

The acetyltransferase 60 kDa *trans*-acting regulatory protein of HIV type 1-interacting protein (Tip60) interacts with the translocation E26 transforming-specific leukaemia gene (TEL) and functions as a transcriptional co-repressor

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The translocation E26 transforming-specific (ETS) leukaemia (TEL), alias the ETS variant (ETV6), gene is expressed in most human tissues and encodes a transcriptional repressor. The TEL gene is involved in more than 40 different chromosomal translocations associated with haematological malignancies. As little is known about the function of intact TEL, we searched for TEL-interacting proteins by yeast two-hybrid screening. Among the interacting partners, we identified the histone acetyltransferase protein Tip60 [60 kDa *trans*-acting regulatory protein of HIV type 1 (Tat)-interacting protein]. The interaction was reproduced *in vitro*, and in mammalian cells we mapped the interaction regions in TEL to the ETS domain and those in Tip60 to the MYST ('MOZ, Ybf2/Sas3, SAS2 and Tip60', where MOZ stands

for male absent on the first, SAS for something about silencing and Ybf2 for identical with SAS2) region. Detailed analysis of the Tip60 MYST domain by introduction of point mutations revealed that an N-terminal C₂HC zinc finger was essential for interaction with TEL. Finally, we showed that Tip60 functions in a reporter system as a co-repressor in TEL-mediated transcription repression.

Key words: E26 transforming-specific transcription factor, histone acetyltransferase, translocation E26 transforming-specific leukaemia (TEL), 60 kDa *trans*-acting regulatory protein of HIV type 1-interacting protein (Tip60), transcription.

INTRODUCTION

The translocation E26 transforming-specific (ETS) leukaemia (TEL) gene was identified originally as a fusion partner to the platelet-derived growth factor receptor β at t(5;12)(q33;p13) chromosomal translocation associated with chronic myelomonocytic leukaemia [1] and was subsequently denoted the ETS variant (ETV6) gene. TEL is expressed in most of the human and mouse tissues [2,3], and is essential during embryonic development since inactivation in mice by knock-out techniques causes severe defects in the developing vascular network of yolk sac and the embryo dies at day E10.5–11.5 [3]. TEL is disrupted in a large number of chromosomal translocations involving various tyrosine kinases {Abelson mouse leukaemia viral oncogene homologue ABL(9q34) [4], Abelson-related gene ARG(1p13) [5], Janus kinase JAK2(9p24) [6]}, tyrosine kinase receptor C TRKC(15q24) [7] and transcription factors {acute myeloid leukaemia (AML) 1/Runt-related transcription factor 1 (21q22) [8–10], ecotropic viral integration site EVI1(3q26) [11] and caudal-type homeobox transcription factor CDX2(13q12) [12]}. TEL contains two functional domains: (i) the N-terminal pointed (PNT) homodimerization domain that is homologous with a region in the *Drosophila* protein pointed [1] and (ii) a C-terminal ETS DNA-binding domain (DBD) [2,13]. In translocations involving the tyrosine kinases, the TEL 5'-region including the PNT domain is fused to the tyrosine kinases, and PNT facili-

tates dimerization of the fusion protein, leading to constitutive activation of tyrosine kinase. In other translocations (TEL/ecotropic viral integration site EVI1 and TEL/caudal-type homeobox transcription factor CDX2), the contribution of TEL is believed to be as an active promoter, because no functional TEL domains are included in the fusion protein. In the most frequent TEL translocation [t(12;21)(p13;q22)], the TEL N-terminal region (residues 1–336) including PNT is fused to the AML1 DBD and transactivation domains (residues 21–480). This translocation is associated with the most common paediatric B-cell acute lymphoblastic leukaemia (ALL) [14]. The non-translocated TEL allele is deleted frequently in the TEL/AML1 type of ALL, suggesting a role for TEL as a tumour suppressor [9,10,15], and is supported by two observations: (i) TEL expression reverses Ras-induced transformation of NIH3T3 cells [16,17] and (ii) TEL inhibits the expression of the metalloproteinase stromelysin-1 by directly binding to the promoter, which is important in tissue remodelling and tumour cell invasion. TEL functions as a repressor of transcription [18] and the repression is associated with the binding of the co-repressors mSin3A and silencing mediator for retinoid and thyroid receptor/nuclear co-repressor [19–21].

Tip60 [60 kDa *trans*-acting regulatory protein of HIV type 1 (Tat)-interacting protein] was identified as an interaction partner for the HIV-1 Tat protein and was shown to increase Tat transactivation of the HIV-1 promoter [22]. Tip60 has a chromatin

Abbreviations used: ALL, acute lymphoblastic leukaemia; AML1, acute myeloid leukaemia 1; AR, androgen receptor; CHROMO, chromatin organization modifier; DBD, DNA-binding domain; EBS, ETS-binding sites; Esa1, essential sas family acetyltransferase 1; ER, oestrogen receptor; ERE, oestrogen response element; ETS, E26 transforming-specific (domain); GST, glutathione S-transferase; HAT, histone acetyltransferase; MOZ, male absent on the first; SAS, something about silencing; Tat, *trans*-acting regulatory protein of HIV type 1; Tip60, 60 kDa Tat-interacting protein; Ybf2, identical with SAS2; MYST, 'MOZ, Ybf2/Sas3, SAS2 and Tip60'; NR, nuclear receptor; PNT, pointed domain; SEAP, secreted alkaline phosphatase; TEL, translocation ETS leukaemia (gene); VP16, virus protein 16; X-Gal, 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside.

