPRALRMEEDSIRL
PAHLRLQPIYWSRDDVAQWLKWAENEFSLRPIDSNTFEMNGKALLLLTKE
DFRYRSPHSGDVLYELLQHILKQRKPRILFSPFFHPGNSIHTQPEVILHQ
NHEEDNCVQRTPRPSVDNVHHNPPTIELLHRSRSPITTNHRPSPDPEQRP
LRSPLDNMIRRLSPAERAQGPRPHQENNHQESYPLSVSPMENNHCPASSE
SHPKPSSPRQESTRVIQLMPSPIMHPLILNPRHSVDFKQSRLSEDGLHRE
GKPINLSHREDLAYMNHIMVSVSPPEEHAMPIGRIADCRLLWDYVYQLLS
DSRYENFIRWEDKESKIFRIVDPNGLARLWGNHKNRTNMTYEKMSRALRH
YYKLNIIRKEPGQRLLFRFMKTPDEIMSGRTDRLEHLESQELDEQIYQED
EC SAM/pointed domain(38-123) ETS domain(338-422)

C.

Source plasmids	Insert	Mutated sites	Name of mutant
pECFP-C1-ETV6	ETV6	S106A	pECFP-C1-ETV6(S106A)
pECFP-C1-ETV6	ETV6	S257A	pECFP-C1-ETV6(S257A)
pECFP-C1-ETV6	ETV6	S323A	pECFP-C1-ETV6(S323A)
pM1- ETV6	ETV6	S323A	pM1-ETV6(S323A)
pEYFP-N1-TIP60β	TIP60β	G380D, L385S	pEYFP-N1-TIP60β(DRS)
pGAD-GH-TIP60β	TIP60β	G380D, L385S	pGAD-GH-TIP60β(DRS)

4.2 Interaction of ETV6 with TIP60β and PIN1

The interaction between ETV6, its deletion mutants and TIP60β and its HAT domain mutants were performed in the yeast two-hybrid system. This method was also used for assaying the interaction between ETV6 (including the various deletion mutants of ETV6) and PIN1 and its various mutants.

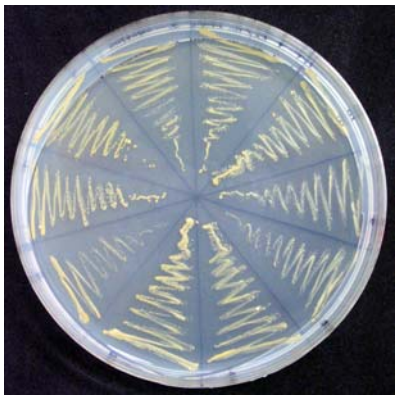
4.2.1 Interaction between ETV6 and TIP60β and the TIP60β HAT mutant

In previous work in our group (performed by Jasmina Putnik and Michael Kickstein) the interaction between ETV6 and TIP60β had been analyzed in

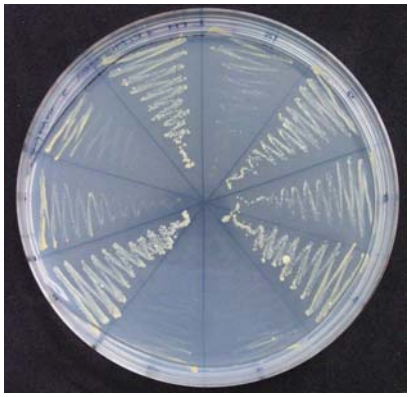
some detail using the yeast two hybrid system. It was shown that the full length TIP60β is able to interact with full length ETV6, the central domain of ETV6(aa118-345) and the C-terminal portion of the central domain (aa282-345). Here these findings were extended to show that the 25 amino acids from 314 to 336 of ETV6 are sufficient for interaction with full length TIP60β (Fig. 4.3).

Previously, we had also shown that deletion mutants of TIP60β were unable to interact with full length ETV6. However, these deletion mutants were able to interact with the C-terminal portions of the ETV6 central domain. In fact, we could show that the MYST domain zinc finger of TIP60β was sufficient for interaction with these C-terminal portions of the ETV6 central domain. To explain this remarkable interaction pattern between the ETV6 and TIP60β deletion mutants, we hypothesized that interaction between ETV6 and TIP60β was a two step process: In a first step a ETV6 might be acetylated by TIP60β which would result in a confirmational change of ETV6; then the zinc finger of the TIP60β MYST domain would be able to interact with an exposed interaction interface in the ETV6 central domain. To test this hypothesis, the TIP60β DRS mutant, which lacks acetyltransferase activity, was tested for interaction with ETV6 and several ETV6 deletion mutants in the yeast two hybrid systems. Interestingly, the TIP60β-DRS mutant was not able to

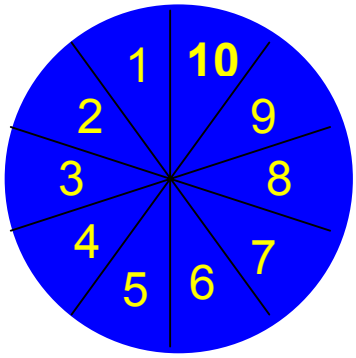
interact with full length ETV6 or with the central domain (aa118-345) of ETV6. However, the HAT deficient TIP60β mutant was still able to interact with the C-terminal half (aa219-345) and quarter (aa 282-345) of the the central domain of ETV6 (Fig 4.4). This is exactly the same interaction pattern which was seen with the TIP60β deletion mutants including the mutant containing only the TIP60β MYST domain zinc finger. These observations agree with what would be expected if the HAT activity of TIP60β is needed to change the conformation of ETV6 to allow efficient interaction.

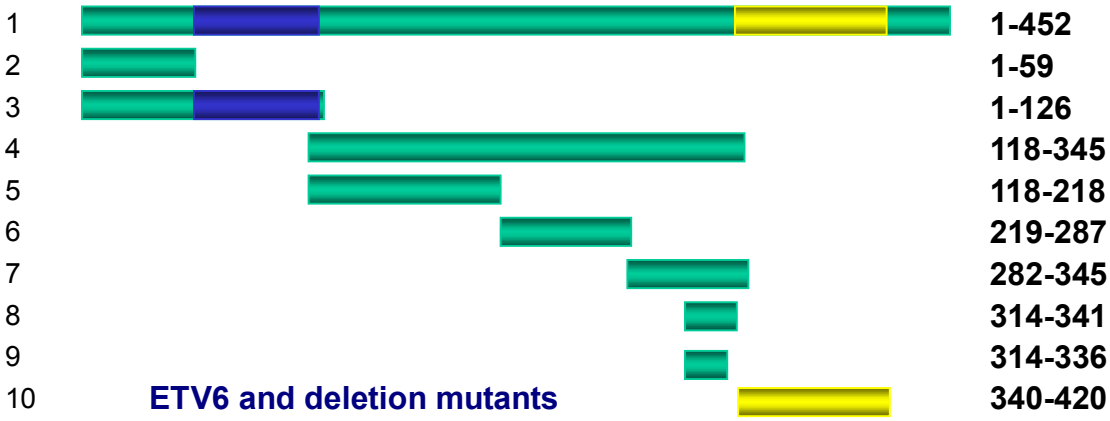


-L-W



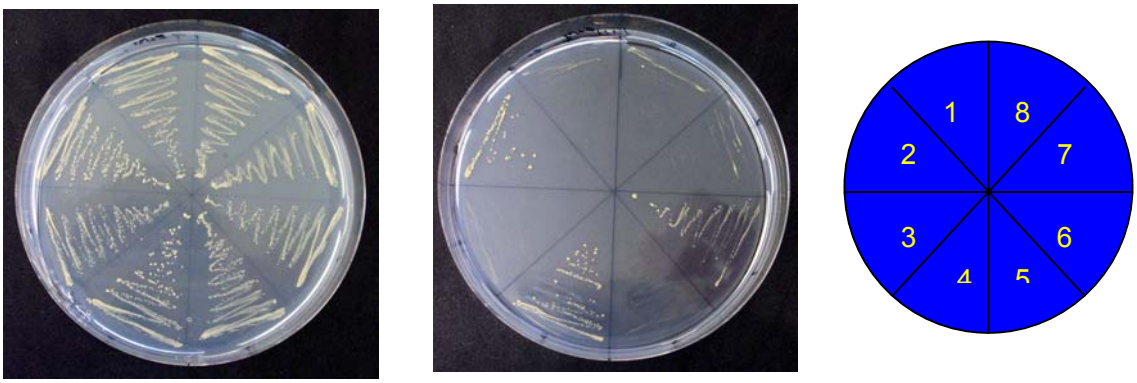
-L-W-H





No.	ETV6 or deletion mutant expressing plasmid	TIP60β expressing plasmid	Interaction
1	pGBT9-ETV6	PGAD-GH-TIP60β	+
2	pGBT9-ETV6 (1-59)	PGAD-GH-TIP60β	-
3	pGBT9-ETV6 (1-126)	PGAD-GH-TIP60β	-
4	pGBT9-ETV6 (118-345)	PGAD-GH-TIP60β	+
5	pGBT9-ETV6 (118-218)	PGAD-GH-TIP60β	-
6	pGBT9-ETV6 (219-287)	PGAD-GH-TIP60β	-
7	pGBT9-ETV6 (282-345)	PGAD-GH-TIP60β	+
8	pGBT9-ETV6 (314-341)	PGAD-GH-TIP60β	+
9	pGBT9-ETV6 (314-336)	PGAD-GH-TIP60β	+
10	pGBT9-ETV6 (340-420)	PGAD-GH-TIP60β	-

Fig. 4.3 Mapping of the TIP60β interaction domain of ETV6. Yeast strain CG1945 was cotransformed with a pGBT9 plasmid expressing ETV6 or the indicated ETV6 mutants as a fusion protein with the GAL4-DBD and with pGAD-GH-TIP60β, which expressed TIP60β as a fusion protein with the activation domain of GAL4 (GAL4-AD). The plate on the left hand side lacks amino acids tryptophan and leucine and allows only growth of cotransformants. The plate on the right hand side which is also lacking the amino acid histidine only allows growth of cotransformants in which interaction between the GAL4-DBD and the GAL4-AD fusion proteins occurs. Clones no. 1, 4, 6, 8 and 9 show growth on the left hand plate indicating interaction between TIP60β and the following portions of ETV6: full length (no. 1), ETV6(118-345) (no. 4), ETV6(282-345) (no. 7), ETV6(314-341) (no. 8) and ETV(314-336) (no. 9). The plates were assayed after 4 days of incubation at 30°C.



-L-W -L-W-H

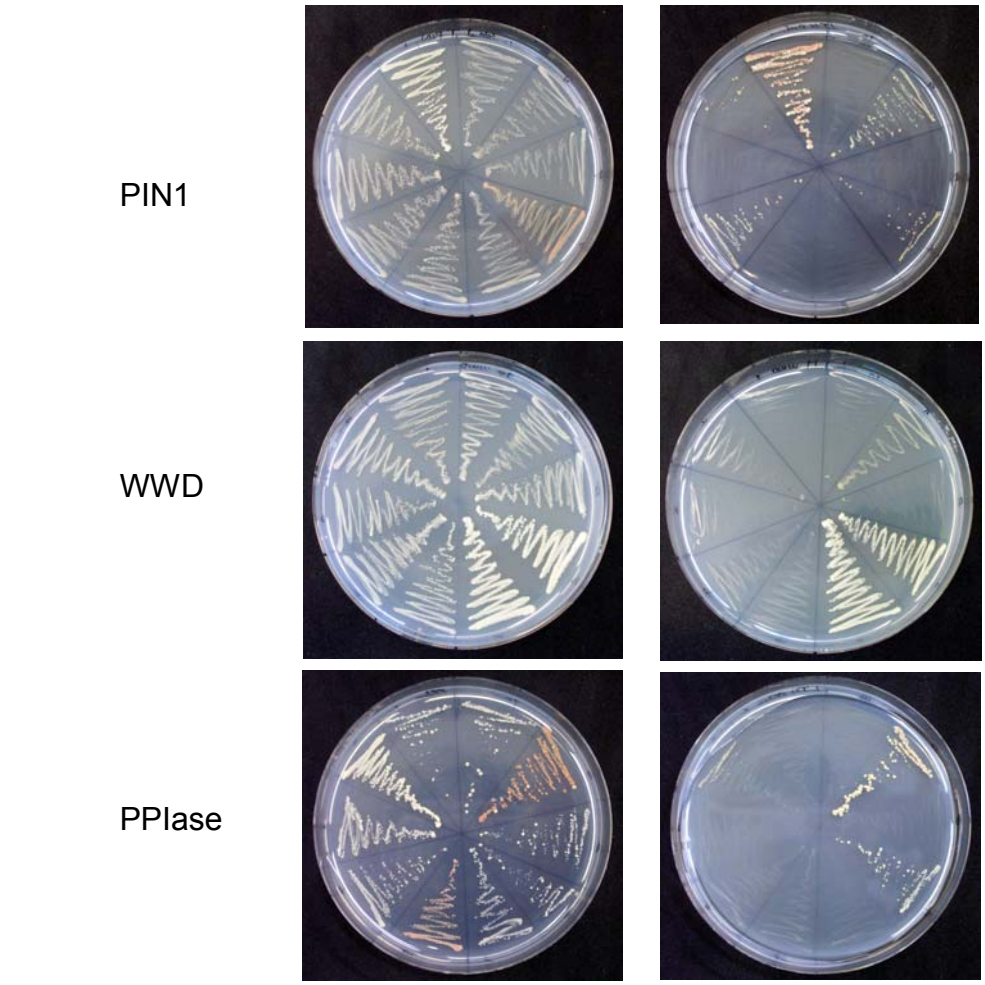
No.	ETV6 or deletion mutant expressing plasmid	TIP60β-DRS expressing plasmid	Interaction
1	ETV6	pGAD-GH-TIP60βDRS	-
2	ETV6 (118-345)	pGAD-GH-TIP60βDRS	+
3	ETV6 (118-218)	pGAD-GH-TIP60βDRS	-
4	ETV6 (219-345)	pGAD-GH-TIP60βDRS	+
5	ETV6 (219-287)	pGAD-GH-TIP60βDRS	-
6	ETV6 (282-345)	pGAD-GH-TIP60βDRS	+
7	ETV6 (314-341)	pGAD-GH-TIP60βDRS	-
8	ETV6 (314-336)	pGAD-GH-TIP60βDRS	-

Fig. 4.4 Interaction between ETV6 and the HAT deficient TIP60β mutant TIP60β DRS. The same cotransformation assay in yeast strain CG1945 as in Fig. 4.3 was performed. This time the HAT deficient TIP60β DRS mutant was expressed as fusion protein with the GAL4-AD. The left plate shows the transformation control and the right hand plate is the assay for protein-protein interaction. Interestingly, there is no interaction of the HAT deficient TIP60β protein with full length ETV6 or with the N-terminal of central domain of ETV6 (aa118-218). However, the HAT deficient TIP60β can still interact with the central domain of ETV6, C terminal half and the C-terminal quarter of the central domain (No. 2, No.4 and No. 6). The plates were assayed after 4 days of incubation at 30°C.

4.2.2 Interaction between ETV6 and PIN1

The yeast two-hybrid system was used as described above to analyze the

interaction between ETV6 and PIN1. Full length PIN1 interacted with full length ETV6, the N terminus of ETV6 (ETV6(1-59), ETV6(1-126)), and the central area of ETV6, ETV6(219-345) and ETV6(282-345). The WW domain of PIN1 interacted with the isolated N-terminal and C-terminal halves of central domain of ETV6, ETV6(118-218) and ETV6(219-345) but not with the complete central domain (118-345). The PPIase domain of PIN1 showed interaction with the C-terminal half (219-345) and the C-terminal quarter (282-345) of the central domain of ETV6 (Fig. 4.5). These results implied that there might be two potential binding sites for PIN1 on ETV6: one in the N-terminus of ETV6 including the *pointed* domain (aa1-126) and one in the C-terminal half of the central region (aa 282-345). The fact that the isolated WW domain is also interacting with the N-terminus of the central domain might be an artefact of the assay due to interaction of the WW domain with proline rich regions. Similar to the interaction pattern observed with ETV6 and TIP60β, the isolated PIN1 domains are not able to interact with full length ETV6. This might be explained by conformational changes that are necessary for efficient interaction to occur for which both the WW and the PPIase domains of PIN1 are required.



ETV6 and deletion mutants

		WW–PPIase	WW	PPIase
1	1-452	+++	--	--
2	1-59	±	--	±
3	58-126	–	–	–
4	1-126	++	--	--
5	118-345	–	–	–
6	118-218	–	++	--
7	219-345	++	++	++
8	219-287	–	–	–
9	282-345	++	--	++
10	340-420	–	–	–

No.	GAL4-DBD Fusion	GAL4-AD fusion PIN1	GAL4-AD fusion PIN1 WWD	GAL4-AD fusion PIN1 PPIase
1	ETV6	+++	-	-
2	ETV6 (1-59)	±	-	±
3	ETV6 (58-126)	-	-	-
4	ETV6 (1-126)	++	-	-
5	ETV6 (118-345)	-	-	-
6	ETV6 (118-218)	-	++	-
7	ETV6 (219-345)	++	++	++
8	ETV6 (219-287)	-	-	-
9	ETV6 (282-345)	++	-	++
10	ETV6 (420-452)	-	-	-

+ denotes growth on plates lacking tryptophan, leucine, and histidine (SD, –W, -L, -H)

Fig. 4.5 Analysis of ETV6 – PIN1 interaction. As in figures 4.3 and 4.4 yeast strain CG1945 was co-transformed with a plasmid expressing ETV6 or one of nine ETV6 deletion mutants as a GAL4-DBD fusion protein and with a plasmid expressing full length PIN1, the WW domain of PIN1 or the PPIase domain of PIN1 as a fusion protein with the GAL4-AD. Interaction between the proteins was assayed by growth on SD plates lacking histidine, tryptophane and leucine. See text for description of results. The plates were assayed after 4 days of incubation at 30°C.

4.3 Subcellular co-localization of ETV6 with TIP60β and PIN1

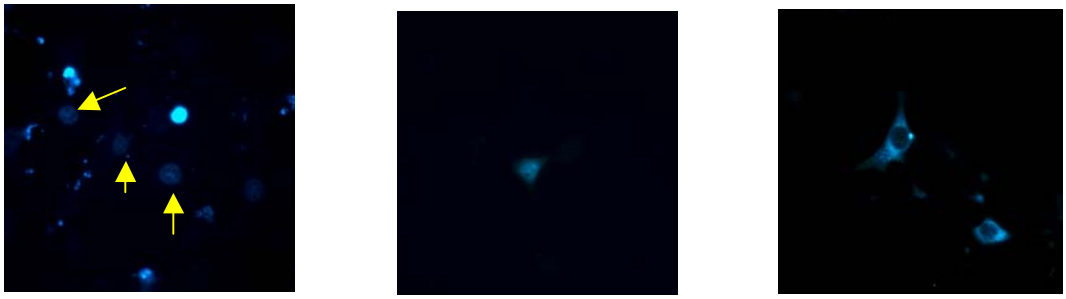
4.3.1 Subcellular localization of ETV6, TIP60β and PIN1

4.3.1.1 Subcellular localization of ETV6

To examine the subcellular localization of ETV6, a CFP-ETV6 fusion protein

was expressed in mouse fibroblast cells (NIH3T3) after transient transfection. The transfected cells were observed with fluorescence microscopy (Zeiss). Both nuclear and cytoplasmic localization of CFP-ETV6 were observed with some cells exhibiting exclusively nuclear localization of CFP-ETV6 while others had both nuclear and cytoplasmic staining. There appeared to be some correlation between the expression level of the CFP-ETV6 fusion protein and its subcellular localization. In cells with moderate to low expression CFP-ETV6 was localized predominantly in the nucleus. The nuclear staining with CFP-ETV6 was finely granular and spared the nucleoli (Fig. 4.6A). When the CFP-ETV6 fusion protein was expressed at high levels, it would localize in the cytoplasm as well (Fig. 4.6A). It appeared as if there is a certain limit as to how much CFP-ETV6 can be accommodated in the nucleus. If that limit is exceeded any additional protein would remain in the cytoplasm of the cell. However, we do not have firm proof that the expression level of CFP-ETV6 is the only factor that determines its subcellular distribution.

A.



B

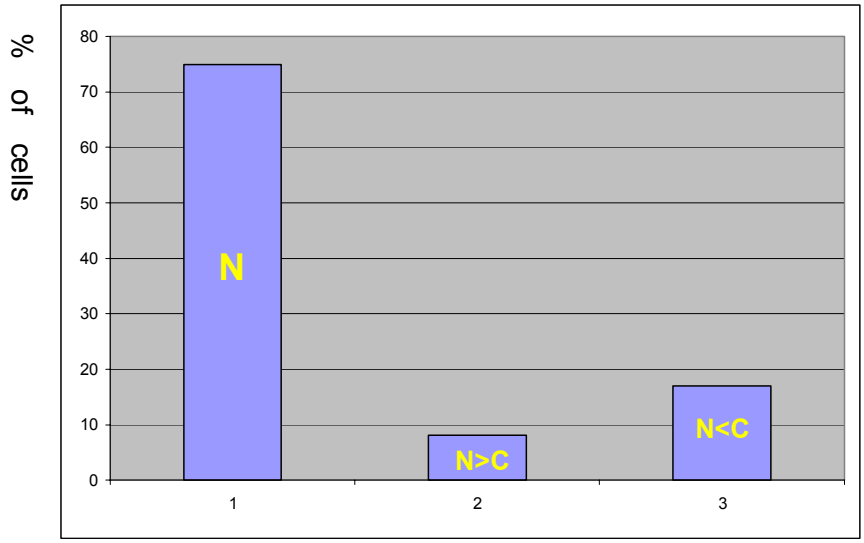
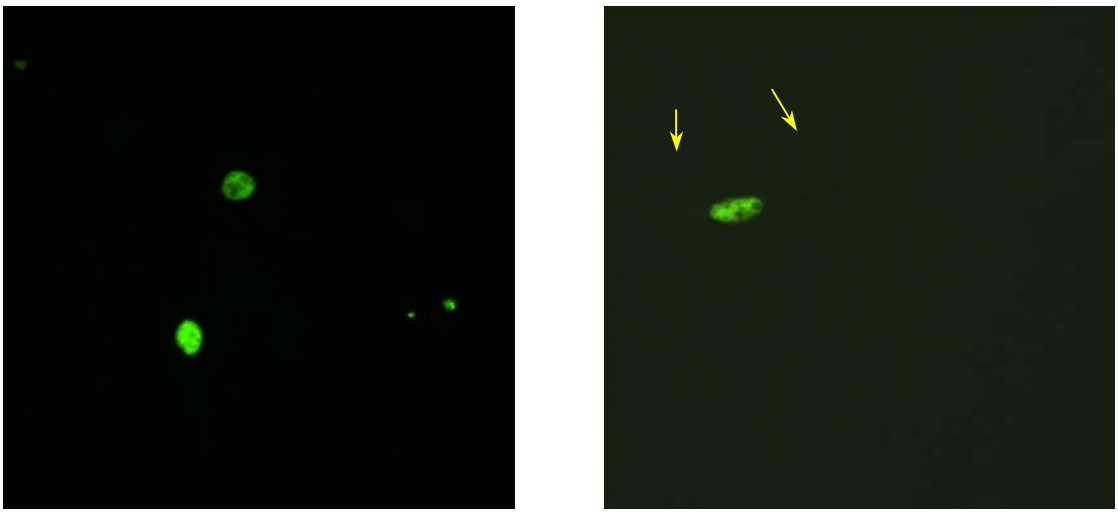


Fig. 4.6 Subcelluar localization of CFP-ETV6 in murine fibroblast cells. **A:** NIH3T3 cells were transiently transfected with a CFP-ETV6 expressing plasmid. The upper pictures were taken with a 400x objective with a filter combination to allow CFP visualization. The exposure time for the left picture is 1000 ms and shows most of the CFP-ETV6 protein localized in the nucleus. The middle picture has an exposure time of 400 ms and shows CFP-ETV6 with predominant nuclear localization. The right picture was taken at 150 ms exposure time and shows most of the CFP-ETV6 localized in the cytoplasm. There is still CFP-ETV6 in the nucleus but it cannot be seen very well at this short exposure time. Arrows point to CFP-ETV6 in nucleus. The pictures were acquired as grey scale images by the CCD camera and pseudo-colored in OpenLab to reflect the color of the two fluorescences as seen through the microscope. **B:** Distribution of transfected cells showing ETV6 in the nucleus only (N), more ETV6 in the nucleus than in the cytoplasm (N>C) and more ETV6 in the cytoplasm than in the nucleus (N<C). Approximately 100 transfected cells were evaluated and counted.

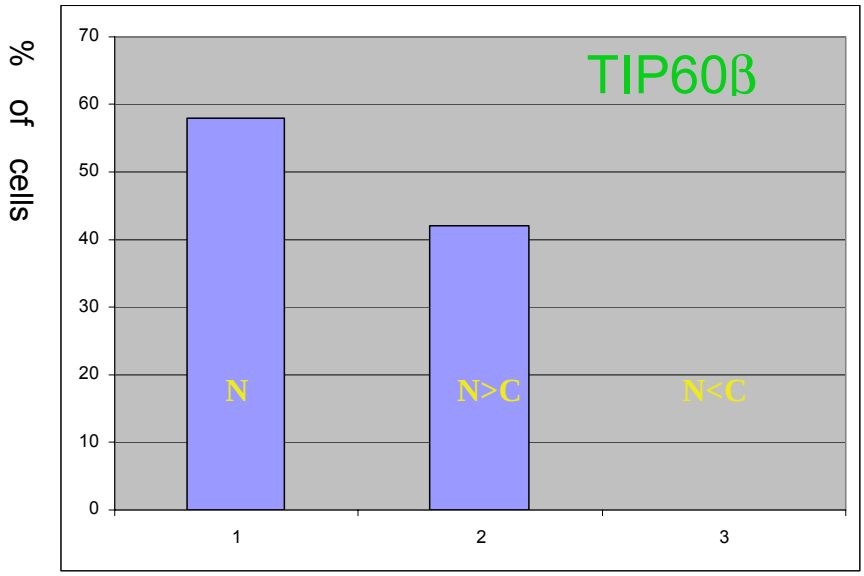
4.3.1.2 Subcellular localization of TIP60β

The subcellular localization of TIP60β was examined by expressing a TIP60β yellow fluorescent protein (YFP) fusion protein in NIH3T3 cells. In most cells, TIP60β-YFP was observed exclusively in the nucleus. Only in some cells with very high expression of TIP60β-YFP a small amount of TIP60β-YFP could be observed in the cytoplasm (Fig. 4.7).

A.



B.



Nucleus (N), cytoplasm (C)

TIP60β-YFP, 400x, YFP channel, Openlab, NIH3T3 cell line

Nucleus (N), cytoplasm (C)

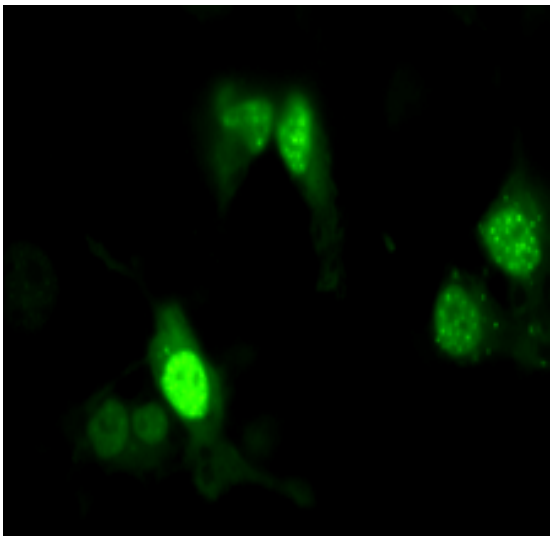
Fig. 4.7 Subcelluar localization of TIP60β. **A:** NIH3T3 cells were transiently transfected with a plasmid expressing TIP60β-YFP. The pictures were taken with a 40x objective (final magnification about 400x) and an excitation and emission filter set for YFP fluorescence. The picture on the left side shows TIP60β-YFP in the nucleus. The picture on the right side shows some TIP60β-YFP in the cytoplasm in a cell with a high level of TIP60β-YFP expression (arrow points to TIP60β-YFP in the cytoplasm). The pictures were acquired as grey scale images by the CCD camera and pseudo-colored in OpenLab to reflect the color of the two fluorescences as seen through the microscope. **B**: Distribution of transfected cells showing TIP60β-YFP in the nucleus only (N) and cells with some TIP60β-YFP in the cytoplasm (N>C). Approximately 100 transfected cells were evaluated and counted.

4.3.1.3 Subcellular localization of PIN1

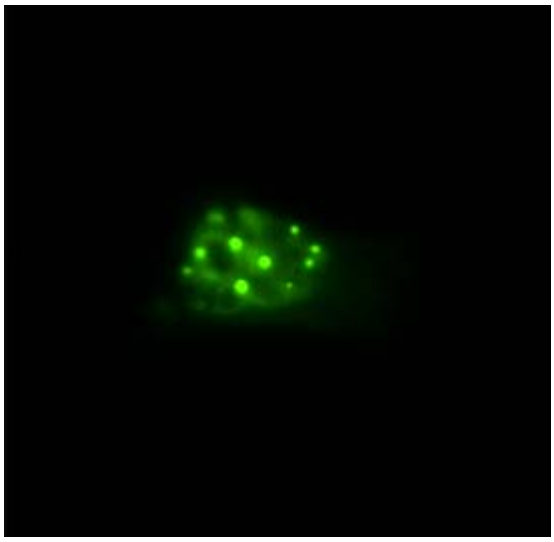
The subcellular localization of PIN1 was assayed in the same way as for ETV6 and TIP60β. An N-terminal PIN1-YFP fusion protein was expressed transiently in NIH3T3 cells. PIN1-YFP was always found in the nucleus of

transfected cells with most cells showing also some PIN1-YFP in the cytoplasm. The nuclear distribution of PIN1-YFP was diffuse with sparing of the nucleoli. In some nuclei foci of PIN1-YFP were observed (Fig. 4.8). There was some correlation with the amount of PIN1-YFP expression in a cell and its appearance in the cytoplasm (Fig. 4.8. C).

A.



B.



C.

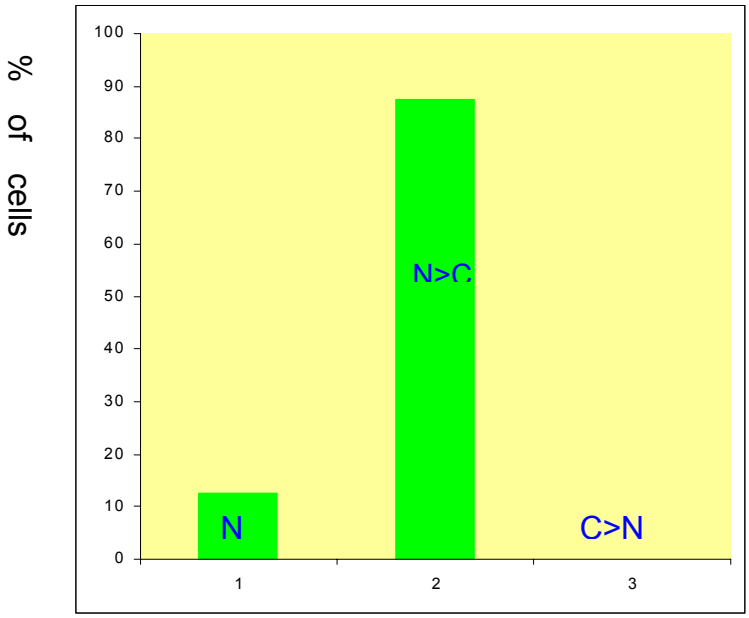


Fig. 4.8 Subcelluar localization of PIN1. **A:** NIH3T3 cells were transiently transfected with a plasmid expressing PIN1-YFP. The pictures were taken with a 40x objective (final magnification about 400x) and an excitation and emission filter set for YFP fluorescence. The picture on the left hand side shows PIN1-YFP in the nucleus and cytoplasm. These cells have a rather high level of PIN1-YFP expression. **B:** The picture on the right side shows a cell with almost exclusive nuclear distribution of PIN1-YFP. Nuclear foci of PIN1-YFP can be observed in this cell. The pictures were acquired as grey scale images by the CCD camera and pseudo-colored in OpenLab to reflect the color of the two fluorescences as seen through the microscope. **C:** Distribution of transfected cells showing PIN1 in the nucleus only (N) and cells with some PIN1 in the cytoplasm (N>C). Approximately 100 transfected cells were evaluated and counted.

4.3.2 Co-localization of ETV6 with TIP60β and PIN1

4.3.2.1 Co-localization of ETV6 and TIP60β

To further confirm the interaction between ETV6 and TIP60β, colocalization experiments with fluorescent protein tagged ETV6 and TIP60β were carried out using transient transfection in murine fibroblast cells. CFP and YFP as described above were used as tags for ETV6 and TIP60β, respectively. These colors were chosen for the colocalization experiments because there is no spectral overlap between these colors with the excitation and emission filter sets available for these colors on our microscope. These experiments showed that CFP-ETV6 and TIP60β-YFP have comparable distribution in the nucleus (Fig. 4.9, left column). Also, in cells with high levels of CFP-ETV6 expression and predominant cytoplasmatic localization of CFP-ETV6 there

was still colocalization of CFP-ETV6 and TIP60β-YFP in the nucleus (Fig. 4.9 middle and right column). These observed colocalizations are in agreement with the data from the yeast two hybrid experiments (Fig. 4.3) and the GST-pull-down experiment (Putnik, Ph.D. thesis, 2001). To examine the colocalization of ETV6 and TIP60β in the nucleus in more detail, volume deconvolution of Z-stack images (Z spacing 0.2 μm) taken from co-transfected cells was performed. Deconvolution is a mathematical operation that allows the partial removal of light from out-of focus objects, resulting in images that resemble those obtained with a confocal microscope. In figure 4.10 the results of such a deconvolution performed separately for the CFP and YFP channel from a single cell are shown. It can be clearly seen that the CFP-ETV6 pattern matches almost exactly that of TIP60β-YFP.

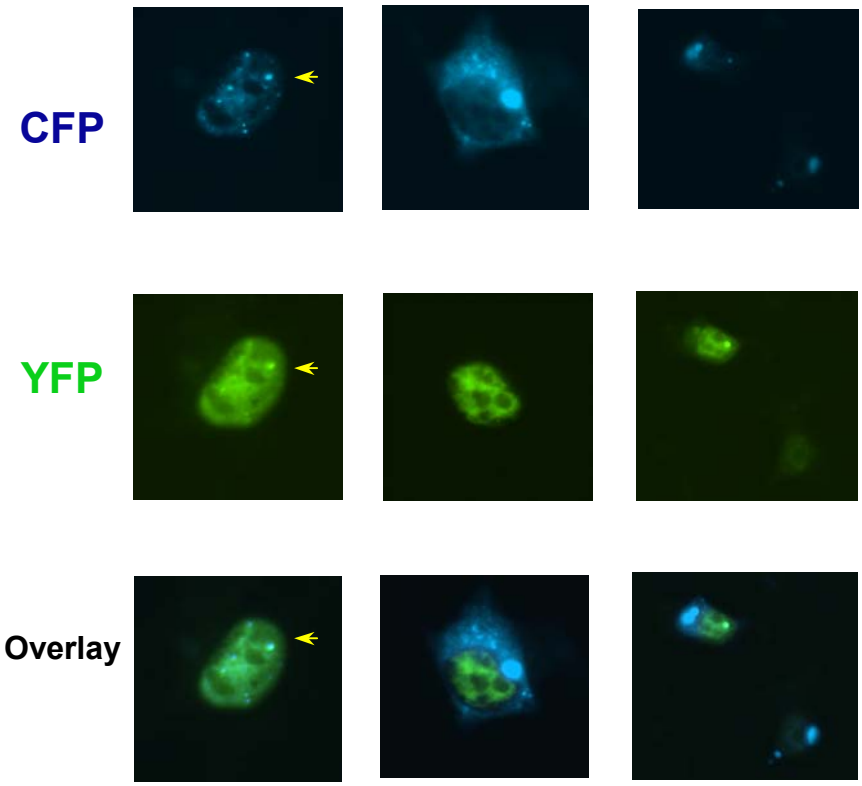
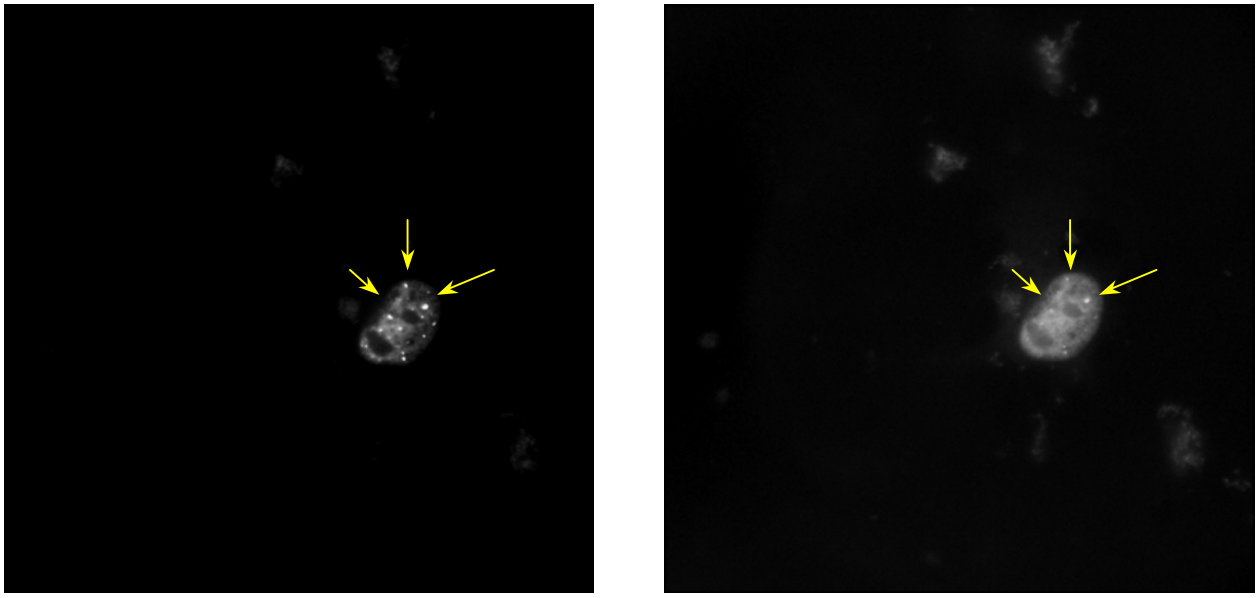


Fig. 4.9 Co-localization of ETV6 and TIP60β in NIH3T3 cells. NIH3T3 cells were transiently co-transfected with plasmids expressing CFP-ETV6 and TIP60β-YFP. The pictures were taken with a 100x objective (final magnification about 1000x). Three different cells are shown (left, middle and right column). The pictures in the upper row were taken with an excitation and emission filter set for CFP fluorescence and the pictures in the middle row were taken with an excitation and emission filter set for YFP fluorescence. The third row shows an overlay of the images from the CFP (blue) and YFP (yellow) images. The pictures were acquired as grey scale images by the CCD camera and pseudo-colored in OpenLab to reflect the color of the two fluorescences as seen through the microscope.



CFP- ETV6 (CFP channel) **TIP60β -YFP (YFP channel)**

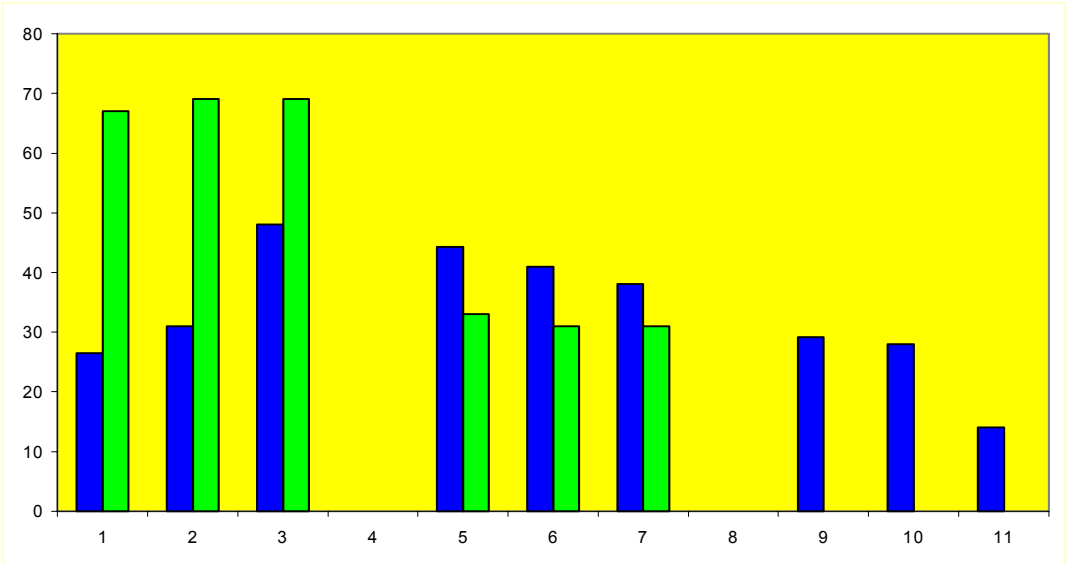
DECONVOLUTED IMAGES

Fig. 4.10 Deconvoluted images of the CFP (left side) and the YFP (right side) channel from a section through an NIH 3T3 cell nucleus contransfected with CFP-ETV6 and TIP60β-YFP expressing plasmids. The images were taken with a 100x objective. It is quite evident that the pattern of nuclear CFP-ETV6 and TIP60β-YFP distribution is almost identical. Arrows point to three prominent bright spots, which are seen in both fluoresecence channels.

4.3.2.2 Coexpression of TIP60β affects subcellular localization of ETV6

When we performed the colocalization studies of ETV6 and TIP60β, we noticed that the co-expression of TIP60β seemed to influence the distribution of ETV6 in the same cell. To examine this phenomenon on a semi-quantitative basis, we varied the ratio of CFP-ETV6 to TIP60β-YFP

expressing plasmids in three different co-transfections and analyzed the nuclear and cytoplasmic distribution of CFP-ETV6 and TIP60β-YFP. We used the following ratios of CFP-ETV6 to TIP60β-YFP: 3:1, 1:1, 1:7. While the relative distribution of cells expressing TIP60β-YFP in the nucleus only (N) to those cells expressing TIP60β-YFP in the nucleus and less in the cytoplasm (N>C) did not change in the different experiments, there was a marked change in the distribution of CFP-ETV6 with a shift towards more cells showing only nuclear localization of CFP-ETV6 the more TIP60β-YFP was used in the transfection experiment (Fig 4.11). This implies that TIP60β is able to influence the subcellular localization of ETV6.



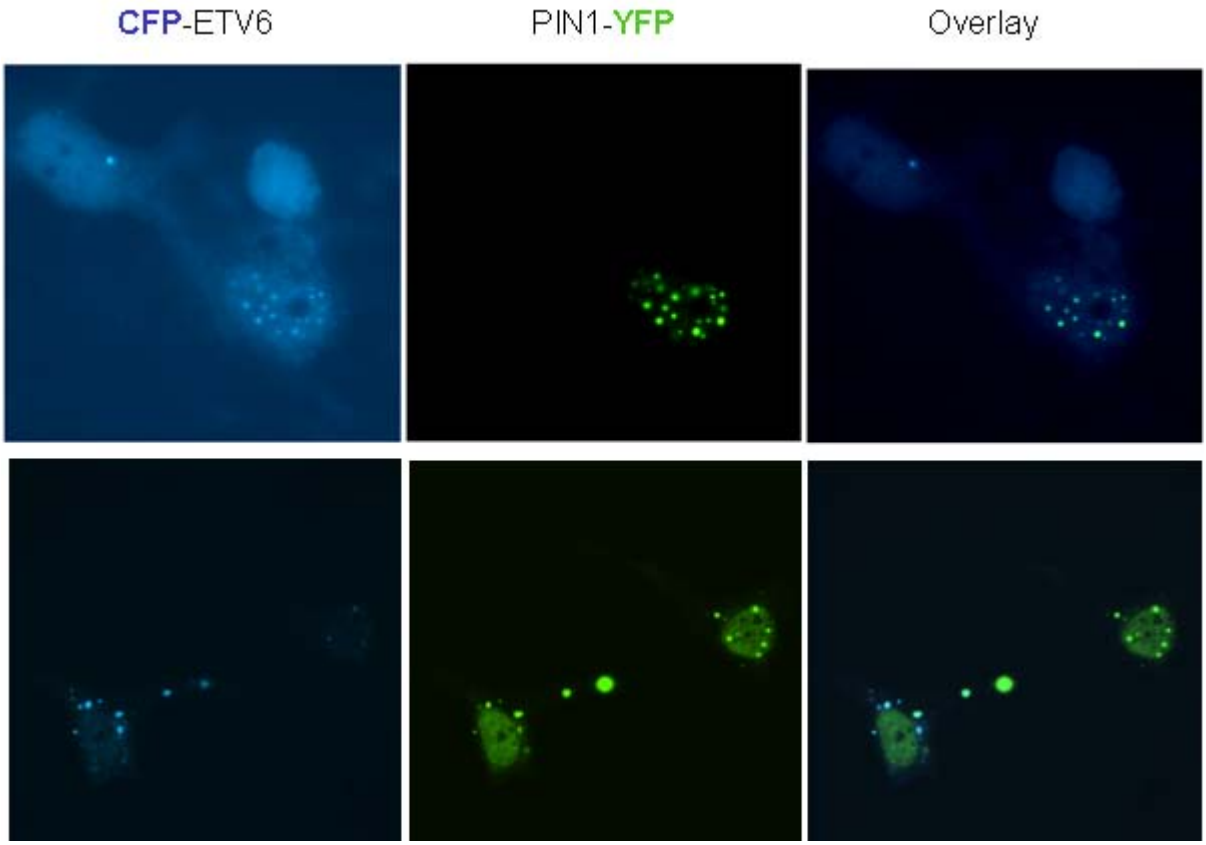
	N (Nucleus)				N>C (Cytoplasm)				C >N		
No.	1	2	3	4	5	6	7	8	9	10	11
CFP-ETV6	3	1	1	0	3	1	1	0	3	1	1
TIP60β-YFP	1	1	7	0	1	1	7	0	1	1	7

Fig. 4.11 TIP60β-YFP affects the subcellular localization of CFP-ETV6. The pECFP-C1-ETV6 plasmid and the pEYFP-N1-TIP60β plasmids were cotransfected at different ratios (3:1, 1:1 and 1:7) into NIH3T3 cells. It can be seen that there is a shift towards more nuclear localization of CFP-ETV6 as the amount of TIP60β-YFP expressing plasmid increases. About 100 cells co-expressing both fusion proteins were counted in each experiment. Percentages for CFP-ETV6 are represented as blue bars, percentages for TIP60β-YFP are shown as green bars.

4.3.2.3 Co-localization of ETV6 and PIN1

To examine whether ETV6 would colocalize with PIN1, the plasmids pECFP-C1-ETV6 and pEYFP-N1-PIN1 were co-transfected into NIH3T3 cells. As can be seen in figure 4.12 both proteins show a very similar nuclear distribution pattern. In addition, PIN1-YFP is found in the cytoplasm in more than 80% of the cells examined. The similarity of the nuclear distribution of CFP-ETV6 and PIN1-YFP strongly suggests that both proteins physically interact *in vivo*.

A.



B.

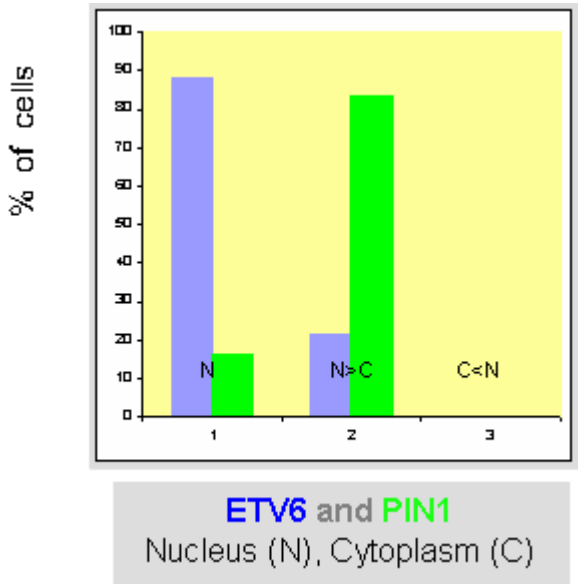


Fig. 4.12 Co-localization of ETV6 and PIN1 in NIH3T3 cells. **A**: NIH3T3 cells were transiently co-transfected with plasmids expressing CFP-ETV6 and PIN1-YFP. The

pictures were taken with a 63x objective (final magnification about 630x). The left image was taken with an excitation and emission filter set for CFP fluorescence and the the middle image was taken with an excitation and emission filter set for YFP fluorescence. The right image shows an overlay of the CFP (blue) and YFP (yellow) images. CFP-ETV6 and PIN1-YFP colocalize in nucleus where both proteins show a very similar pattern of bright speckles. **B**: Diagram showing distribution of the relative nuclear (N) and cytoplasmatic distribution of CFP-ETV6 and YFP-PIN1 in this experiment. CFP-ETV6 was found only in the nucleus in 88% of the cells, while in more than 80% of the cells YFP-PIN1 could be observed in both nucleus and to a lesser extent in the cytoplasm. Percentages for CFP-ETV6 are represented as blue bars, percentages for PIN1-YFP are shown as green bars.

4.3.3 Effect of TIP60β and PIN1 on the function of ETV6

4.3.3.1 ETV6 is a transcriptional repressor

It is known that ETV6 acts as a strong transcriptional repressor when assayed in various reporter gene systems. We therefore wanted to examine whether the observed protein interactions of ETV6 with TIP60β and PIN1 would affect the repression function of ETV6. The repression function of ETV6 was assayed using two different reporter gene systems.

In the first system, ETV6 and various deletion mutants of ETV6 were expressed as a fusion proteins with a heterologous DNA binding domain, the DNA binding domain of the yeast transcription factor GAL4 (GAL4-DBD). In this system the reporter plasmid contained the firefly luciferase gene under the control of part of the Herpes Simplex Virus thymidine kinase promoter

and five upstream activating sequences (UAS) which are binding sites for the GAL4-DBD (GAL4(5)-tk-Luc) (Fig. 3.2A). The tk-promoter of this reporter plasmid will direct expression of moderate levels of luciferase if transfected into mammalian cells.

In the second system, ETV6 was expressed as a fusion protein with the cyan fluorescent protein. As a reporter plasmid construct expressing the firefly luciferase gene under the control of the ETV6-responsive stromelysin-1 promoter was used (pTR344). pTR334 contains 344 base pairs of the stromelysin-1 promoter including an ets binding site (EBS) and two *ets* potential binding sites (TBS). As a control reporter plasmid, a contstruct containing a truncated stromelysin-1 promoter without the EBS and TBS was used (pTR89). In this system the effect of ETV6 on the transcription of the reporter gene was dependent on the function of the native DNA binding domain of ETV6 (Fig. 3.2B).

To demonstrate that ETV6 mediated transcriptional repression was dependent on the function of histone deacetylases, trichostatin-A, an inhibitor of HDACs, was added 24 hours after transfection at a concentration of 75 nM to the cells. All experiments were performed at least in triplicate. A plasmid containing the *Renilla* luciferase gene (Fig. 3.2C) with a minimal promoter

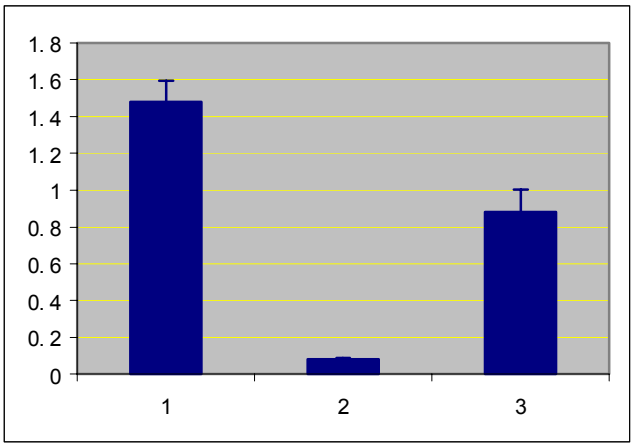
was cotransfected as a transfection control. Firefly luciferase values were normalized to the *Renilla* luciferase values.

As can be seen in Fig 4.13, a GAL4DBD-ETV6 fusion protein mediates strong repression of the luciferase expression (18 fold). The C-terminal region of the central ETV6 domain which had been shown to interact with TIP60β still mediates about a two-fold repression of luciferase expression.

Using the stromelysin-1 promoter based reporter plasmids about four-fold repression could be achieved when using 0.3µg of pECFP-C1-ETV6 plasmid. A good expression and repression (3.7 fold) of the firefly luciferase gene was evident from the pTR344 plasmid (bars 1 and 2). The serine to alanine mutants at position 106 and 257 of ETV6 showed slightly higher repression (6.2 fold and 7.8 fold) than ETV6. The ETV6(S332A) mutant had a similar repression ability like wild type ETV6 (Fig. 4.14).

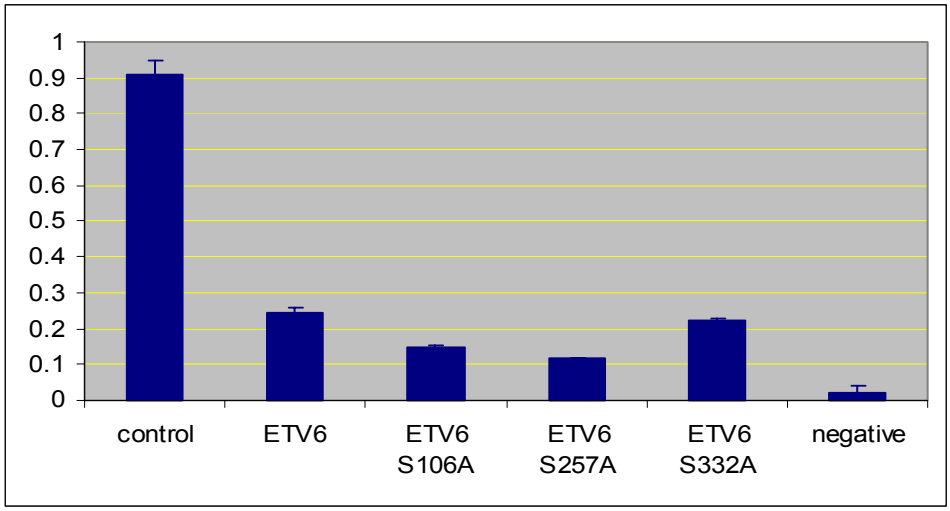
Addition of TSA to the medium led to a partial relieve of the transcriptional repression mediated by GAL4DBD-ETV6 (Fig 4.15 compare bars 1 to 4). This effect has been described before (Chakrabarti and Nucifora, 1999; Lopez et al., 1999). However, the transcriptional repression mediated by GAL4DBD-ETV6(282-345) could be completely abolished by the addition of TSA (compare bars 2 and 5 in Fig. 4.15). This is the TIP60β interaction

domain of ETV6. These findings can be interpreted in the following way: transcriptional repression by ETV6 is mediated through a HDAC dependent and a HDAC independent pathway. The repression mediated through amino acids 282-345 of ETV6 is completely dependent on HDAC function.



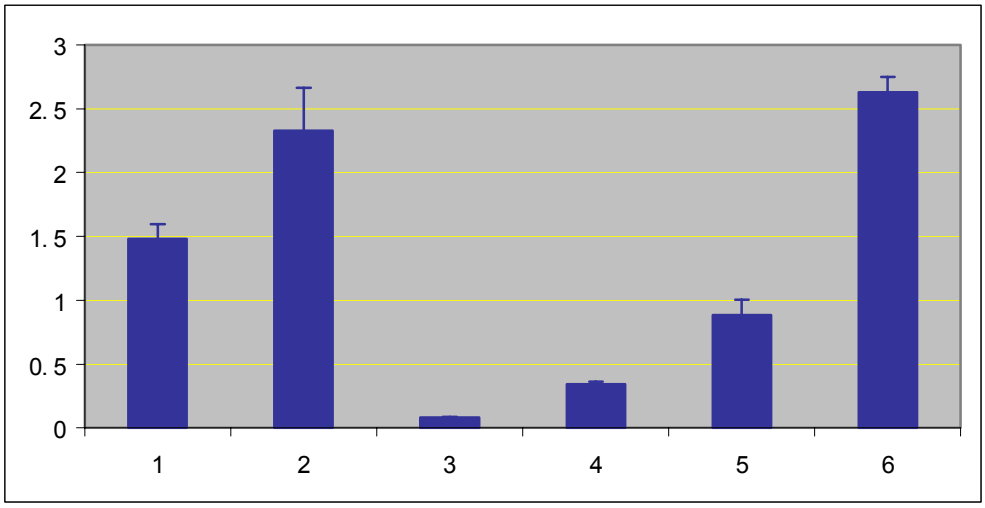
Plasmid	1	2	3
pTK Luc	+	+	+
pM1 ETV6		+	
pM1 ETV6 (282-345)			+

Fig. 4.13 GAL4(5)-tk-Luc based reporter gene assay for ETV6 mediated transcriptional repression. Full length ETV6 and amino acids 282-345 of ETV6 (the TIP60β interaction domain of ETV6) fused to the GAL4DBD were tested for their ability to repress transcription of a luciferase reporter gene in HEK293 cells. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.



Plasmid	1	2	3	4	5	6
pTR 334	+	+	+	+	+	
pECFP C1 ETV6		+				
pECFP C1 ETV6(S106A)			+			
pECFP C1 ETV6(S257A)				+		
pECFP C1 ETV6(S332A)					+	

Fig. 4.14 Stromelysin-1 promoter based reporter gene assay for ETV6 and ETV6 serine to alanine mutant mediated transcriptional repression. CFP-ETV6 and three serine to alanine mutants(CFP-ETV6(S106A), CFP-ETV6(S257A) and CFP-ETV6 (S332A)) were tested for their ability to repress transcription of a luciferase reporter gene in HEK293 cells. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.



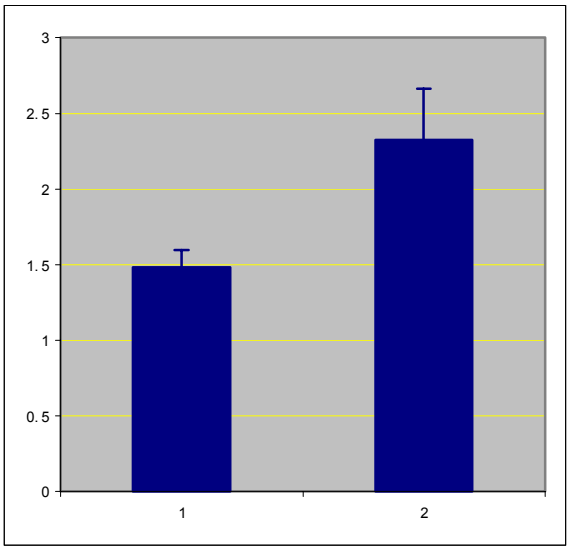
Plasmid	1	2	3	4	5	6
pTK Luc	+	+	+	+	+	+
pM1 ETV6			+	+		
pM1 ETV6 (282-345)					+	+
TSA (75 nM)		+		+		+

Fig. 4.15 GAL4(5)-tk-Luc based reporter gene assay demonstrating the effect of the HDAC inhibitor TSA on ETV6 mediated transcriptional repression. Full length ETV6 and amino acids 282-345 of ETV6 (the TIP60β interaction domain of ETV6) fused to the GAL4DBD were tested for their ability to repress transcription of a luciferase reporter gene in HEK293 cells in the presence and absence of 75 nM TSA. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.

4.3.3.2 Function of TIP60β in the reporter assays

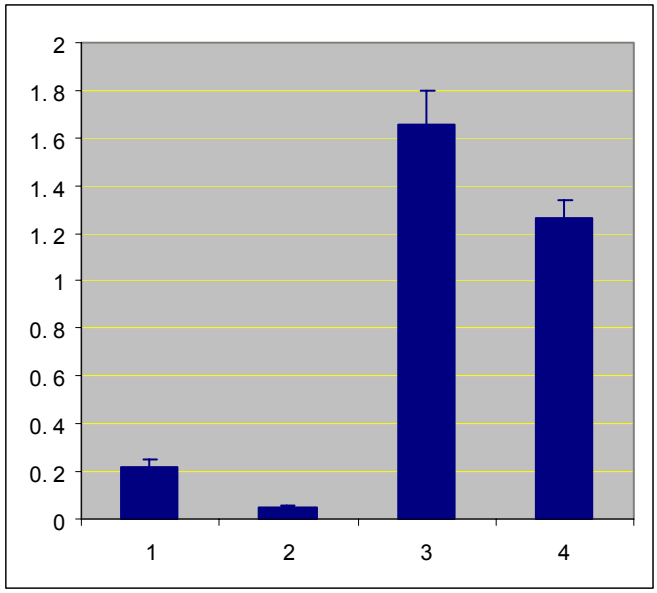
Depending on its interaction partners, TIP60 functions as a transcriptional co-activator or as a transcriptional corepressor. Interestingly, we found that the expression of a TIP60β-YFP fusion protein shows opposite effects in the

two reporter systems used in this study. While there was a slight (1.6 fold) activation of transcription when the GAL4(5)-tk-Luc reporter plasmid was used (Fig 4.16) there was a slight repression (down to 70% of the control value) of transcriptional activity when the stromelysin-1 promoter based reporters were used (pTR344 and pTR89) (Fig 4.17). Although, these effects were not very large they were consistently found. Since TIP60 is not known to have a DNA binding domain and this effect is also seen when pTR89 which does not have any *ets* binding sites is used, these effects are most likely indirect general effects on transcription.



Plasmid	1	2
pTK- Luc	+	+
pEYFP-N1-TIP60β		+

Fig. 4.16 Expression of a TIP60β-YFP fusion protein leads to enhanced activity of firefly luciferase with the GAL4(5)-tk-Luc based reporter in HEK293 cells. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.



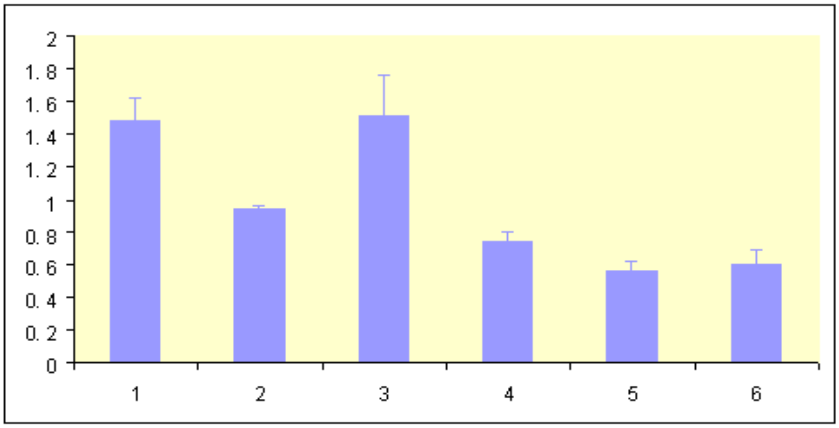
Plasmid	1	2	3	4
pTR 89	+	+		
pTR 334			+	+
pEYFP-N1-TIP60β		+		+

Fig. 4.17 Expression of a TIP60β-YFP fusion protein leads to a decrease in firefly luciferase activity with the stromelysin-1 promoter based reporter (pTR344 and pTR89) in HEK293 cells. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.

4.3.3.3 The HAT deficient mutant of TIP60β lacks transcriptional activity

As shown in figure 4.17, TIP60β-YFP shows modest transcriptional repression activity when pTR334 is used as a reporter gene. When the histone acetyltransferase deficient DRS mutant of TIP60β-YFP was used, no difference in the transcriptional activity of the luciferase reporter gene was

observed (compare bar 1 and 3 in Fig. 4.18). This indicates that the acetyltransferase function of TIP60β is required for its transcriptional effects.



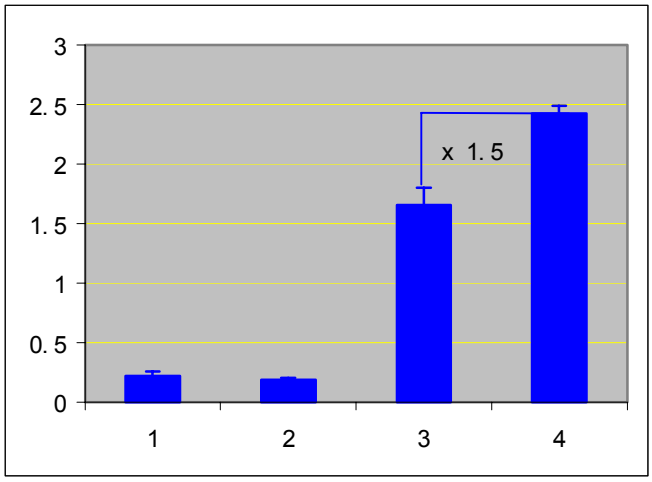
Plasmid	1	2	3	4	5	6
pTR 334	+	+	+	+	+	+
pEYFP N1-TIP60β		+			+	
pEYFP N1-TIP60β (DRS)			+			+
pECFP C1-ETV6				+	+	+

Fig. 4.18 Expression of the HAT deficient TIP60β(DRS)-YFP fusion protein does not change the firefly luciferase activity with the stromelysin-1 promoter based reporter (pTR344) in HEK293 cells. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.

4.3.3.4 Effect of PIN1 in the stromelysin-1 promoter based reporter assay

Although PIN1 has no DNA binding site, the expression of PIN1-YFP resulted in a 1.5 fold increased expression of the firefly luciferase reporter gene when

the pTR334 reporter plasmid was used in HEK293 cells (Fig. 4.19). The pTR344 reporter plasmid contains an *ets* (EBS) and two potential *ets* binding sites (TBS). These sites are absent in pTR89. It is thus conceivable that the positive effect of PIN-YFP on the transcription of the reporter gene is mediated through regulation of a cellular *ets* factor by PIN1.



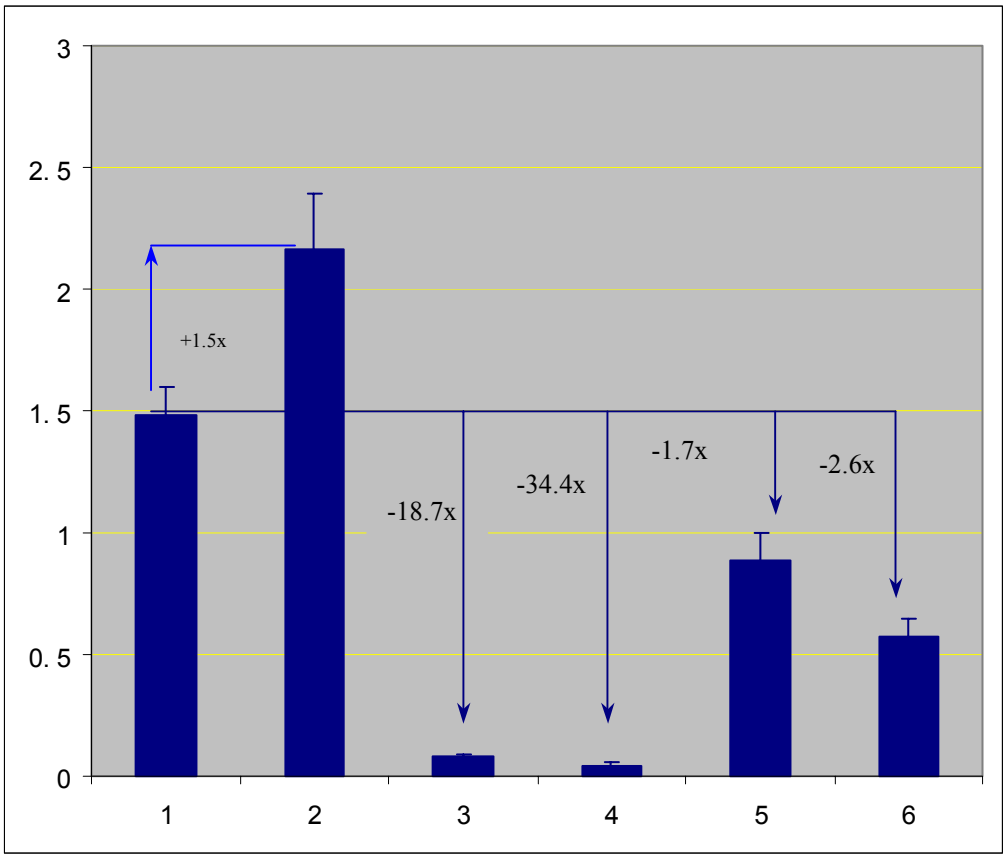
Plasmid	1	2	3	4
pTR 89	+	+		
pTR 334			+	+
pEYFP-N1-PIN1		+		+

Fig. 4.19 Expression of a PIN1-YFP fusion protein leads to an increase in firefly luciferase activity with the stromelysin-1 promoter based reporter pTR334 but not with the pTR89 reporter, which lacks *ets* and ETV6 DNA binding sites, in HEK293 cells. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.

4.3.4 The transcriptional repression function of ETV6 is modified by TIP60β and PIN1

4.3.4.1 TIP60β is a co-repressor of ETV6 in the GAL4(5)-pTK-Luc reporter system

When TIP60β-YFP is coexpressed with GAL4DBD-ETV6, the transcription of the firefly luciferase reporter gene from the GAL4(5)-tk-Luc plasmid is strongly repressed (about 34 fold). This repression is about twice as strong as seen with GAL4DBD-ETV6 alone (Fig 4.20, No. 3 and 4). The same enhanced repression of the reporter gene upon coexpression of TIP60β-YFP is seen when only the TIP60β interaction domain of ETV6 (aa282-345) fused to the GAFL4DBD is expressed. GAL4DBD-ETV6(282-345) shows a very modest repressional activity of about 1.7 fold. However, when TIP60β-YFP is coexpressed, this repression is enhanced considerably (Fig 4.20, No. 5 and 6).

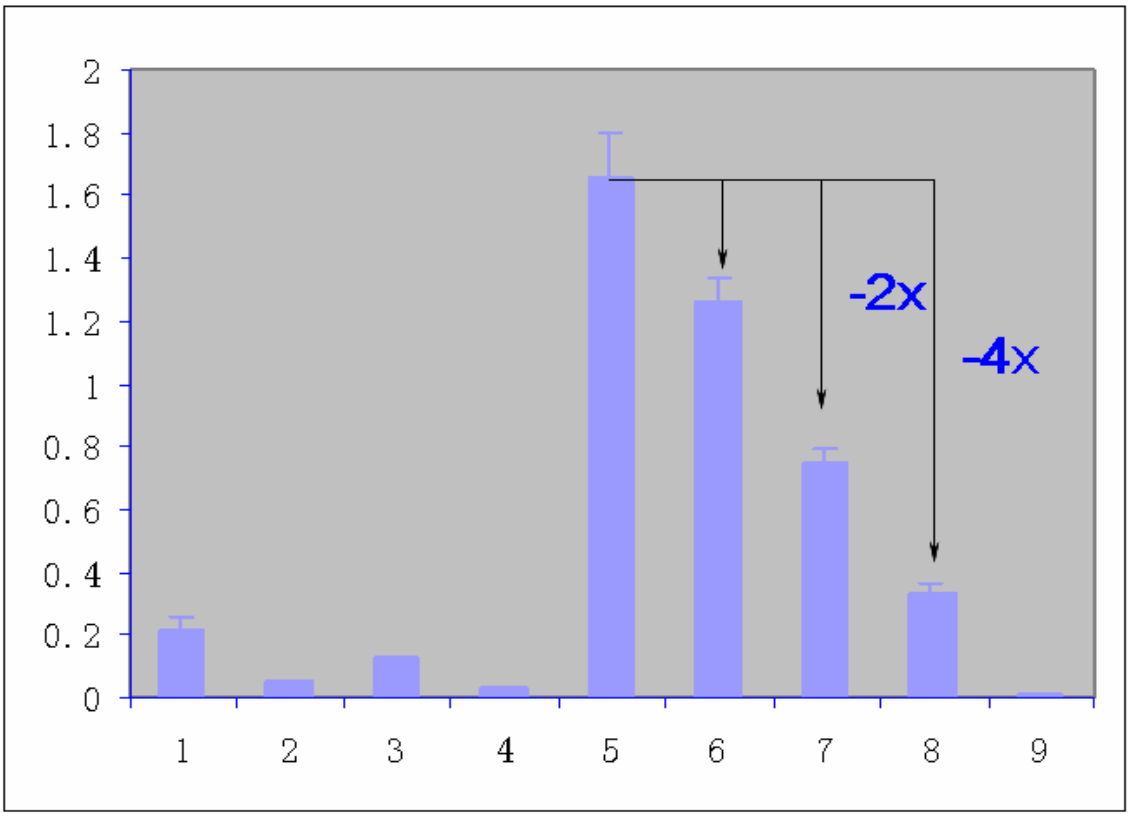


Plasmid	1	2	3	4	5	6
GAL4(5)-TK-Luc	+	+	+	+	+	+
pM1-ETV6			+	+		
pM1-ETV6 (282-345)					+	+
pEYFP-N1-TIP60β		+		+		+

Fig. 4. 20 Coexpression of a GAL4DBD-ETV6 or a GAL4DBD-ETV6(282-345) fusion protein with TIP60β-YFP leads to enhanced repression of the firefly luciferase reporter gene from the GAL4(5)-tk-Luc reporter plasmid in HEK293 cells. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.

4.3.4.2 TIP60β is a co-repressor of ETV6 in the stromelysin-1 based reporter system

The results of the experiments in Fig 4.20 clearly show that TIP60β acts as a corepressor of ETV6 when ETV6 is fused to a heterologous DNA binding domain. To analyze whether this corepressor activity would also be found in situations where ETV6 uses its *ets* domain for DNA binding, coexpression studies of ETV6 and TIP60β were performed in the stromelysin-1 based reporter system. The expression of TIP60β-YFP leads to a modest 1.5 fold repression of reporter gene activity and the expression of CFP-ETV6 to a 2 fold repression when the pTR344 reporter was used (Fig. 4.21, No. 5, 6 and 7). However, when both CFP-ETV6 and TIP60β-YFP were coexpressed a syngergistic increase of repression to 4 fold was observed (Fig. 4.21, No. 8). When this experiment was repeated with a reporter that lacked both the EBS and TBS (pTR89), similar results were observed, however at a much lower overall level of reporter gene expression (Fig. 4.21, No 1 to 4)

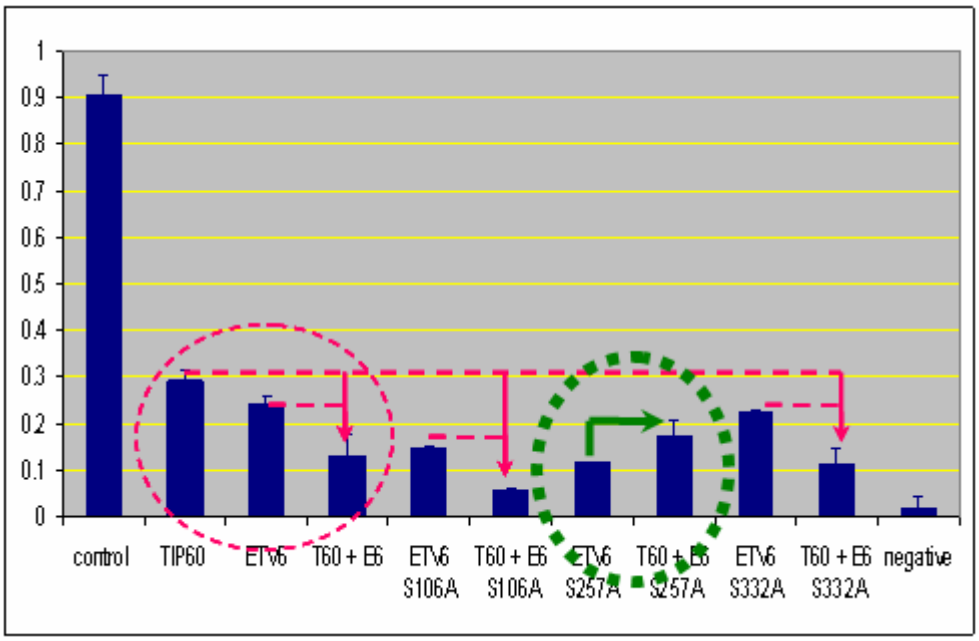


pTR89	x	x	x	x					
pTR334					x	x	x	x	
pECFP C1 ETV6			x	x			x	x	
pEYFP N1 TIP60β		x		x		x		x	

Fig. 4. 21 Coexpression of a CFP-ETV6 fusion protein with TIP60β-YFP leads to enhanced repression of the firefly luciferase reporter gene from the stromelysin-1 promoter luciferase reporter plasmid in HEK293 cells. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.

4.3.4.3. The phosphorylation of Ser257 on ETV6 is important for the interaction with TIP60β

The results in Fig. 4.20 and Fig. 4.21 clearly show that TIP60β is a transcriptional corepressor of ETV6. The experimental results shown in Fig. 4.14 demonstrate that two of the CFP-ETV6 serine to alanine mutants (S106A and S257A) have slightly stronger repressional activity than CFP-ETV6. The S332A mutant showed a similar repressional activity like the wild type ETV6. Ser257 of ETV6 is known as a p38 dependent phosphorylation site (Arai et.al., 2002). Therefore the ability of TIP60β to function as a corepressor for these serine to alanine mutant CFP-ETV6 proteins was examined (Fig. 4.22). When the CFP-ETV6 serine to alanine mutants and TIP60β-YFP were coexpressed, corepressor activity of TIP60β could only be seen for the S106A and the S332A mutants (compare no. 5 and 6, and no. 9 and 10) but not for the S257A mutant (compare no. 7 and 8). This implies that the phosphorylation of Ser257 on ETV6 is important for this functional interaction with TIP60β.



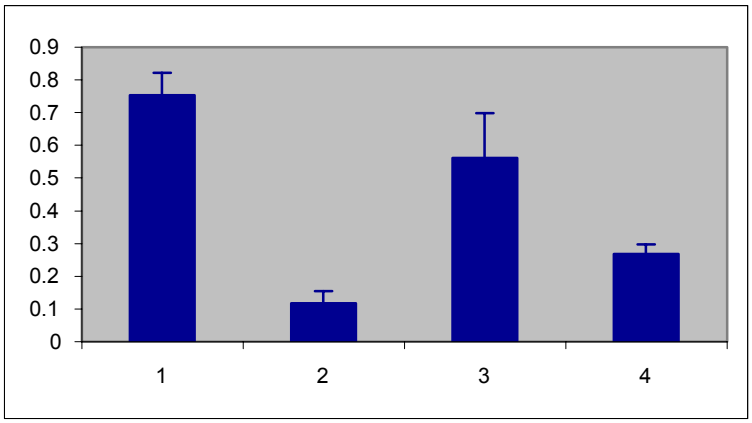
Plasmid	1	2	3	4	5	6	7	8	9	10	11
pTR 334	+	+	+	+	+	+	+	+	+	+	
pEYFP N1-TIP60 β		+		+		+		+		+	
pECFP C1 ETV6		+	+	+							
pECFP C1 ETV6(S106A)					+	+					
pECFP C1 ETV6(S257A)							+	+			
pECFP C1 ETV6(S332A)									+	+	

Fig. 4.22 Stromelysin-1 promoter based reporter gene assay for corepressor activity of TIP60β and ETV6 serine to alanine mutants in HEK293T cells. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.

4.3.4.4 PIN1 reduces the transcriptional repression of ETV6

When PIN1-YFP was coexpressed with the GAL4DBD-ETV6 fusion protein the ability of GAL4DBD-ETV6 to repress transcription of the firefly luciferase

reporter gene from the GAL4(5)-tk-Luc reporter was reduced from 7 fold repression in the absence of PIN1-YFP to only about two fold in the the presence of PIN1-YFP (Fig 4.23, compare No. 1 and 2 and No. 3 and 4). The expression of PIN1-YFP alone led to a slight reduction in reporter gene expression (Fig 4.23; compare No. 1 and 3).

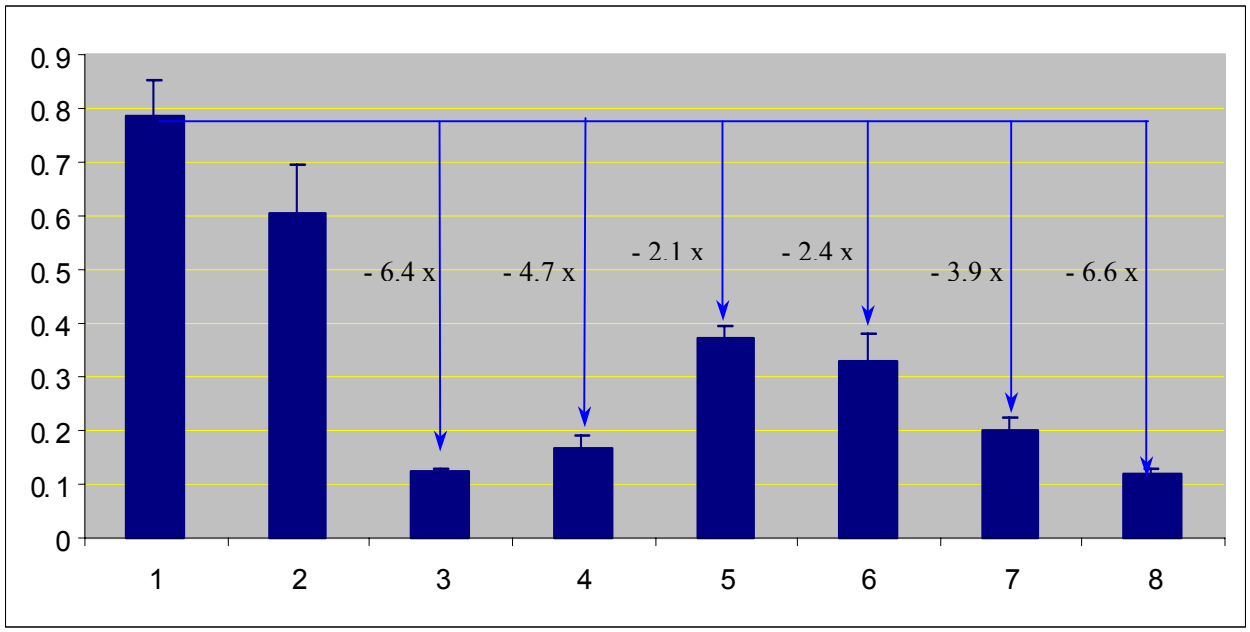


Plasmid	1	2	3	4
pGAL4(5)- TK Luc	+	+	+	+
pM1-ETV6		+		+
pEYFP-N1-PIN1			+	+

Fig. 4. 23 Coexpression of a GAL4DBD-ETV6 fusion protein with PIN1-YFP leads to reduced repression of the firefly luciferase reporter gene from the GAL4(5)-tk-Luc reporter plasmid in HEK293 cells. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.

4.3.4.5. Effect of PIN1 on the function of the *pointed* domain and the function of C terminal half of the central domain of ETV6

PIN1 reduces the transcriptional repression of ETV6 in the GAL4(5)-tk-Luc reporter system. Using two deletion mutants of ETV6 fused to the GAL4DBD, we tried to map the domain of ETV6 that is responsible for this effect. The first deletion mutant of ETV6 that was tested contained the C terminal half of the central domain (aa282-345); this is also the TIP60β interaction domain of ETV6. While the repression of GAL4DBD-ETV6 was slightly reduced by the coexpression of YFP-PIN1 (Fig. 4.24 compare No. 1 and 3, and No. 3 and 4), there was no appreciable effect of coexpression of PIN1-YFP for this mutant. With or without PIN1-YFP, expression of GAL4DBD-ETV6(282-345) resulted in a two fold reduction of the firefly luciferase expression (Fig. 4.24 compare No. 1 and 5, and No. 2 and 6). However, the situtation was slightly different when the GAL4DBD-ETV6 (1-126), which included the *pointed* domain of ETV6, was tested with or without PIN1-YFP: there was a very slight increase of repressional acitivity of the GAL4DBD-ETV6(pointed) fusion in the presence of PIN1-YFP (Fig. 4.24, compare No. 1 and 7, and No. 2 and 8).

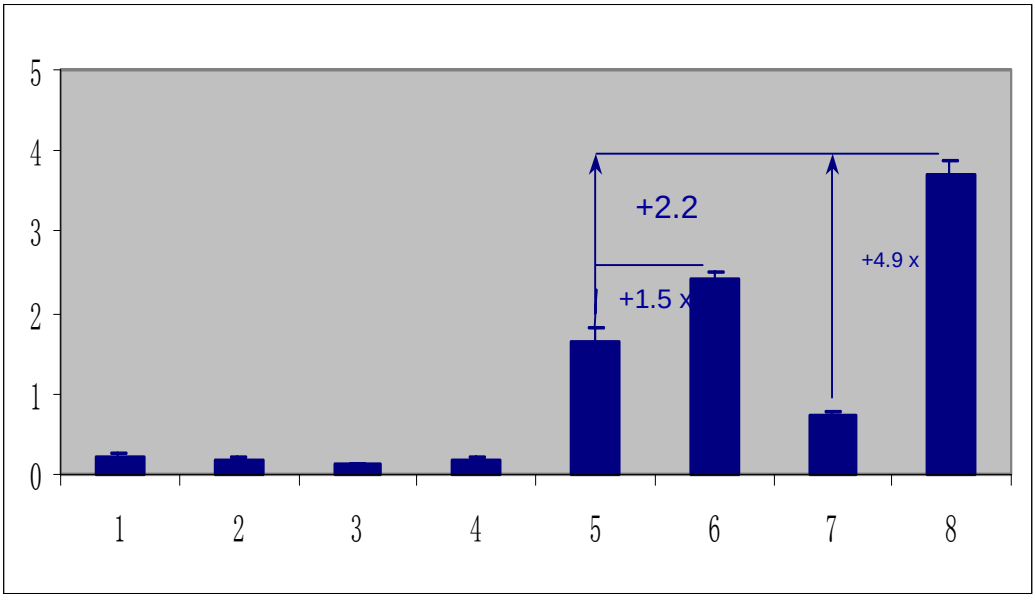


Plasmid	1	2	3	4	5	6	7	8
pGAL4(5)-TK Luc	+	+	+	+	+	+	+	+
pM1-ETV6			+	+				
pM1-ETV6 (282-345)					+	+		
pM1-ETV6 (1-126)							+	+
pEYFP-N1-PIN1		+		+		+		+

Fig. 4.24 Effect of PIN1-YFP expression on the transcriptional acitivity of GAL4DBD-ETV6 deletion mutants (aa 282-345 and aa 1-126) on the expression of the firefly luciferase reporter gene from the GAL4(5)-tk-Luc reporter plasmid in HEK293 cells. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.

4.3.4.6 Coexpression of PIN1 transforms ETV6 from a transcriptional repressor to a transcriptional activator

To examine the effect of PIN1-YFP expression on the function of ETV6 in a more physiological promoter setting, the stromelysin-1 promoter based reporter system was used. In this system the transcriptional activity of ETV6 is dependent on the DNA binding domain of ETV6. As was shown above (Fig. 4.19), expression of PIN1-YFP results in a slight increase in the expression of the firefly luciferase reporter gene from the pTR344 plasmid (Fig. 4.25, compare No. 5 and 6, 1.5 fold). Also as shown before, expression of CFP-ETV6 leads to a repression of the transcription of the reporter gene (Fig 4.25, compare No. 5 and 7). However, when PIN1-YFP and CFP-ETV6 are coexpressed, a moderate transcriptional activation (2.2 fold) is observed which is much stronger than when PIN1-YFP is expressed alone (Fig. 4.25, No. 8). This means that PIN1 does not only abolish ETV6 mediated transcriptional repression, but that PIN1 converts ETV6 from a transcriptional repressor to a transcriptional activator.



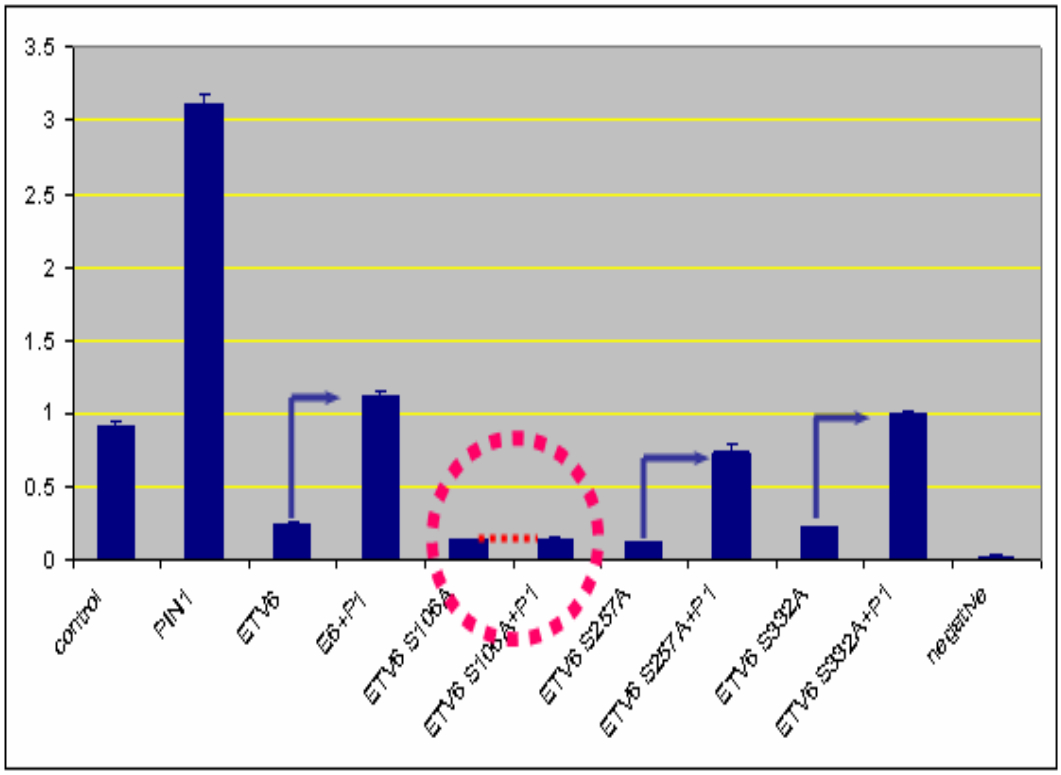
Plasmid	1	2	3	4	5	6	7	8
pTR 89	+	+	+	+				
pTR 334					+	+	+	+
pECFP C1 ETV6			+	+			+	+
pEYFP N1PIN1		+		+		+		+

Fig. 4. 25 Coexpression of a CFP-ETV6 fusion protein with PIN1-YFP leads to greatly enhanced expression of the firefly luciferase reporter gene from the stromelysin-1 promoter luciferase reporter plasmid in HEK293 cells. ETV6 is turned from a transcriptional repressor to a transcriptional activator. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.

4.3.4.7 The ETV6 Ser-Pro motif at position 106-107 is a potential binding site for PIN1

The binding sites of PIN1 in its substrates have been identified as a Ser/Thr-Pro bond. The results of our ETV6-PIN1 interaction experiments

implied that the SAM/*pointed* domain and the central domain of ETV6 contain the PIN1 interacting sites (see Fig. 4.5). Serine to alanine mutants at the Ser-Pro bonds at positions 106, 257 and 332 of ETV6, which represent potential PIN1 interaction motifs, were tested in the stromelysin-1 promoter based reporter gene system for their functional interaction with PIN1 (Fig. 4.26). PIN1 is able to completely block the repressional acitivity of ETV6 and in some cases even turn ETV6 into an activator of transcription. While the repressional acitivity of the ETV6 serine to alanine mutants at positions 257 and 332 were blocked by PIN1 (compare no. 7 and 8, and no 9 and 10 in Fig. 4.26), this was not the case for the ETV6 serine to alanine mutant at position 106 (compare no. 5 and 6 in Fig. 4.26). The ETV6 (S106A) mutant was completely resistant to the influence of PIN1 and exhibited full repressional activity. These findings imply that a conformational change through prolyl-cis trans isomerization at the Ser106-Pro107 bond mediated through PIN1 after phosphorylation of ETV6 at serine 106 cause ETV6 to lose its repressional activity.

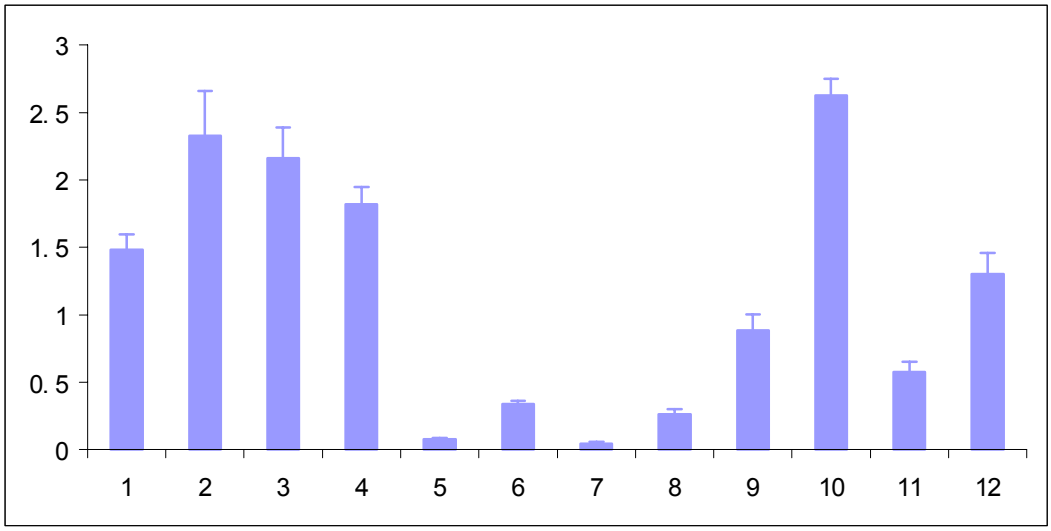


Plasmid	1	2	3	4	5	6	7	8	9	10	11
pTR334	+	+	+	+	+	+	+	+	+	+	+
pECFP C1 ETV6			+	+							
pECFP C1 ETV6 S106A					+	+					
pECFP C1 ETV6 S257A							+	+			
pECFP C1 ETV6 S332A									+	+	
pEYFP N1PIN1		+		+		+		+		+	

Fig. 4.26 Stromelysin-1 promoter based reporter gene assay to examine the effects of PIN1 on ETV6 serine to alanine mutants in HEK293T cells. If residue 106 of ETV6 is mutated from a serine into an alanine PIN1 is no longer able to block ETV6 mediated repression. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.

4.3.4.8 The corepression function of TIP60β for ETV6 is independent of HDACs

Transcriptional repression by a transcription factor is often mediated through the recruitment of corepressors which themselves recruit histone deacetylases. In Fig. 4.15 it could be shown that part of the trancriptional repression function of ETV6 can be relieved by blocking HDACs with trichostatin A (TSA). Using the GAL4(5)-tk-Luc reporter system we examined whether the corespressor effect of TIP60β is dependent on HDAC acitivity. Even in the presence of TSA, the expression of TIP60β-YFP still causes corepression (Fig. 4.27, compare No. 3, No. 5 and 7, and No. 4, No. 6 and 8). This independence of TIP60β corepressor acitivity was even more obvious when only the TIP60β interaction domain of ETV6 was expressed as a fusion with GAL4DBD (GAL4DBD-ETV6(282-345). Even when HDACs were inhibited by TSA, TIP60β-YFP represses transcription of the reporter gene together with GAL4DBD-ETV6(282-345) (Fig. 4.27, compare No. 10 and 12). Neither TIP60β-YFP (Fig. 4.27, No.4) nor GAL4DBD-ETV6 (282- 345) alone (Fig. 4.27, No. 10) were able to repress transcription in the presence of TSA.



Plasmid	1	2	3	4	5	6	7	8	9	10	11	12
pTK Luc	+	+	+	+	+	+	+	+	+	+	+	+
pM1 ETV6					+	+	+	+				
pM1 ETV6 (282-345)									+	+	+	+
pEYFP N1TIP60β			+	+			+	+			+	+
TSA		+		+		+		+		+		+

Fig. 4. 27 GAL4(5)-tk-Luc based reporter gene assay demonstrating the independence of the TIP60β corepressor effect from HDACs. Full length ETV6 and amino acids 282-345 of ETV6 (the TIP60β interaction domain of ETV6) fused to the GAL4DBD and TIP60β-YFP were tested for their ability to repress transcription of a luciferase reporter gene in HEK293 cells in the presence and absence of 75 nM TSA. The mean and standard deviation of the normalized firefly luciferase values from three experiments are shown. Transfected plasmids are shown below the bar graph. The raw data of this experiment are given in the Appendix. See text for explanation and interpretation.

4.3.5 The role of ETV6, TIP60β and PIN1 in cell cycle regulation

PIN1 has been shown to play an important role in cell cycle regulation. Overexpressing of PIN1 causes cell cycle arrest in the G2 phase of the cell

cycle (Lu et al., 1996). This part of my work will focus on the effects of overexpression of ETV6, TIP60β and PIN1 on the cell cycle of HEK 293T cells.

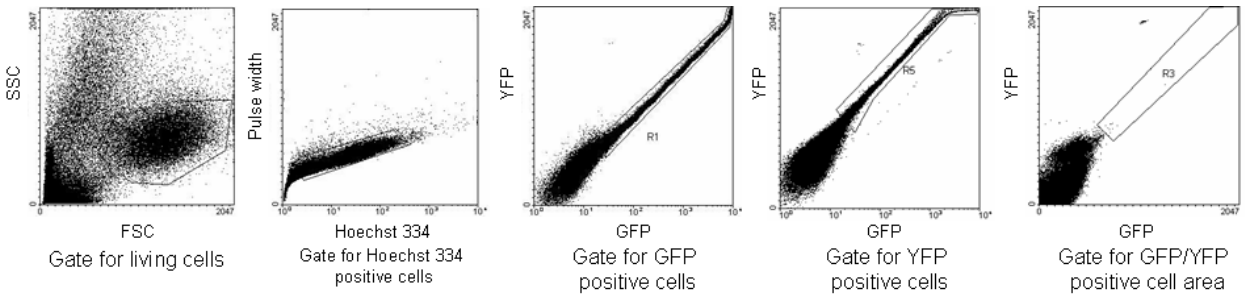
4.3.5.1 The effect of the expression of the fluorescence proteins GFP and YFP on HEK 293T cell cycle regulation

Since the ETV6, TIP60β and PIN1 proteins used in this experiment are fused to the GFP or YFP fluorescent proteins, the effect of GFP and YFP expression on the cell cycle of 293T cells was tested first. For the analysis of the cell cycle, the DNA content of the cells was measured by Hoechst 334 staining. The primary FACS data were analyzed in the following way: First life cells were gated using forward and side scatter. Then the GFP, YFP or GFP/YFP postive cells were gated and their DNA contents was measured with the Hoechst stain (Fig. 4.28 A.).

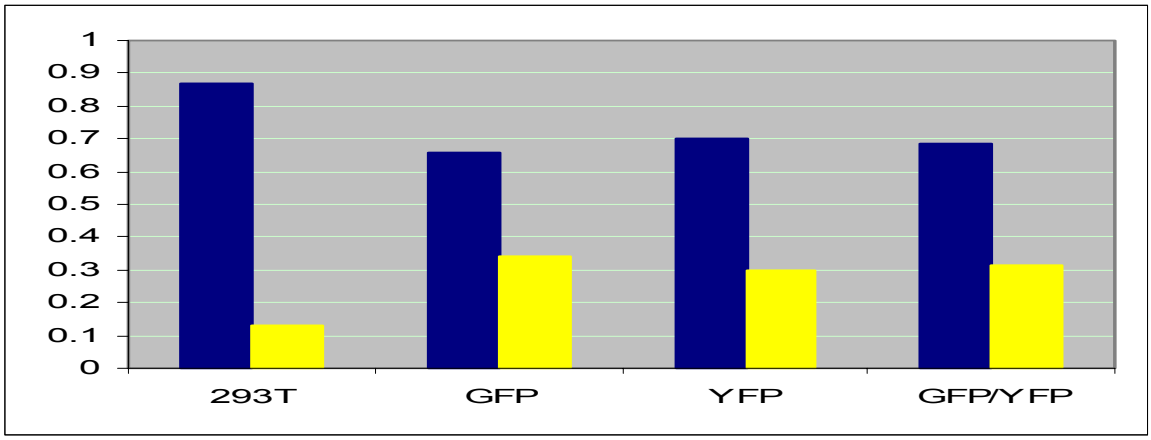
When the transfected cells were observed under the micoroscope around 90% were GFP or YFP positive. However, the FACS analysis was only able to identify around 20% of cells as positive. This can probably be explained by different expression levels of GFP and YFP in the cells. This means that about 70% of GFP or YFP negative cells as identified by FACS were also

transfected and expressed the fluorescent proteins to a lesser extent. When we analyzed the Hoechst stain in the FACS GFP positive and FACS GFP negative groups no difference in the relative distribution of cells in the different cell cycle phases was observed (Fig. 4.28D). This meant that the GFP or YFP negative cells that were identified by FACS could not be used as a control in our cell cycle experiments. We therefore used mock transfected cells as controls. The initial experiments showed that the expression of the fluorescent proteins GFP, YFP, and GFP/YFP increased the proportion of cells in S/G2/M phase from 13% to between 30 to 34% (Fig. 4.28 B and C).

A.

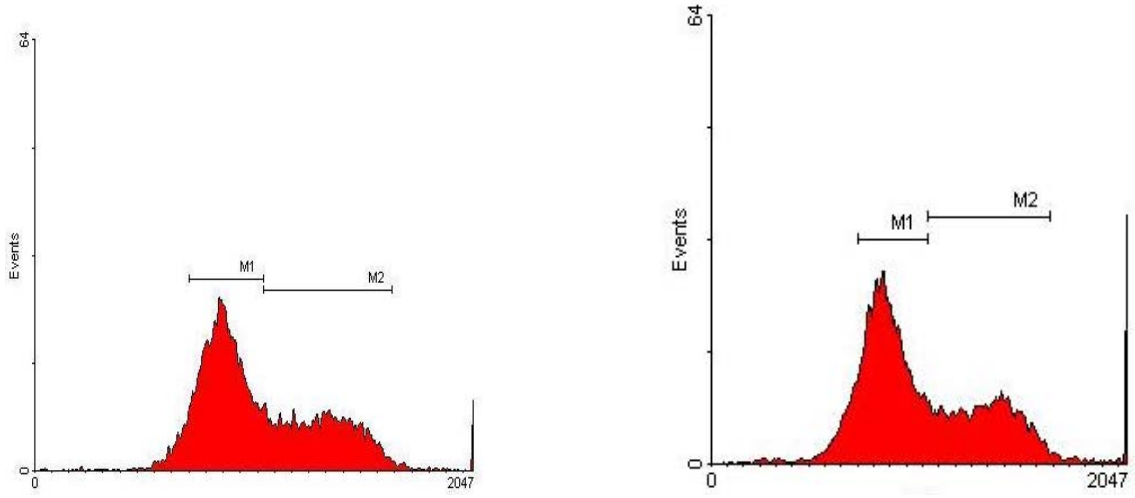


B.

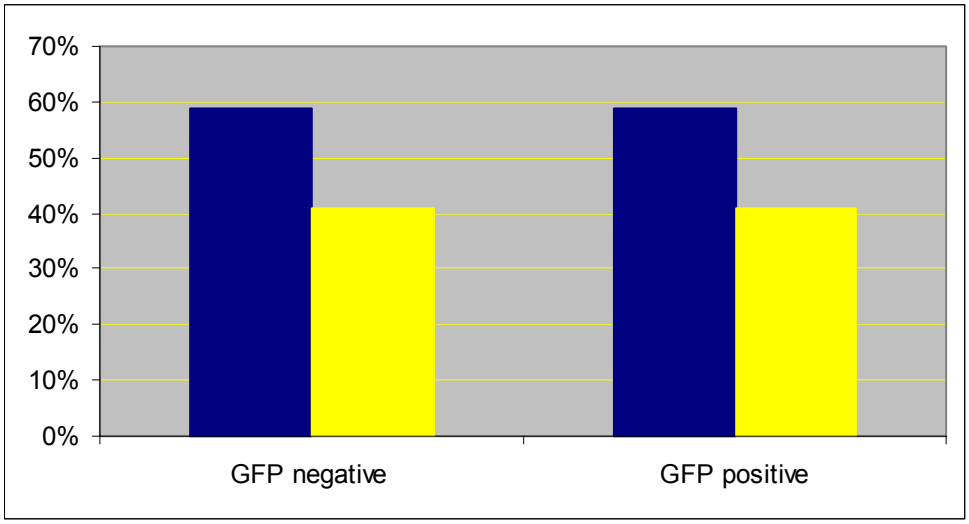


	1	2	3	4
Cycle phase	293T	GFP	YFP	GFP+YFP
G1(blue)	86.88%	65.59%	70.06%	68.51%
S+G2/M (yellow)	13.12%	34.41%	29.94%	31.49%

C.



D.



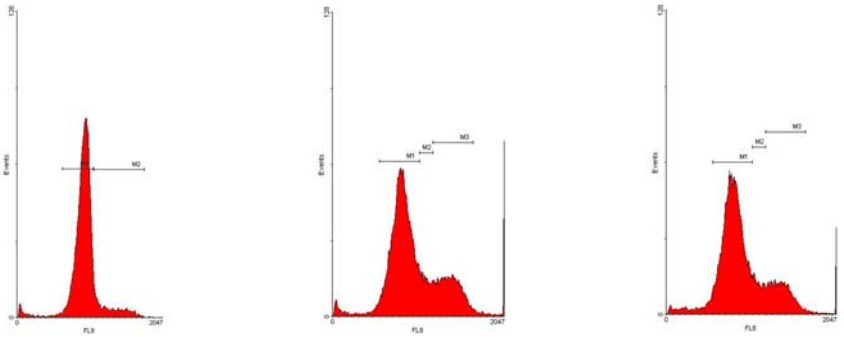
	1	2
Cycle phase	GFP negative	GFP positive
G1(blue)	59%	59.03%
S+G2/M (yellow)	41%	40.97%

Fig. 4.28 Effect of overexpression of GFP and YFP on the cell cycle of 293T cells. 293T cells were transfected with pEGFP-C1, pEYFP-N1 or both plasmids together. The 293T control and transfected positive cells were harvested after 48 hours and stained with propidium iodide (PI) and Hoechest 334. A) The process for analyzing the primary data B) Histograms for the Hoechest 334 stain of GFP, YFP or GFP and YFP positive cells. C) Bar graph showing the percentage of cells ins G1 phase (blue) and S/G2/M phases (yellow). D) The contribution of FACS GFP positive and FACS GFP negative groups to cell cycle phases

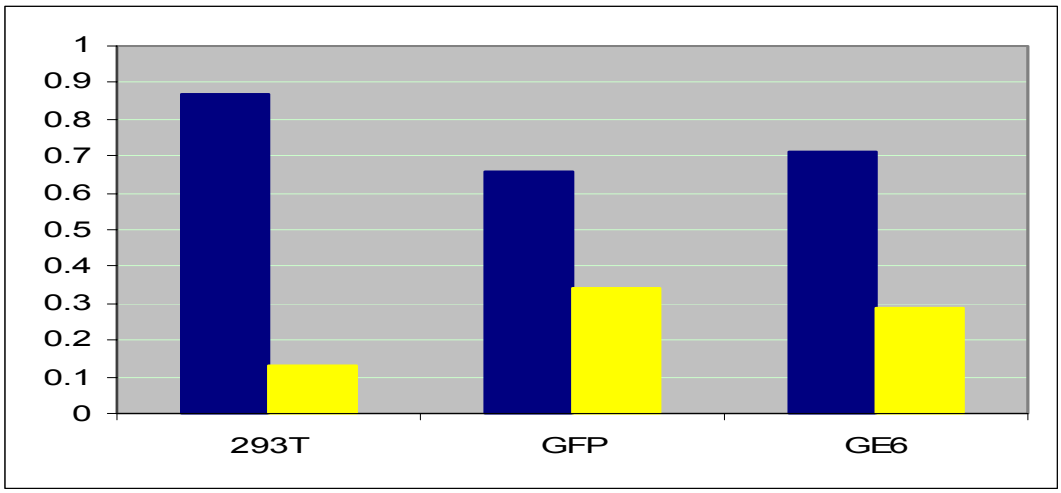
4.3.5.2 Effect of ETV6 on the cell cycle

The expression of the GFP-ETV6 fusion protein in 293T cells caused a slight increase (from 65 to 71%) in the proportion of cells in G1 phase (Fig. 4.29A and B, lane 3), 6% more than GFP expressed cells (Fig. 4.29B, lane 2).

A.



B.



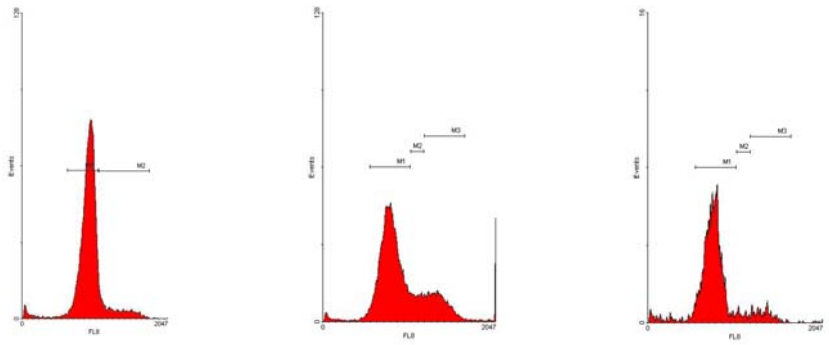
	1	2	3
Cycle phase	293T	GFP	GFP-ETV6
G1(blue)	86.88%	65.59%	71.03%
S+G2/M (yellow)	13.12%	34.41%	28.97%

Fig. 4.29 Effect of overexpression of GFP-ETV6 on the cell cycle of 293T cells. 293T cells were transfected with pEGFP-ETV6 or pEGFP. The cells were harvested after 48 hours and stained with propidium iodide (PI) and Hoechst 334. A) Histograms for the Hoechst stain of GFP-ETV6, or GFP positive cells. B) Bar graph showing the percentage of cells ins G1 phase (blue) and S/G2/M phases (yellow).

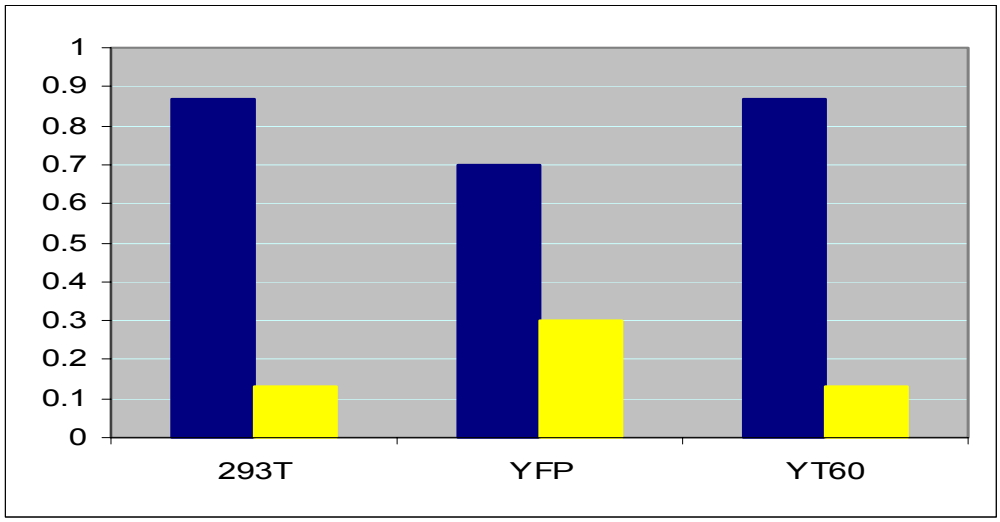
4.3.5.3 Effect of TIP60β on the cell cycle

The expression of TIP60β-YFP in 293T cells caused a marked increase of cells in G1 phase (Fig. 4.30 A, B lane 3) compared to YFP expressing 293T cells (Fig. 4.30A, B lane 2). The analysis of TIP60β-YFP expressing cells showed that 86.65% of the cells were in G1 phase, with just 13.25% in S/G2/M phase (Fig. 4.30 B).

A.



B.



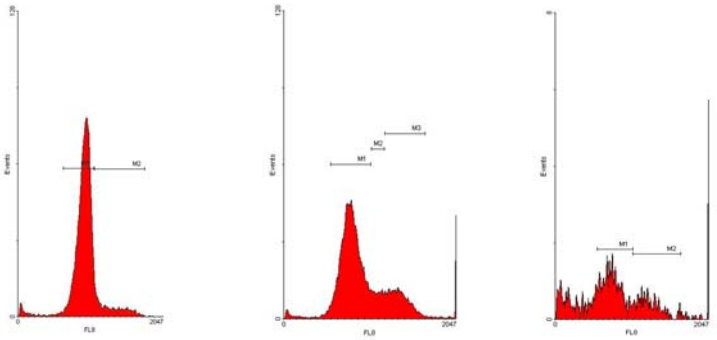
	1	2	3
Cycle phase	293T	YFP	TIP60β-YFP
G1(blue)	86.88%	70.06%	86.65%
S+G2/M (yellow)	13.12%	29.94%	13.35%

Fig. 4.30 Effect of overexpression of TIP60β-YFP on the cell cycle of 293T cells. 293T cells were transfected with pEYFP-N1- TIP60β or pEYFP. The cells were harvested after 48 hours and stained with propidium iodide (PI) and Hoechst 334. A) Histograms for the Hoechst stain of TIP60β-YFP, or YFP positive cells. B) Bar graph showing the percentage of cells ins G1 phase (blue) and S/G2/M phases (yellow).

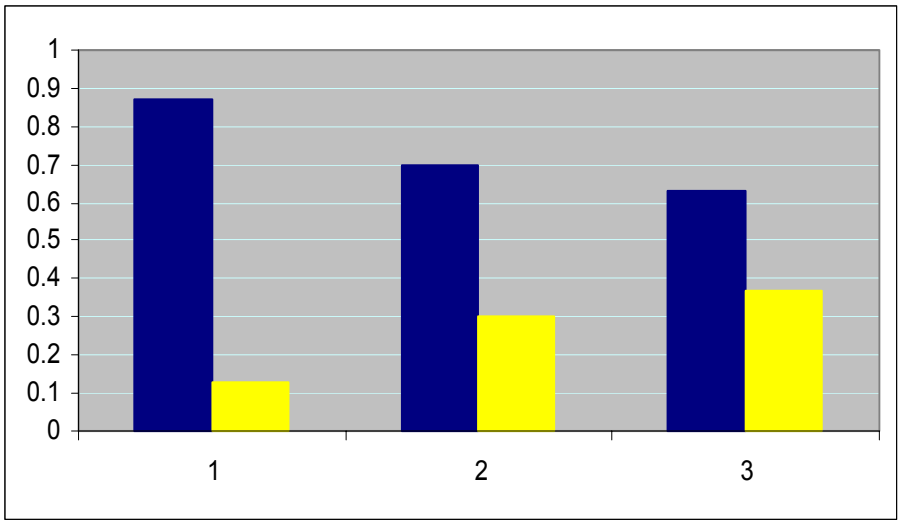
4.3.5.4 Effect of PIN1 on the cell cycle

The overexpression of PIN1 is known to arrest cells in G2 phase . Our data of PIN1-YFP transfected 293T cells showed a similar result. 63.29% of PIN1-YFP positive cells were in G1 phase and 36.71% were in S/G2/M (Fig. 4.31 A and B).

A.



B.



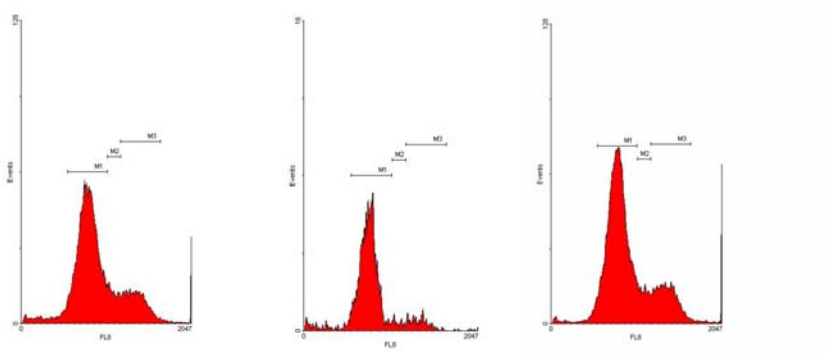
	1	2	3
Cycle phase	293T	YFP	PIN1-YFP
G1(blue)	86.88%	70.06%	63.29%
S+G2/M (yellow)	13.12%	29.94%	36.71%

Fig. 4.31 Effect of overexpression of PIN1-YFP on the cell cycle of 293T cells. 293T cells were transfected with pEYFP-N1-PIN1 or pEYFP. The cells were harvested after 48 hours and stained with propidium iodide (PI) and Hoechst 334. A) Histograms for the Hoechst stain of PIN1-YFP or YFP positive cells. B) Bar graph showing the percentage of cells ins G1 phase (blue) and S/G2/M phases (yellow).

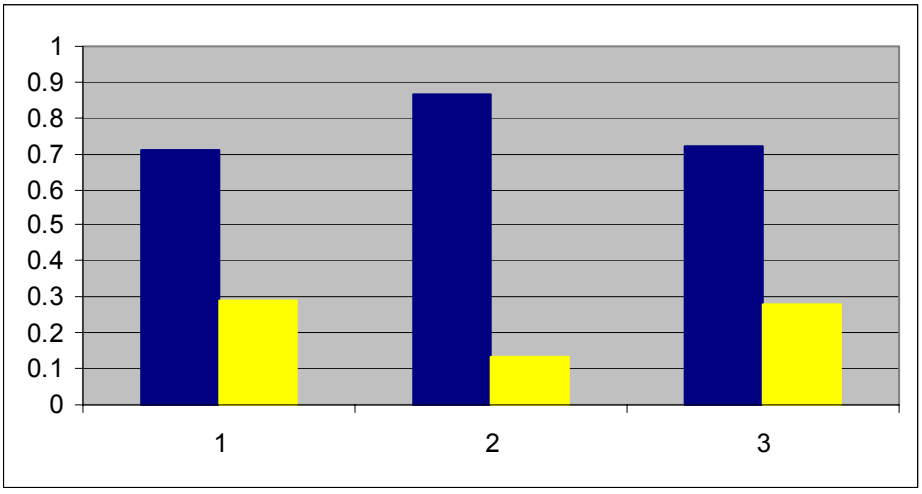
4.3.5.5 Effect of coexpression of GFP-ETV6 and TIP60β-YFP on the cell cycle

Coexpression of GFP-ETV6 and TIP60β-YFP in 293T cells had almost the same effect as expressiong GFP-ETV6 only (Fig. 4.32 A). 72.31% of GFP-ETV6 and TIP60β-YFP positive cells were in G1 phase and 27.69% cells were in S/G2/M phase (Fig. 4.32 B).

A.



B.



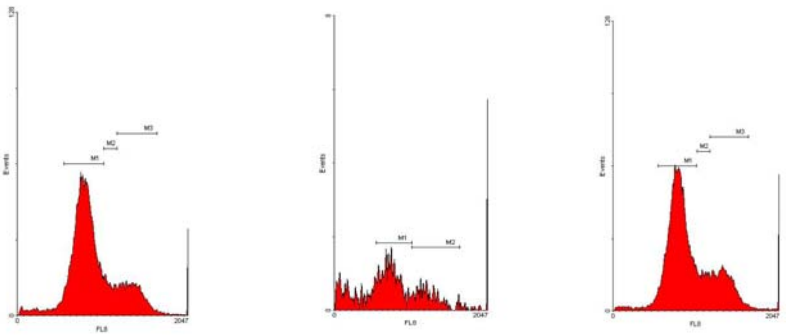
	1	2	3
Cycle phase	GFP-ETV6	TIP60β-YFP	GFP-ETV6+ TIP60β-YFP
G1(blue)	71.03%	86.65%	72.31%
S+G2/M (yellow)	28.97%	13.35%	27.69%

Fig. 4.32 Effect of coexpression of GFP-ETV6 and TIP60β-YFP on the cell cycle of 293T cells. 293T cells were transfected with pEGFP-C1-ETV6, pEYFP-N1-TIP60β and with both plasmids together. The cells were harvested after 48 hours and stained with propidium iodide (PI) and Hoechst 334. A) Histograms for the Hoechst stain of GFP-ETV6, TIP60β-YFP, or for the double positive cells. B) Bar graph showing the percentage of cells ins G1 phase (blue) and S/G2/M phases (yellow).

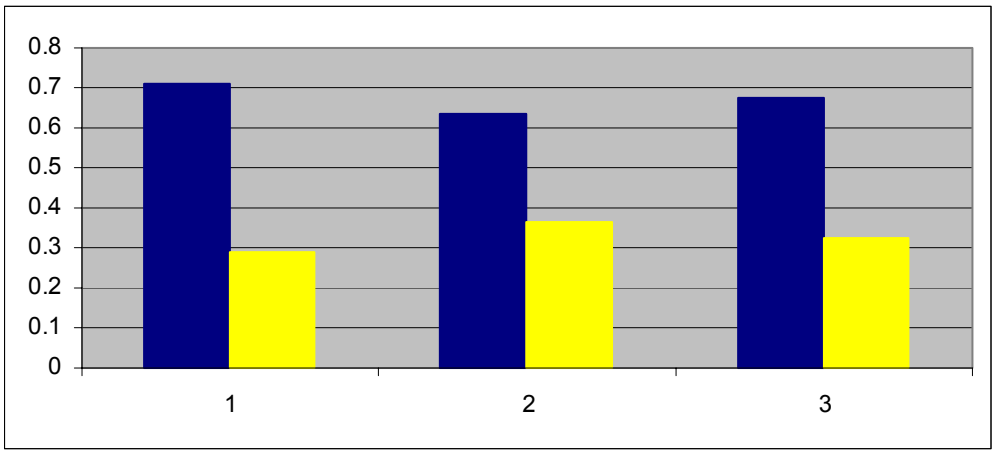
4.3.5.5 Effect of coexpression of GFP-ETV6 and PIN1-YFP on the cell cycle

Coexpression of GFP-ETV6 and PIN1-YFP resulted in a slight increase of cells in the S/G2/M phase compared to cells expressing GFP-ETV6 alone (32% versus 29%). The percentage of cells in S/G2/M phase was not as high as in the cells which expressed PIN1-YFP only (Fig. 4.33).

A.



B.



	1	2	3
Cycle phase	GFP-ETV6	PIN1-YFP	GFP-ETV6+ PIN1-YFP
G1(blue)	71.03%	63.29%	67.59%
S+G2/M (yellow)	28.97%	36.71%	32.41%

Fig. 4.33 Effect of coexpression of GFP-ETV6 and PIN1-YFP on the cell cycle of 293T cells. 293T cells were transfected with pEGFP-C1-ETV6, pEYFP-N1-PIN1 and with both plasmids together. The cells were harvested after 48 hours and stained with propidium iodide (PI) and Hoechst 334. A) Histograms for the Hoechst stain of GFP-ETV6, PIN1-YFP, or for the double positive cells. B) Bar graph showing the percentage of cells in G1 phase (blue) and S/G2/M phases (yellow).

5. Discussion

5.1 ETV6

The ETV6 protein can be divided into three functional domains: the *pointed* or SAM (sterile alpha motif) domain, the central domain and the *ets* DNA binding domain.

The SAM/*pointed* domain of ETV6 is a dimerization and oligomerization domain for ETV6 and several ETV6 fusion proteins (e.g. ETV6-NTRK3). The crystal structure of the *pointed* domain oligomer shows a helical head-to-tail structure. ETV6-*pointed* domain oligomers were also observed under the electron microscope. The formation of these polymeric structures by SAM/*pointed* domains in two different transcriptional repressors (ETV6 and polyhomeotic) suggests a conserved mode of repression involving a higher order chromatin structure (Kim et al., 2001; Kim et al., 2002). The *pointed* domain mediated dimerization is essential for the constitutive activation of the ETV6-tyrosine kinase fusion protein (Golub et al., 1996). More recent studies on ETV6-NTRK3 show that the *pointed* domain is important for the oligomerization of this fusion protein *in vivo*. When the *pointed* domain of ETV6-NTRK3 was replaced by an inducible dimerization domain of the

FK506 binding protein (FKBP), this new fusion protein failed to transform NIH3T3 cells when dimerization was not induced. So the transforming potential of ETV6-NTRK3 which is found in different types of solid tumors depends on the *pointed* domain mediated oligomerization. An ETV6-NTRK3 fusion protein with a mutated *pointed* domain did not dimerize *in vivo* and showed no transforming potential (Tognon et al., 2004). These results show that the *pointed* domain plays an important role for the function of ETV6 and of ETV6 fusion proteins.

The *pointed* domain is not only important for ETV6 oligomerization but is also an important protein-protein interaction interface. ETV6 interactions with the *ets* protein FLI1 (Kwiatkowski et al., 1998) and the SUMO conjugating enzyme UBC9 are mediated through the *pointed* domain (Chakrabarti and Nucifora, 1999; Chakrabarti et al., 1999). The polycomb group protein H-L(3)MBT (human lethal(3)malignant brain tumor) interacts with ETV6 through the SAM domain as well. H-L(3)MIBT is a transcriptional regulator with a C-terminal SPM domain which is structurally related to the SAM/*pointed* domain. The SPM domain of H-L(3)MIBT interacts with ETV6 *in vitro* and *in vivo*. This interaction enhanced the transcriptional repression caused by ETV6 on a stromelysin-1 promoter luciferase reporter gene (Boccuni et al., 2003). The *pointed* domain of ETV6 is sumoylated at lysine

99 by UBC9. SUMO-1 (small ubiquitin-like modifier) conjugation enhances the nuclear export of ETV6 which leads to reduced ETV6 transcriptional repression activity (Wood et al., 2003).

In our study, the *pointed* domain of ETV6 did not interact with TIP60β in the yeast system (Fig. 4.3). However, the N terminal 126 amino acids of ETV6 which include the *pointed* domain, showed interaction with PIN1 in the yeast two-hybrid system (Fig. 4.5). In the mammalian two hybrid system, the N-terminal domain which included the *pointed* domain showed a much stronger interaction with PIN1 than full length ETV6. (Putnik, Ph.D. thesis, 2001). The interaction between PIN1 and the ETV6 SAM/*pointed* domain is probably different from the interactions of ETV6 with FLI1 or H-L(3)MBT which use their own SAM or SAM like domains for binding. There is no SAM-like domain in PIN1. PIN1 probably recognizes the phosphorylated serine at postition 106 in the ETV6 *pointed* domain, which precedes a proline. This is the typical recognition motif for PIN1 binding (Joseph et al., 2003). Interestingly, the ETV6(S106A) mutant had a stronger repression activity than wild type ETV6 and this mutant was not responsive to PIN1 coexpression (Fig. 4.26). It appears thus very likely that the serine at position 106 in the *pointed* domain is very important for modulating the function of ETV6.

The other well-known functional domain of ETV6 is the 85 amino acid long *ets* DNA binding domain. This domain specifically binds to DNA segments with a GGAA/T core recognition sequence. The only leukemia-associated fusion protein where the *ets* domain is present in the critical fusion protein is the MN1-ETV6 fusion. The MN1-ETV6 fusion protein, which is found in acute myeloid leukaemia (AML), consists of an N-terminus derived from MN1 (Meningioma 1) and a C-terminus comprising the *ets* domain of ETV6. This fusion protein can transform NIH3T3 cells and its transformation potential is dependent on both the N-terminal MN1 portion and the ETV6 *ets* domain. Interestingly, this fusion protein showed transcriptional activator function, which also depended on both the domains from MN1 and the *ets* domain of ETV6 (Buijs et al., 2000). We did not detect any interaction between the isolated *ets* domain of ETV6 and TIP60β (Fig. 4.3) or PIN1 (Fig. 4.5).

The *pointed* domain and the central domain of ETV6 are present in most fusion proteins (especially in the very common ETV6/AML1 fusion). This implies that the central domain of ETV6 might play a significant role in the function of ETV6 and the fusion proteins. It could be demonstrated that this region interacts with a repression protein complex including SMRT (Silencing mediator of retinoic acid and thyroid hormone receptor) and mSin3A, which act as transcriptional co-repressors for ETV6/TEL. (Chakrabarti and Nucifora,

1999; Fenrick et al., 1999). Another protein that interacts with the central domain of ETV6 is N-CoR (Nuclear receptor co-repressor) (Guidez et al., 2000). In our studies, the central domain of ETV6 is an important domain for the interaction with both TIP60β and PIN1. Mapping of the TIP60β interacting domain of ETV6 in yeast shows that the whole central domain of ETV6 interacts with full length TIP60β. The TIP60β interacting domain of ETV6 can be narrowed down to the C-terminal part of the central domain (aa 282-345) and to a 23 amino acid long stretch of this region (aa 314-336) (Fig. 4.3). Nordentoft and Jorgensen reported in 2003 that the C-terminal region of the central domain and the *ets* domain of ETV6 contribute to the interaction with TIP60 as assayed in yeast. Mapping of the PIN1 interaction domain of ETV6 in the yeast two-hybrid system also showed that the C-terminal portion of the central domain (aa 282-345) could interact with PIN1 (Fig. 4.5). The serine to alanine point mutant at position 257 of ETV6 showed increased transcriptional repression activity in the stromelysin-1 promoter-based reporter assay compared to the wild type pTR334 luciferase gene reporter system (Fig. 4.14). This result suggests that Ser257 in the central domain of ETV6 might be an important site for the modulation of ETV6 repression function by PIN1.

Up to now, about 8 ETV6 interacting proteins have been shown to bind at the

pointed domain or the central domain of ETV6. These interactions influence the cellular localization or the transcriptional function or both of ETV6 (Fig. 5.1).

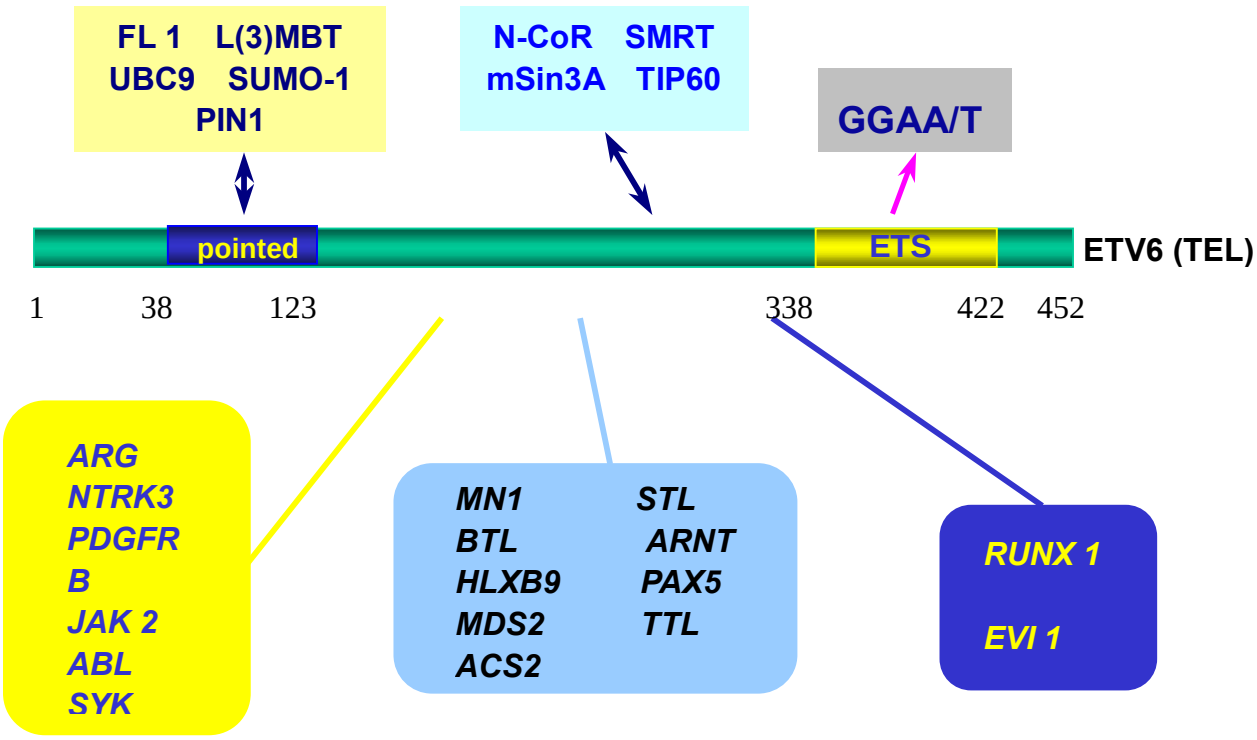


Fig. 5.1 ETV6 interacting proteins (upper part of figure) and ETV6 fusion partners (lower part of figure)

5.2 TIP60β and the ETV6-TIP60β interaction

TIP60 is a MYST domain histone acetyltransferase which catalyzes the acetylation of histones H4 and H2. Several recent studies have shown that TIP60 has not only histones as substrate but that it also interacts with numerous proteins in the cytoplasm and the nucleus. A TIP60 protein

complex was described which contains more than 13 proteins and has histone acetylase, ATPase, DNA helicase and structural DNA binding activities. This complex was shown to be involved in DNA repair and apoptosis (Ikura et al., 2000). The MYST domain of TIP60 consists of a Zinc finger and a histone co-enzyme A binding site. The MYST domain plays an important role in the function of TIP60 as a protein-interaction domain (zinc finger) and as the acetyltransferase domain. TIP60 acts as a transcriptional co-regulator. It is a transcriptional co-repressor for STAT3, CREB, ZEB and ETV6/TEL or a transcriptional co-activator for HIV1 TAT, the class I nuclear hormone receptors, for APP (beta -Amyloid Precursor Protein) and the AR (androgen receptor).

TIP60 has been shown to affect the stability and the subcellular localization of its interacting partner PIRH2, which also interacts with p53 (Logan et al., 2004). TIP60 and p53 are both modified by the ubiquitin ligase MDM2 (Katayama et al., 2004; Legube et al., 2002).

TIP60 was identified as an ETV6 interacting protein (Bohlander et al., 2001; Nordentoft and Jorgensen, 2003).

It could be shown that the phosphorylation of TIP60 is enhanced by drug

induced arrest of cells in G2/M phase. Phosphorylation of TIP60 is important for its acetytransferase activity (Lemercier et al., 2003). Our analysis showed that expression of TIP60β can increase the proportion of 293T cells in G1 phase (Fig. 4.30). Coexpression of TIP60β and ETV6 reduced the number of cells in G1 phase again (Fig. 4. 33).

Our co-localization data showed that the interaction between TIP60β and ETV6 influences the subcellular localization of ETV6. When more TIP60β was expressed, more ETV6 was localized in the nucleus together with TIP60β (Fig. 4.11). The subnuclear localization of ETV6 is regulated by sumoylation of ETV6. Sumoylation of ETV6 enhances nuclear export of the protein (Wood et al., 2003). Our data suggest that the interaction between TIP60β and ETV6 might lead to an enhanced transcriptional repression by keeping ETV6 in the nucleus for a longer time.

We could demonstrate that TIP60β is a transcriptional co-repressor of ETV6 using two different reporter gene assays. First a reporter plasmid was used in which the firefly luciferase reporter gene was under the control of GAL4 binding sites and the Herpes Simplex virus thymidine kinase promoter. This plasmid expresses luciferase at a relatively high level. Upon expression of ETV6 fused to the GAL4 DNA binding domain (GAL4DBD) a strong

repression of luciferase expression was observed. This repression was slightly enhanced by cotransfecting TIP60β suggesting a co-repressor role for TIP60β (Fig 4.20). When only the TIP60β interaction domain of ETV6 (aa282-345) fused to the GAL4DBD was expressed the repression observed was very mild. However, co-expression of TIP60β with the GAL4DBD-ETV6(282-345) showed a clear co-repression activity of TIP60β. This co-repression function of TIP60 for ETV6 was described in a similar system by Nordentoft and Jorgensen (Nordentoft and Jorgensen, 2003). Expression of TIP60β alone (a TIP60β-YFP fusion protein) led to a slight increase in reporter gene repression. Since TIP60β is not known to have a DNA binding domain we assume that in this case TIP60β acts as a co-activator for one (or several) of the transcription factors that are recruited to the Herpes Simplex virus thymidine kinase promoter in the reporter plasmid. There are numerous examples for TIP60 acting as a co-activator (see above and (Baek et al., 2002; Brady et al., 1999; Halkidou et al., 2003)).

The co-repressors SMRT, mSin3A/B and N-CoR recruit histone deacetylases (HDAC). Since ETV6 recruits these corepressors, inhibition of HDACs by TSA decreases ETV6-mediated transcriptional repression (Chakrabarti and Nucifora, 1999). When HDACs were blocked by TSA, the small TIP60β interacting domain of ETV6 (aa282-345) did not show any repressional

activity. Indeed, it slightly activated the expression of the reporter gene (Fig 4.20). However, upon co-expression of TIP60β with GAL4DBD-ETV6(282-345) under TSA conditions a clear co-repressional activity of TIP60β was observed. In other words, blocking endogenous HDACs unmasked the co-repressor acitivity of TIP60β. This observation also implies that the co-repression of TIP60β is not mediated through HDACs alone. In fact, as discussed below, an acetyltransferase (AT) dead mutant of TIP60β has reduced co-repressor activity implying an interesting new mechanism of TIP60-mediated transcriptional co-repression. These results suggest that the transcriptional repression activity of ETV6 might be mediated by both HATs and HDACs.

To examine the functional consequences of the interaction between ETV6 and TIP60β in a more physiological setting a second reporter gene assay was used. In this system ETV6 has to use its own DNA binding domain to bind to the reporter plasmid. This requirement also meant that only ETV6 as a whole could be used in order to be sure that the *ets* domain retained its three dimensional structure and was able to bind DNA. In this second system the expression of the luciferase reporter gene was controlled by a fragment of the rat stromelysin-1 promoter which contains an *ets* DNA binding sequences (EBS) and two *ets* potential binding sites (TBS) (Fenrick et al.,

2000). In this system a clear co-repressor activity of TIP60β for ETV6 could be demonstrated (Fig. 4.21 and Fig. 4.22). It could also be shown that the acetyltransferase (AT) activity of the MYST domain of TIP60β is essential for this co-repression activity. An AT dead mutant of TIP60β (TIP60β-DRS) showed no co-repressor activity (Fig. 4.18). Since we could show that an AT dead mutant of TIP60β does not interact with ETV6 (Fig 4.4) it could be speculated that this failure to interact with ETV6 is the reason for the loss of TIP60β-mediated co-repression. The ETV6 serine to alanine point mutants at position 106 (S106A) and at position 332 (S332A) showed both an increased repressional activity when TIP60β was coexpressed (Fig. 4.22). The S106A mutant showed an overall stronger repression both with and without TIP60β expression. This observation could be due to the fact that the PIN1 regulatory site is not functioning anymore (see below) but that interaction with TIP60β and hence corepression is still possible. The stronger overall repression with the S106A mutant would then be due to the lack of the anti-repression activity of cellular PIN1. On the other hand the S257A mutant shows no significant change in repression when TIP60β is expressed. This could be due to lack of interaction of ETV6 with TIP60β because of the S257A mutant. These interesting observations imply that ETV6 function is controlled by several different posttranslational modifications at different sites of the molecule.

We as well others (Nordentoft and Jorgensen, 2003) have shown that the MYST domain zinc finger of TIP60 is essential for ETV6-TIP60 interaction. Nordentoft and Jorgensen showed that a point mutant, which destroys the MYST domain zinc finger of TIP60, abolished interaction with ETV6. We have previously shown that the MYST domain zinc finger of TIP60β alone is able to interact with the C-terminal portion of the central domain of ETV6 but is unable to interact with the complete central domain of ETV6 or full length ETV6 (Putnik, Ph.D. thesis, 2001; Putnik and Bohlander, 1999). In this thesis it could be demonstrated that TIP60β AT function is also required for interaction of TIP60β with full length ETV6. A TIP60β AT dead mutant failed to interact with full length ETV6 (Fig. 4.4) but was still able to interact with the same portions of ETV6 that also showed interaction with the zinc finger of TIP60β. Taken together, these results suggest that the interaction between ETV6 and TIP60β might involve two steps: 1) acetylation of ETV6; this acetylation would change the 3D conformation of ETV6 so that the domains that are recognized by the TIP60β zinc finger become exposed; 2) stable binding of ETV6 by the TIP60β zinc finger.

Our results show that TIP60β is an important co-factor, which can modulate the transcriptional activity of ETV6. Since TIP60 itself is up-regulated after UV damage (Legube et al., 2002) and has been shown to be involved in the

p53 response (Berns et al., 2004). ETV6 might also be important in these pathways.

5.3 PIN1 and the ETV6-PIN1 interaction

Phosphorylation of proteins at serine or threonine residues which are followed by a proline is found in the regulation of many cellular functions such as cell cycle control, transcriptional regulation, differentiation and proliferation. ETV6 has been shown to be a nuclear phosphor protein (Poirel et al., 1997). The phosphorylation sites of ETV6 were mapped to serine 22 and serine 257 (Arai et al., 2002). While serine 22 of ETV6 is constitutively phosphorylated, serine 257 was shown to be inducibly phosphorylated by the p38 MAPK (mitogen activated protein kinase).

PIN1, which has been mentioned to interact with ETV6 by us (Putnik Ph.D. thesis) and others (Nordentoft and Jorgensen, 2003), is a peptidyl prolyl cis trans isomerase. PIN1 binds substrate proteins at phosphorylated serine or threoine residues followed by a proline with its WW domain. PIN1 then induces a cis – trans isomerization reaction of the peptidyl prolyl bond thereby changing the conformation of the substrate protein. It could be shown that the isolated WW domain of PIN1 exhibits the same binding

properties towards the phosphorylated target proteins as full length PIN1 (Lu, et al., 2002). In contrast, the PPIase (peptidyl prolyl isomerase) domain of PIN1 had only low or no affinity to the substrate (Verdecia, et al., 2000).

We were also able to observe this lower affinity of the PPIase domain of PIN1 for ETV6. PIN1 full length as well as the isolated WW and PPIase domains showed the same interaction pattern with different ETV6 deletion mutants. The interactions of the PPIase domains with the ETV6 deletion mutants were slightly weaker. Interaction of PIN1 was observed with full length ETV6, the first 126 amino acids of ETV6, which include the *pointed* domain, and the C-terminal half of the central domain. Interestingly, there was no interaction between PIN1, the WW domain or the PPIase domain and the central domain of ETV6. The fact that PIN1 and its two domains interacted again with the C-terminal half of the central domain could be explained by steric hindrance or blockage of the PIN1 interaction interface in the C-terminal portion of the central domain by the N-terminal portion of the central domain. This situation is reminiscent of the interaction pattern observed between ETV6 and TIP60β. The inducibly phosphorylated serine 257 – Pro could be the target of PIN1 interaction in the C-terminal half of the central domain. Putnik could show that the strongest interaction between ETV6 and PIN1 occurs if aa 1-126 (including the *pointed* domain) of ETV6 are used in the

mammalian two hybrid system (Putnick, Ph.D. thesis, 2001). Similar results were obtained in this work with the yeast two hybrid system (Fig. 4.5). Since it could also be shown that the first 59 amino acids of ETV6 (which contain the reported Serine 22 phosphorylation site) slightly interact with PIN1 (Fig. 4.5), the interaction of PIN1 and the N-terminus of ETV6 (aa 1-126) might occur at the Ser106-Pro or the Ser22-Pro bond (Fig. 5.2). So three ETV6 serine to alanine mutants at position106, Ser257 and Ser332 were constructed and tested in stromelysine-1 promoter-based reporter gene system (Fig. 4.14, Fig. 4.22 and Fig. 4.26).

Fig. 5.2 Four potential PIN1 binding sites in ETV6

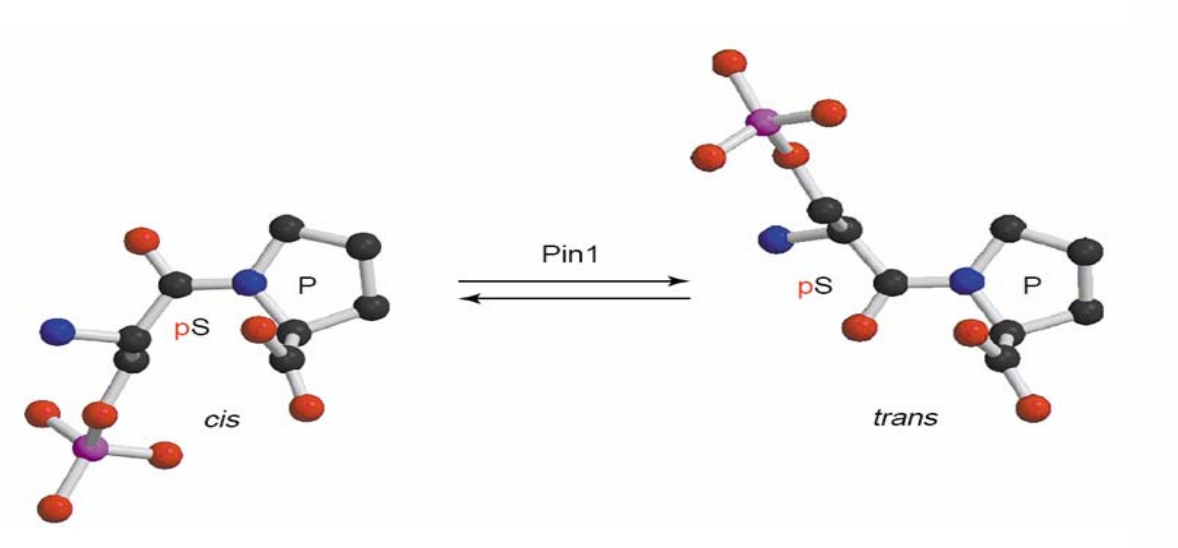
The subcellular localization studies that we performed with a PIN1-YFP protein showed that PIN1 can be found in the cytoplasm and the nucleus with more staining in the nucleus. The bright green spots of PIN1-YFP were always prominently observed in the nucleus. This distribution could be caused by the overexpresseion of PIN1-YFP as well by its interacting with one or several of its different substrate proteins. The co-localization experiments of ETV6 (CFP-ETV6) and PIN1-YFP showed that both proteins

exhibit a similar staining pattern in the nucleus suggesting physical interaction of PIN1 and ETV6 (Fig 4.12). In some nuclei a striking dot like pattern of PIN1-YFP was observed. The same pattern could be seen in these nuclei in the distribution of CFP-ETV6. At the present time we are not sure whether this dot like pattern of PIN1-YFP represents an over-expression artefact or whether it has some physiological relevance. This suggests that PIN1 modulates the transcriptional function of ETV6 in the nucleus. This is in line with the observation that PIN1 can inhibit the binding of beta-catenin to APC and thereby lead to an increased localization of beta-catenin in the nucleus (Ryo, Nakamura et al., 2001).

On a functional level the interaction of ETV6 and PIN1 had quite profound consequences for the well-known function of ETV6 as a transcriptional repressor. When PIN1 was co-transfected in the GAL4-UAS HSV thymidine kinase based reporter system the repressional activity of ETV6 was greatly inhibited (Fig 4.23). When PIN1 was co-expressed with ETV6 in the stromelysin-1 promoter based reporter system, ETV6 was turned from a repressor of transcription to a clear activator of transcription (Fig. 4.25). This is a very interesting observation since it implies that ETV6 might function as an activator of transcription under the appropriate cellular conditions and that PIN1 is critically involved in regulating the function of ETV6 (Zhang C.D. et al.,

2004). When the three ETV6 serine to alanine point mutants were tested with PIN1 in the reporter gene assays, the repressional activity of the mutants ETV6(S257A) and ETV6(S332A) was still relieved. However, the transcriptional repression of the ETV6(S106A) mutant could not be relieved by PIN1 coexpression (Fig. 4.26). This indicates that phosphorylation at serine 106 is essential for the effect that PIN1 has on ETV6 function. If serine 106 cannot be phosphorylated anymore, PIN1 is probably unable to isomerize Ser106-Pro107 bond. It can be hypothesized that PIN1 causes a conformational switch in the ETV6 protein through peptidyl-prolyl isomerization at Ser106 – Pro bond which converts ETV6 from a transcriptional repressor to a transcriptional activator. Since it is known that PIN1 interacts with many other proteins, it can be assumed that these proteins are regulated in a similar fashion by PIN1 (see reviews (Lu et al., 2002; Lu, 2004; Shaw, 2002; Zhou et al., 1999)).

A.



Lu K.P. et al., 2002

B.

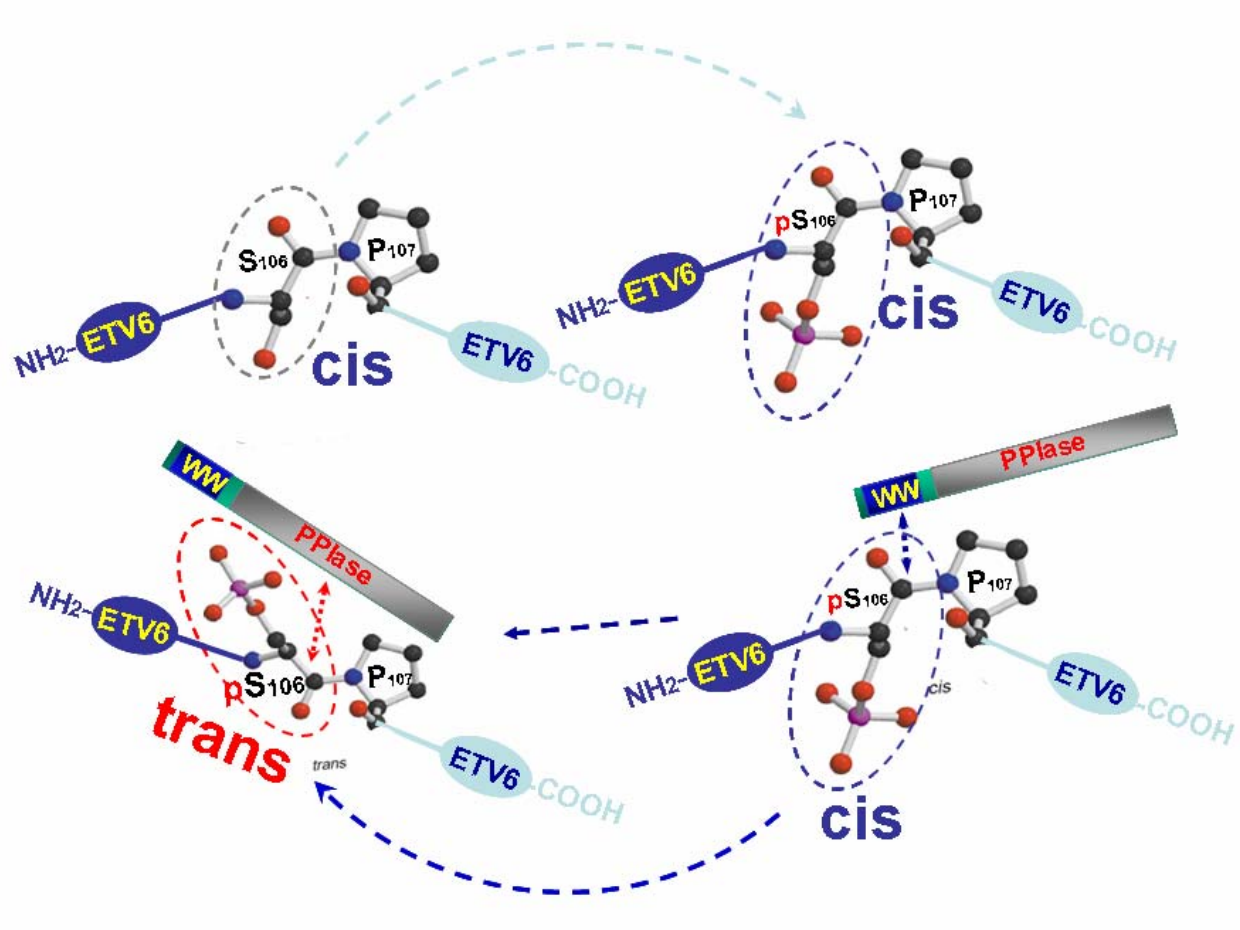


Fig. 5.3 Hypothetical model showing the effect of PIN1 peptidyl prolyl isomerization on the conformation of ETV6. A) Effect of PIN1 cis to trans isomerization on an isolated Ser-Pro bond. B. Diagram showing hypothetical effect of PIN1 action on the conformation of ETV6. The cis to trans isomerization of ETV6, which has to be preceded by serine phosphorylation, completely changes the conformation of the ETV6 protein. In this example the serine-proline bond at position 106 of ETV6 is affected by isomerization.

Lu K.P. showed in 1996 that the depletion of PIN1 in HeLa cells induced mitotic arrest. Overexpression of PIN1 in Hela cells caused cells to arrest in G2 phase (Lu et al., 1996). In our experiments, PIN1 caused an increase in the proportion of S/G2/M phase cells in 293T cells. This observation is compatible with an arrest of the cells in S, G2 or M phase (Fig. 4.31). This effect of PIN1 expression was also visible when ETV6 was coexpressed. Although accumulation in S/G2/M phase was slightly less in these coexpression experiments (Fig. 4.33).

Summary

The *ETV6* gene is involved in many chromosomal translocations forming different fusion genes in both myeloid and lymphoid leukemias as well as in some solid tumors. *ETV6* is a member of the *ets* family of transcription factors and has been shown to be a transcriptional repressor. There is also evidence that *ETV6* is a tumor suppressor gene in certain leukemias. ETV6 interacts with TIP60β and PIN1. TIP60 (HIV-1 Tat interacting protein, 60 kDa), a member of the MYST family of histone acetyltransferases, is a transcriptional co-activator or co-repressor depending on the interacting transcription factor. PIN1, an important peptidyl-prolyl cis/trans isomerase, is the only known enzyme to specifically isomerize phosphorylated serine or threonine - proline bonds. This isomerization reaction changes the conformation of the protein substrate and is a central signaling mechanism controlling normal cell proliferation and malignant transformation. In this work the interactions between ETV6 and TIP60β and between ETV6 and PIN1 were analyzed in detail.

Using the yeast two hybrid system the TIP60β interacting domain of ETV6 was mapped to the C terminal half of the central domain of ETV6. The interaction between full length ETV6 and TIP60β was shown to be dependent

on an intact acetyltransferase activity (HAT) of TIP60β. Interestingly, the HAT deficient TIP60β mutant is still able to interact with the C terminal half of the ETV6 central domain. These results imply a complicated mechanism of ETV6-TIP60β interaction which might involve acetylation of ETV6 by TIP60β and a subsequent conformational change of ETV6. In transient co-transfection experiments using fluorescently tagged proteins ETV6 and TIP60β were co-localized in the nucleus. Reporter gene assays demonstrated that TIP60β is a corepressor of ETV6. This co-repressor activity of TIP60β was especially noticable when a reporter plasmid based on the ETV6-responsive stromelysin-1 promoter was used. TIP60β corepressor activity was dependent on an intact TIP60 acetyl- transferase domain. TIP60β did not exhibit corepessor activity for a serine to alanine mutant at position 257 of ETV6. This result could be caused by a lack of interaction between ETV6(S257A) and TIP60β which might be due to a requirement for a phosphorylation / isomerization dependent conformational change of ETV6 in this region for TIP60β interaction. The expression of GFP-ETV6 had no discernible effect on the proportion of cells in the different phases of the cell cycle as compared to the expression of GFP or YFP. The expression of a TIP60β-YFP construct led to a marked reduction of the proportion of cells in the S/G2/M phase of the cell cycle. Coexpression of both GFP-ETV6 and TIP60β-YFP restored the normal distribution of cycling cells.

Similar studies were performed to analyze the interaction between ETV6 and PIN1. The PIN1 interaction domain of ETV6 was mapped to the *pointed* domain and to the C-terminal half of the central region of ETV6 in the yeast two hybrid system. In both regions, PIN1 consensus binding sites containing a serine/threoine – proline sequence are present. It could be shown that both the WW domain and the PPIase domain of PIN1 participate in the interaction. Co-localization of ETV6 and PIN1 was seen in the nucleus of transiently transfected cells. On a functional level, co-expression of PIN1 with ETV6 markedly reduced the repressor activity of ETV6 when ETV6 was fused to a heterologous DNA binding domain (GAL4DBD). When the stromelysin-1 promoter based reporter gene system was used, co-expression of PIN1 not only relieved ETV6 mediated repression but actually turned ETV6 from a transcriptional repressor to a transcriptional activator. A serine to alanine mutant at position 106 of ETV6 was completely unresponsive to PIN1 coexpression which indicates that S106 of ETV6 is the main site of PIN1 interaction. These findings show that ETV6 activity can be regulated in very decisive ways by interaction with PIN1. It could be that this regulation of ETV6 through PIN1 is a result of ETV6 peptidyl-prolyl cis-trans isomerization and hence a conformational change of ETV6. Expression of YFP-PIN1 led to a slight increase in the proportion of cells in the S/G2/M phase. Like for TIP60β,

coexpression of GFP-ETV6 led to a restoration of the distribution of cycling cells.

Zusammenfassung

Das ETV6-Gen ist an vielen chromosomalen Translokationen beteiligt und bildet viele verschiedene Fusionsgene. ETV6-Fusionsgene findet man sowohl bei myeloischen und lymphatischen Leukämien als auch bei gewissen soliden Tumoren. ETV6 ist ein Mitglied der *ets* Familie von Transkriptionsfaktoren. Es gibt Hinweise darauf, daß ETV6 auch ein Tumorsuppressorgen bei Leukämien ist.

ETV6 interagiert mit TIP60 und PIN1. TIP60 (HIV-1 Tat interacting protein, 60 kDa), eine MYST–Domänen Histonacetyltransferase, ist ein transkriptioneller Ko-Aktivator oder Ko-Repressor je nachdem mit welchem Transkriptionsfaktor es interagiert. PIN1, eine wichtige peptidyl-prolyl cis trans Isomerase, ist das einzige bekannte Enzym, das spezifisch phosphorylierte Serin/Threonin–Prolin Peptidbindungen isomerisiert. Diese Isomerisierungsreaktion verändert die Konformation des Proteinsubstrats und ist ein zentraler Signaltransduktionsmechanismus der die normale Zellproliferation und die maligne Transformation kontrolliert.

In dieser Arbeit wurde die Interaktion zwischen ETV6 und TIP60 und zwischen ETV6 und PIN1 im Detail analysiert.

Im Yeast Two Hybrid System konnte die TIP60-Interaktionsdomäne von ETV6 auf die C-terminale Hälfte der zentralen Region von ETV6 kartiert werden. Es konnte gezeigt werden, daß für die Interaktion zwischen dem vollständigen ETV6-Protein und TIP60 die Acetyltransferasefunktion (HAT) von TIP60 notwendig war. Interassanterweise, ist die HAT defiziente TIP60-Mutante noch in der Lage mit der C terminalen Hälfte der zentralen Region von ETV6 zu interagieren. Diese Ergebnisse legen nahe, daß der Interaktion zwischen ETV6 und TIP60 ein komplizierter Mechanismus zugrunde liegt, der möglicherweise acetylierungsabhängige Konformationsänderungen von ETV6 voraussetzt. In transienten Kotransfektionsexperimenten mit mit fluoreszierenden Proteinen markiertem ETV6 und TIP60 konnte eine Kolokalisation der beiden Proteine im Zellkern beobachtet werden. In Reportergenassays konnte gezeigt werden, daß TIP60 ein transkriptioneller Korepressor für ETV6 ist. Diese Korepressoreigenschaft von TIP60 für ETV6 wurde besonders in Reportergenassays sichtbar, die auf dem ETV6–responsiven Stromelysin-1 Promotor basierten. Die TIP60-Korepressorfunktion war auf eine intakte HAT-Domäne von TIP60 angewiesen. TIP60 wies keine Korepressoraktivität für eine Punktmutante von ETV6, die an Position 257 ein Alanin anstatt eines Serins hatte, auf. Dies könnte auf eine fehlende Interaktion zwischen ETV6(S257A) und TIP60 zurückzuführen sein.

Möglicherweise ist die TIP60-ETV6 Interaktion auf eine Phosphorylierung / Isomerisierungs abhängige Konformationsänderung von ETV6 in diesem Bereich angewiesen. Die Expression von CFP-ETV6 hatte keinen signifikanten Einfluß auf die Zellzyklusregulation im Vergleich zur Expression von CFP oder YFP alleine. Die Expression von TIP60-YFP führte zu einer merklichen Abnahme des Anteils der Zellen, die sich in der S/G2M Phase befanden. Die Koexpression von CFP-ETV6 und TIP60-YFP führte wieder zu einer normalen Verteilung der Zellen in den Phasen des Zellzyklus.

Die Interaktion von ETV6 mit PIN1 wurde mit ähnlichen Experimenten untersucht. Im Yeast Two Hybrid System wurde die PIN1 Interaktionsdomäne von ETV6 auf die *pointed* Domäne und auf die C-terminale Hälfte der zentralen Region von ETV6 kartiert. In beiden Regionen sind PIN1 Konsensus-Bindungsstellen vorhanden, die eine Serin/Threonin–Prolin–Peptidbindung enthalten. ETV6 und PIN1 konnten im Zellkern von transient transfizierten Zellen kolokalisiert werden. Funktionell konnte gezeigt werden, daß die Ko-Expression von PIN1 mit ETV6, die transkriptionelle Repressorfunktion von ETV6, wenn ETV6 als Fusion mit einer heterologen DNA Bindungsdomäne exprimiert wurde (GAL4DBD), deutlich reduzierte. In einem Reportergenassay, der auf dem ETV6-responsiven Stromelysin-1 Promoter basiert, verwandelte sich ETV6

durch Ko-Expression von PIN1 von einem Repressor zu einem Aktivator der Transkription. Diese Ergebnisse zeigen, daß ETV6 in seiner Funktion in ganz entscheidender Weise durch die Interaktion mit PIN1 reguliert wird. Es könnte sein, daß die PIN1-bedingte Regulation von ETV6 ein Resultat einer peptidyl-cis-trans Isomerisierungsreaktion an ETV6 ist, und somit durch eine Änderung der Konformation von ETV6 bedingt ist. Die Expression von YFP-PIN1 führte zu einer geringfügigen Zunahme der Zellen, die sich in der S/G2/M Phase des Zellzyklus befanden. Wie bei TIP60, führte die Koexpression von CFP-ETV6 zu einer Normalisierung der Zellen in den Phasen des Zellzyklus.

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Abbreviations Used

α	anti/alpha
aa	amino acid
Ab	antibody
ALL	acute lymphoblastic leukemia
AP	alkaline phosphatase
ATP	adenosine triphosphate
BCR	B cell receptor
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CML	chronic myeloid leukemia
CMV	cytomegalovirus
Coom.	coomassie
ddH2O	doubly distilled water
DAPI	4´,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EB	ethidium bromide
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
ets	erythroblast transformation specific
ETV6	ets translocation variant 6
FCS	fetal calf serum
GFP	green fluorescent protein
GH	growth hormone
GST	glutathione-S-transferase
h	human
HAT	histone acetyltransferases
HDAC	histone deacetylase enzymes
HLH	helix-loop-helix
hr	hour
HRP	horseradish peroxidase
Ig	Immunoglobulin
IPTG	isopropyl-1-thio-β-D galactopyranoside

IR	insulin receptor
IRES	internal ribosomal entry site
ITAM	immunoreceptor tyrosine-based activation motif
kb	kilobase
kDa	kilo dalton
LB	Luria-Bertani medium
2-ME	2-mercapto ethanol
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MCS	multiple cloning site
MDS	myelodysplastic syndrome
MOZ	monocytic leukemia zinc finger protein
mRNA	messenger RNA
MW	molecular weight
NIMA1	never in mitosis A
O.D.	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFRβ	platelet derived growth factor receptor β
PEG	polyethylene glycol
PI	propidium iodide
PIN1	protein interaction with NIMA1
PMSF	phenylmethylsulfonyl fluoride
PoV	sodium pervanadate
RNA	ribonucleic acid
RT	room temperature
SAM	sterile alpha motif
SDS	sodium dodecyl sulphate
SEAP	secreted alkaline phosphatase
sec	second
SIRP	signal regulating protein
SREs	serum response elements
STAT	signal tranducer and activator of transcription
TAE	tris acetate buffer
TBS	tris buffered saline
TCR	T-cell receptor
TF	transcription factor
TIP60β	human HIV tat interaction protein, Mw 60 KDa
Tm	melting temperature

TNFα	tumour necrosis factor alpha
Tris-Cl	tris hydrochloride
U	unit of enzymatic activity
UBC9	ubiquitin-conjugating enzyme 9
UV	ultraviolet
v/v	volume-per-volume
X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside

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Research and Publications

Publication list:

ETV6 (TEL) Interacts with the MYST Domain Histone Acetyltransferase TIP60
Jasmin Putnik*, Chang-Dong Zhang*, Sigrun Bartels, Michael Kickstein, Stefan K. Bohlander
Genes Chromosomes Cancer, resubmitted, *both authors contributed equally

SALL1, the gene mutated in Townes-Brocks syndrome, encodes a transcriptional repressor which interacts with TRF1/PIN2 and localizes to pericentromeric heterochromatin

Christian Netzer, Leonie Rieger, Alessandro Brero, Chang-Dong Zhang, Markus Hinzke, Jurgen Kohlhase, Stefan K. Bohlander
Human Molecular Genetics, 2001 Dec 15; 10(26): 3017-24.

Presentations:

The peptidyl prolyl cis trans isomerase PIN1 interacts with ETV6 and converts ETV6 from a transcriptional repressor to a transcriptional activator
Changdong Zhang, Jasmina Putnik, Stefan K. Bohlander
Oral presentation at the 9th EHA (European Society of Hematology) annual meeting, Jun. 2004, Geneva, Swissland, ***The Hematology Journal*** (2004) 5, S209
This presentation received a travel award.

The Acetyltransferase Activity of TIP60β is required for TIP60β-ETV6 Interaction and Influences the Subcellular Localization of ETV6
Chang-Dong Zhang, Stefan K. Bohlander
Poster presentation at the 45th ASH (American Society of Hematology) annual meeting, Dec. 2003, San Diego, U.S.A. ***Blood***, 2003 Nov 16; 102(11): 857a

Characterization of the interaction of ETV6 with the Histone Acetyltransferase TIP60 and Peptidyl-prolyl cis-trans Isomerase PIN1

Chang-Dong Zhang, Jasmin Putnik, Stefan K. Bohlander
Oral presentation at the 13th European Students' Conference, Oct. 2002, Berlin, Germany

Study on affinity ligands of human granulocyte colony-stimulating factor
Chang-Dong Zhang, Hui Zhou, Chun Ji, Hui-Peng Chen, Wei Li
Oral presentation at the 3rd International Symposium on Frontiers of Protein Chemistry and Technology, Aug. 1998, Changchun, P. R. China

Appendix

The raw data of Fig.4.14			
	1	2	3
Plasmid (µg)	negative	ETV6	pM1-TEL(282-345)
GAL4 tk LUC	0.3	0.3	0.3
null PRL	0.05	0.05	0.05
pM1	0.3		
pM1-TEL		0.3	
pM1-TEL(282-345)			0.3
Relative Light Units (RLU)			
Luciferase activity(firefly)	84.71	6.776	37.93
	57.02	6.8	53.26
	59.03	6.442	53.97
Luciferase activity(Renilla)	52.7	77.08	50.91
	39.32	92.89	56.67
	42.67	83.3	56.08
Ratio (firefly/Renilla)	1.607	0.088	0.745
	1.45	0.073	0.94
	1.383	0.077	0.962
Standard deviation	0.115	0.008	0.119
Average Ratio	1.48	0.079	0.882

The raw data of Fig.4.15						
	1	2	3	4	5	6
Plasmid (µg)	control	ETV6	ETV6 (S106A)	ETV6 (S257A)	ETV6 (S332A)	negative
pTR 334	0.3	0.3	0.3	0.3	0.3	
null PRL	0.05	0.05	0.05	0.05	0.05	
CFP C1-TEL		0.3				
CFP C1-TEL S106A			0.3			
CFP C1-TEL S257A				0.3		
CFP C1-TEL S332A					0.3	
pECFP C1	0.3					
Relative Light Units (RLU)						
Luciferase activity(firefly)	1847	754.9	60.84	641.4	752.1	0.008
	2083	613.3	808.3	780.2	1786	0.006
	1865	582.7	578.6	681.9	1161	0
Luciferase activity(Renilla)	2145	3144	406.2	5466	3335	0.007
	2208	2624	5720	6555	8257	0.132
	2033	2230	3891	5873	5079	0.089
Ratio (firefly/Renilla)	0.861	0.24	0.15	0.117	0.226	0.0112
	0.944	0.234	0.141	0.119	0.216	0.046
	0.917	0.261	0.149	0.116	0.229	0.002
Average Ratio	0.907	0.245	0.147	0.117	0.224	0.02
Standard deviation	0.042	0.014	0.005	0.002	0.007	0.023

The raw data of Fig.4.15						
	1	2	3	4	5	6
Plasmid (µg)	control	ETV6	ETV6 (S106A)	ETV6 (S257A)	ETV6 (S332A)	negative
pTR 334	0.3	0.3	0.3	0.3	0.3	
null PRL	0.05	0.05	0.05	0.05	0.05	
CFP C1-TEL		0.3				
CFP C1-TEL S106A			0.3			
CFP C1-TEL S257A				0.3		
CFP C1-TEL S332A					0.3	
pECFP C1	0.3					
	1847	754.9	60.84	641.4	752.1	0.008
Luciferase activity(firefly)	2083	613.3	808.3	780.2	1786	0.006
	1865	582.7	578.6	681.9	1161	0
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Luciferase activity(Renilla)	2208	2624	5720	6555	8257	0.132
	2033	2230	3891	5873	5079	0.089
	0.861	0.24	0.15	0.117	0.226	0.0112
Deviation Ratio	0.944	0.234	0.141	0.119	0.216	0.046
	0.917	0.261	0.149	0.116	0.229	0.002
Average Ratio	0.907	0.245	0.147	0.117	0.224	0.02
Error bar	0.042	0.014	0.005	0.002	0.007	0.023

The raw data of Fig.4.16						
	1	2	3	4	5	6
Plasmid (µg)	negative	negative+TSA	ETV6	ETV6+TSA	pM1-TEL(282-345)	pM1-TEL(282-345)+TSA
GAL4 tk LUC	0.3	0.3	0.3	0.3	0.3	0.3
null PRL	0.05	0.05	0.05	0.05	0.05	0.05
pM1	0.3	0.3				
TSA		1.5ML		1.5ML		1.5ML
pM1-TEL			0.3	0.3		
pM1-TEL(282-345)					0.3	0.3
Relative Light Units (RLU)						
Luciferase activity(firefly)	84.71	28.49	6.776	8.928	37.93	69.84
	57.02	56.25	6.8	9.229	53.26	75.28
	59.03	45.05	6.442	8.24	53.97	70.56
Luciferase activity(Renilla)	52.7	18.67	77.08	26.35	50.91	27.04
	39.32	20.81	92.89	25.57	56.67	29.71
	42.67	20.37	83.3	25.78	56.08	25.52
Ratio (firefly/Renilla)	1.607	2.062	0.088	0.339	0.745	2.583
	1.45	2.703	0.073	0.361	0.94	2.534
	1.383	2.212	0.077	0.32	0.962	2.764
Standard deviation	0.115	0.335	0.008	0.021	0.119	0.121
Average Ratio	1.48	2.326	0.079	0.34	0.882	2.627

The raw data of Fig.4.17		
	1	2
Plasmid (µg)	negative	TIP60β
GAL4 tk LUC	0.3	0.3
null PRL	0.05	0.05
pM1	0.3	0.3
pEYFP N1-TIP60β		0.3
pEYFP N1	0.3	
Relative Light Units (RLU)		
Luciferase activity(firefly)	84.71	18.09
	57.02	20.56
	59.03	15.6
Luciferase activity(Renilla)	52.7	9.456
	39.32	8.691
	42.67	7.068
Ratio (firefly/Renilla)	1.607	1.913
	1.45	2.365
	1.383	2.207
Standard deviation	0.115	0.229
Average Ratio	1.48	2.162

The raw data of Fig.4.18				
	1	2	3	4
Plasmid (μg)	negative	control	TIP60β	TIP60β
pTR89	0.2		0.2	
pTR334		0.2		0.2
null PRL	0.05	0.05	0.05	0.05
pEYFP N1-TIP60β			0.2	0.2
pEYFP N1	0.2	0.2		
Relative Light Units (RLU)				
Luciferase activity(firefly)	8.025	71.89	2.992	64.31
	6.277	66.91	2.751	69.64
	5.906	57.87	1.983	64.63
Luciferase activity(Renilla)	37.32	42.22	53.5	47.79
	24.49	37.85	49.38	56.73
	32.83	38.76	42.94	52.89
Ratio (firefly/Renilla)	0.215	1.703	0.056	1.346
	0.256	1.768	0.056	1.228
	0.18	1.493	0.046	1.222
Standard deviation	0.038	0.144	0.006	0.007
Average Ratio	0.217	1.655	0.053	1.265

The raw data of Fig.4.19			
	1	2	3
Plasmid (µg)	control	TIP60β	TIP60DRS
pTR334	0.2	0.2	0.2
null PRL	0.05	0.05	0.05
pEYFP N1-TIP60β		0.2	
pEYFP N1-TIP60DRS			0.2
pEYFP N1	0.2		
Relative Light Units (RLU)			
Luciferase activity(firefly)	23.68	12.71	13.47
	22.96	16.8	33.22
	21.7	17.96	25.62
Luciferase activity(Renilla)	17.95	13.14	10.95
	14.4	17.74	19.53
	14.16	19.32	15.89
Ratio (firefly/Renilla)	1.319	0.967	1.23
	1.595	0.947	1.701
	1.532	0.93	1.612
Average Ratio	1.482	0.948	1.514
Standard deviation	0.145	0.19	0.25

The raw data of Fig.4.20				
	1	2	3	4
Plasmid (µg)	negative	negative	PIN1	PIN1
pTR89	0.2		0.2	
pTR334		0.2		0.2
null PRL	0.05	0.05	0.05	0.05
pEYFP N1-PIN1			0.2	0.2
pEYFP N1	0.2	0.2		
Relative Light Units (RLU)				
Luciferase activity(firefly)	8.025	71.89	9.746	160
	6.277	66.91	9.247	145.4
	5.906	57.87	8.991	134.2
Luciferase activity(Renilla)	37.32	42.22	48.28	67.89
	24.49	37.85	51.02	59.99
	32.83	38.76	52.04	53.95
Ratio (firefly/Renilla)	0.215	1.703	0.202	2.356
	0.256	1.768	0.181	2.424
	0.18	1.493	0.173	2.488
Standard deviation	0.038	0.144	0.015	0.066
Average Ratio	0.217	1.655	0.185	2.423

The raw data of Fig.4.21						
	1	2	3	4	5	6
Plasmid (µg)	negative	TIP60β	ETV6	ETV6+TIP60β	pM1-TEL(282-345)	pM1-TEL(282-345)+TIP60β
GAL4 tk LUC	0.3	0.3	0.3	0.3	0.3	0.3
null PRL	0.05	0.05	0.05	0.05	0.05	0.05
pM1	0.3	0.3				
pEYFP N1-TIP60		0.3		0.3		0.3
pEYFP N1	0.3		0.3		0.3	
pM1-TEL			0.3	0.3		
pM1-TEL(282-345)					0.3	0.3
Relative Light Units (RLU)						
Luciferase activity(firefly)	84.71	18.09	6.776	4.846	37.93	34.08
	57.02	20.56	6.8	1.561	53.26	40.84
	59.03	15.6	6.442	1.858	53.97	30.12
Luciferase activity(Renilla)	52.7	9.456	77.08	80.19	50.91	57.6
	39.32	8.691	92.89	35.14	56.67	63.7
	42.67	7.068	83.3	74.94	56.08	60.57
Ratio (firefly/Renilla)	1.607	1.913	0.088	0.06	0.745	0.592
	1.45	2.365	0.073	0.044	0.94	0.641
	1.383	2.207	0.077	0.025	0.962	0.497
Standard deviation	0.115	0.229	0.008	0.018	0.119	0.073
Average Ratio	1.48	2.162	0.079	0.043	0.882	0.577

The raw data of Fig. 4.22									
	1	2	3	4	5	6	7	8	9
Plasmid (µg)	control	TIP60β	ETV6	E6 + TIP60β	control	TIP60β	ETV6	E6 + TIP60β	Negative
pTR89	0.2	0.2	0.2	0.2					
pTR334					0.2	0.2	0.2	0.2	
null PRL	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	
CFP C1	0.2	0.2			0.2	0.2			
pECFP - ETV6			0.2	0.2			0.2	0.2	
pEYFP N1-TIP60β		0.2		0.2		0.2		0.2	
pEYFP N1	0.2		0.2		0.2		0.2		
Relative Light Units (RLU)									
Luciferase activity(firefly)	8.025	2.992	0.344	2.253	71.89	64.31	39.81	17.64	0.01
	6.277	2.751	1.699	2.01	66.91	69.64	36.63	18.64	0.023
	5.906	1.983	3.061	1.625	57.87	64.63	32.32	20.58	0.012
Luciferase activity(Renilla)	37.32	53.5	2.781	62.45	42.22	47.79	55.13	59.1	1.934
	24.49	49.38	13.69	66.18	37.85	56.73	49.42	58.46	2.41
	32.83	42.94	23.3	47.27	38.76	52.89	40.79	56.53	2.101
Ratio (firefly/Renilla)	0.215	0.056	0.124	0.036	1.703	1.346	0.722	0.298	0.005
	0.256	0.056	0.124	0.03	1.768	1.228	0.741	0.319	0.009
	0.18	0.046	0.131	0.034	1.493	1.222	0.793	0.364	0.006
Standard deviation	0.038	0.006	0.004	0.003	0.144	0.07	,037	0.034	0.002
Average Ratio	0.217	0.055	0.126	0.033	1.655	1.265	0.752	0.327	0.007

The raw data of Fig.4.23											
	1	2	3	4	5	6	7	8	9	10	11
Plasmid (µg)	control	TIP60β	ETV6	TIP60β+ E6	ETV6 S106A	TIP60β+E6 S106A	ETV6 S257A	TIP60β+E6 S257A	ETV6 S332A	TIP60β+E6 S332A	negative
pTR 334	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	
null PRL	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	
pEYFP N1	0.2		0.2		0.2		0.2		0.2		
pEYFP N1-TIP60β		0.2		0.2		0.2*3=0.6		0.2		0.2	
CFP C1-TEL			0.2	0.2							
CFP C1-TEL 106					0.2	0.2					
CFP C1-TEL 257							0.2	0.2			
CFP C1-TEL 332									0.2	0.2	
pECFP C1	0.2	0.2									
Relative Light Units (RLU)											
Luciferase activity(firefly)	1847	514.2	754.9	62.24	60.84	91.03	641.4	127	752.1	99.12	0.008
	2083	639.2	613.3	59.8	808.3	166.9	780.2	78.31	1786	46.25	0.006
	1865	179.3	582.7	35.06	578.6	160.7	681.9	76.22	1161	21.38	0
Luciferase activity(Renilla)	2145	1810	3144	650.6	406.2	1504	5466	870.3	3335	745.7	0.007
	2208	2325	2624	329.3	5720	2740	6555	367.8	8257	687.8	0.132
	2033	567.1	2230	323.5	3891	2968	5873	481.2	5079	162.4	0.089
Ratio (firefly/Renilla)	0.861	0.284	0.24	0.096	0.15	0.061	0.117	0.146	0.226	0.133	0.0112
	0.944	0.275	0.234	0.182	0.141	0.061	0.119	0.213	0.216	0.067	0.046
	0.917	0.316	0.261	0.108	0.149	0.054	0.116	0.158	0.229	0.132	0.002
Average Ratio	0.907	0.292	0.245	0.129	0.147	0.059	0.117	0.172	0.224	0.111	0.02
Standard deviation	0.042	0.022	0.014	0.047	0.005	0.004	0.002	0.036	0.007	0.038	0.023

The raw data of Fig.4.24				
	1	2	3	4
Plasmid (µg)	negative	ETV6	PIN1	ETV6/PIN1
pTK Luc	0.3	0.3	0.3	0.3
null PRL	0.05	0.05	0.05	0.05
pM1	0.2		0.2	
pM1-TEL		0.2		0.2
pEYFP N1-PIN1			0.4	0.4
pEYFP N1	0.4	0.4		
Relative Light Units (RLU)				
Luciferase activity(firefly)	4.12	0.513	4.512	2.207
	3.448	0.725	7.67	2.185
	3.005	0.462	4.936	2.478
Luciferase activity(Renilla)	4.946	4.994	10.27	9.094
	4.813	4.547	10.83	8.45
	4.229	4.962	9.16	8.27
Ratio (firefly/Renilla)	0.833	0.103	0.439	0.243
	0.716	0.159	0.708	0.259
	0.711	0.093	0.539	0.3
Standard deviation	0.069	0.036	0.136	0.029
Average Ratio	0.753	0.118	0.562	0.267

The raw data of Fig.4.25								
	1	2	3	4	5	6	7	8
Plasmid (µg)	negative	PIN1	ETV6	ETV6+PIN1	ETV6(218-345)	ETV6(218-345)+PIN1	ETV6(1-126)	ETV6(1-126)+PIN1
pTK Luc	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
null PRL	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
pEYFP N1-PIN1		0.2		0.2		0.2		0.2
pEYFP N1	0.2		0.2		0.2		0.2	
pM1-ETV6			0.2	0.2				
pM1-ETV6(218-345)					0.2	0.2		
pM1-ETV6(1-126)							0.2	0.2
pM1	0.2	0.2						
Relative Light Units (RLU)								
Luciferase activity(firefly)	13.72	15.39	3.67	4.912	12.84	9.803	3.978	2.736
	14.8	11.67	3.563	4.123	11.28	8.863	5.039	2.513
	12.35	11.86	3.489	3.95	9.959	8.79	5.507	2.785
Luciferase activity(Renilla)	16.47	21.83	28.94	25.66	33.44	31.61	17.88	22.37
	18.21	20.31	28.48	25.28	29.03	22.94	24.69	22.8
	17.33	21.92	29.25	27.06	28.8	30.09	30.66	21.52
Ratio (firefly/Renilla)	0.833	0.705	0.127	0.191	0.384	0.31	0.223	0.122
	0.813	0.575	0.125	0.163	0.389	0.386	0.204	0.11
	0.713	0.541	0.119	0.146	0.346	0.292	0.18	0.129
Average Ratio	0.786	0.607	0.124	0.167	0.373	0.329	0.202	0.12
Standard deviation	0.064	0.087	0.004	0.023	0.024	0.05	0.022	0.01

The raw data of Fig.4.26									
	1	2	3	4	5	6	7	8	9
Plasmid (µg)	control 1	PIN1	ETV6	E6 + P1	control 2	PIN1	ETV6	E6 + P1	Negative
pTR89	0.2	0.2	0.2	0.2					
pTR334					0.2	0.2	0.2	0.2	
null PRL	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	
CFP C1	0.2	0.2			0.2	0.2			
pECFP - ETV6			0.2	0.2			0.2	0.2	
pEYFP N1-PIN1		0.2		0.2		0.2		0.2	
pEYFP N1	0.2		0.2		0.2		0.2		
Relative Light Units (RLU)									
Luciferase activity(firefly)	8.025	9.746	0.344	5.341	71.89	160	39.81	134.9	0.01
	6.277	9.247	1.699	6.04	66.91	145.4	36.63	150.8	0.023
	5.906	8.991	3.061	7.994	57.87	134.2	32.32	137.1	0.012
Luciferase activity(Renilla)	37.32	48.28	2.781	32.41	42.22	67.89	55.13	36.42	1.934
	24.49	51.02	13.69	34.88	37.85	59.99	49.42	38.69	2.41
	32.83	52.04	23.3	39.43	38.76	53.95	40.79	38.99	2.101
Ratio (firefly/Renilla)	0.215	0.202	0.124	0.165	1.703	2.356	0.722	3.703	0.005
	0.256	0.181	0.124	0.173	1.768	2.424	0.741	3.897	0.009
	0.18	0.173	0.131	0.203	1.493	2.488	0.793	3.515	0.006
Standard deviation	0.039	0.015	0.004	0.02	0.144	0.066	0.037	0.191	0.002
Average Ratio	0.217	0.185	0.126	0.18	1.655	2.423	0.752	3.705	0.007

The raw data of Fig.4.27											
	1	2	3	4	5	6	7	8	9	10	11
Plasmid (µg)	control	PIN1	ETV6	E6+P1	ETV6 S106A	ETV6 S106A+P1	ETV6 S257A	ETV6 S257A+P1	ETV6 S332A	ETV6 S332A+P1	negative
pTR 334	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	
null PRL	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	
pEYFP N1-PIN1		0.2		0.2		0.2		0.2		0.2	
pEYFP N1	0.2		0.2		0.2		0.2		0.2		
CFP C1-TEL			0.2	0.2							
CFP C1-TEL 106					0.2	0.2					
CFP C1-TEL 257							0.2	0.2			
CFP C1-TEL 332									0.2	0.2	
pECFP C1	0.2	0.2									
Relative Light Units (RLU)											
Luciferase activity(firefly)	1847	4924	754.9	4560	60.84	358.2	641.4	1617	752.1	4895	0.008
	2083	4300	613.3	3807	808.3	277.4	780.2	2688	1786	4449	0.006
	1865	4569	582.7	3337	578.6	342.3	681.9	3837	1161	2570	0
Luciferase activity(Renilla)	2145	1633	3144	3962	406.2	2177	5466	2081	3335	4948	0.007
	2208	1361	2624	3536	5720	1964	6555	3524	8257	4404	0.132
	2033	1454	2230	3022	3891	2734	5873	5736	5079	2536	0.089
Ratio (firefly/Renilla)	0.861	3.016	0.24	1.151	0.15	0.165	0.117	0.777	0.226	0.989	0.0112
	0.944	3.16	0.234	1.077	0.141	0.141	0.119	0.763	0.216	1.01	0.046
	0.917	3.141	0.261	1.104	0.149	0.125	0.116	0.669	0.229	1.013	0.002
Average Ratio	0.907	3.106	0.245	1.111	0.147	0.144	0.117	0.736	0.224	1.004	0.02
Standard deviation	0.042	0.078	0.014	0.037	0.005	0.02	0.002	0.059	0.007	0.013	0.023

The raw data of Fig.4.28												
	1	2	3	4	5	6	7	8	9	10	11	12
Plasmid (µg)	Control	Control+TSA	TIP60β	TIP60β+TSA	ETV6	ETV6+TSA	ETV6+TIP60β	ETV6+TIP60β+TSA	pM1-TEL(282-345)	pM1-TEL(282-345)+TSA	pM1-TEL(282-345)+TIP60β	pM1-TEL(282-345)+TIP60β+TSA
GAL4 tk LUC	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
null PRL	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
pM1	0.3	0.3	0.3	0.3								
TSA		1.5ML		1.5ML		1.5ML		1.5ML		1.5ML		1.5ML
pEYFP N1-TIP60			0.3	0.3			0.3	0.3			0.3	
pEYFP N1	0.3	0.3			0.3	0.3			0.3	0.3		0.3
pM1-TEL					0.3	0.3	0.3	0.3				
pM1-TEL(282-345)									0.3	0.3	0.3	0.3
Relative Light Units (RLU)												
Luciferase activity(firefly)	84.71	28.49	18.09	34.25	6.776	8.928	4.846	6.514	37.93	69.84	34.08	40.43
	57.02	56.25	20.56	22.2	6.0	9.229	1.561	8.214	53.26	75.20	40.04	36.36
	59.03	45.05	15.6	32.2	6.442	8.24	1.858	7.544	53.97	70.56	30.12	41.75
Luciferase activity(Renilla)	52.7	18.67	9.456	20.09	77.08	26.35	80.19	22.72	50.91	27.04	57.6	30.05
	39.32	20.81	8.691	11.32	92.89	25.57	35.14	29.12	56.67	29.71	63.7	24.69
	42.67	20.37	7.068	18.05	83.3	25.78	74.94	33.69	56.08	25.52	60.57	37.01
Ratio (firefly/Renilla)	1.607	2.062	1.913	1.705	0.088	0.339	0.06	0.287	0.745	2.583	0.592	1.345
	1.45	2.703	2.365	1.962	0.073	0.361	0.044	0.282	0.94	2.534	0.641	1.432
	1.383	2.212	2.207	1.784	0.077	0.32	0.026	0.224	0.962	2.764	0.497	1.128
Standard deviation	0.115	0.335	0.229	0.132	0.008	0.021	0.018	0.035	0.119	0.121	0.073	0.157
Average Ratio	1.48	2.326	2.162	1.817	0.079	0.34	0.043	0.264	0.882	2.627	0.577	1.302