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organization modifier (CHROMO) domain in its N-terminal end and a MYST domain named after its founding members ('MOZ, Ybf2/Sas3, SAS2 and Tip60', where MOZ stands for male absent on the first, SAS for something about silencing and Ybf2 is identical with SAS2) in the C-terminal end. The MYST domain defines Tip60 as belonging to the MYST family of histone acetyltransferase (HAT) proteins that are conserved from yeast to human (see [23] for a review). HATs are associated mostly with transcription activation and function as catalytic subunits of large protein complexes. The model is that the HATs acetylate the basic histone tails, leading to a chromatin structure more accessible for regulatory proteins. The acetylated lysine residues have been proposed to form a protein-binding site for protein containing bromodomains [24,25]. Purified Tip60 acetylates the individual histones H4, H3 and H2A, whereas histones in nucleosome particles are acetylated poorly [26,27]. Tip60 is identified as a HAT subunit of a large HAT complex [28], which is most probably the mammalian homologue of the yeast nucleosomal acetyltransferase (NuA4) complex, which includes the essential sas family acetyltransferase (Esa) 1, supposed to be the functional equivalent of Tip60 [29]. The Tip60 complex readily acetylates nucleosome-assembled histones H4 and H2A. Besides Tip60, the complex also contains two proteins, TAP54 α /Tip49a and TAP54 β /Tip49b (where TAP stands for Tip60-associated protein and Tip49 the 49 kDa TATA-box-binding protein (TBP)-interacting protein), which are related to the *Escherichia coli* RuvB hexameric ring helicase protein that plays a role in the repair of damaged DNA. Supporting this finding, the HAT domain of Tip60 is shown to be required for the effective DNA repair of double-strand breaks and for apoptosis after γ -irradiation of human cells [28]. Moreover, Tip60 binds to the β -amyloid precursor protein (APP) and enhances apoptosis [30]. Recently, it has been shown that the ubiquitin ligase mouse double minute clone 2 oncoprotein targets Tip60 for degradation, in a manner similar to mouse double minute clone 2 oncoprotein-mediated degradation of p53 [31]. Finally, Tip60 functions as a co-activator of class I nuclear hormone receptors [32–34].

In the present study, we report that TEL and Tip60 interact *in vitro* and *in vivo* and show that Tip60 in a reporter system functions as a co-repressor for TEL in the regulation of transcription.

MATERIALS AND METHODS

Yeast two-hybrid screening

Full-length TEL (bait) was fused to the Lex A DBD (amino acids 1–202) in pBTM116, creating pBTM116-TEL. Yeast strain L40 [*MAT α* , *trp1*, *leu2*, *his3*, *ade2*, *LYS2::lexAop*]_{4x}-*HIS3*, *URA3::lexAop*]_{8x}-*LacZ*] was transformed sequentially with pBTM116-TEL and the human leucocyte library (ClonTech Laboratories, Palo Alto, CA, U.S.A.) in accordance with the manufacturer's instructions. The cDNA library is positioned in the prey vector pACT2, where Gal4 AD (768–881 amino acids) form the N-terminal part of the fusion. We screened 1.9×10^6 clones. L40 contains histidine and LacZ reporter genes. Positive clones were selected by prototrophic growth on solid media without histidine, but with 5 mM 3-amino-1,2,3 triazole. Activation of the LacZ gene was verified using β -galactosidase overlay assay: L40 transformants containing the bait-LexA (pBTM116) plasmid and prey-Gal4 AD (pACT2) were grown for 2–3 days on SC-Trp, Leu or SC-Trp, Leu and His plates. Then, 0.5% low-melting agarose in 0.5 M potassium phosphate buffer (pH 7.0) was prepared and cooled to 60 °C, then 0.1% (v/v) SDS, 6% (v/v) 1-methyl-2-pyrrolidone and 0.05% (w/v) 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside (X-Gal) were added. The plates were then

carefully overlaid with agarose. When solidified, the plates were inverted and incubated at 30 °C. Activation of LacZ will hydrolyse X-Gal and will be visualized as a blue colour within a few hours. Prey plasmids from positive clones were purified and cloned into *E. coli* MC1066 [*trpC9830*, *leuB600*, *pyrF::Tn5 lac Δ X74*, *strA*, *galU*, *galK* (*r⁻m⁺*)] and plated on to M9 medium without leucine. The pACT2 prey plasmid was purified and sequenced.

Interaction mapping in yeast

TEL deletions were fused to the LexA DBD (1–202 amino acids) by cloning into pBTM116. Tip60 deletions were fused to Gal4 AD (768–881 amino acids) by cloning into pACT2 (ClonTech Laboratories). All constructs were cloned by PCR using PFU turbo DNA polymerase (Stratagene, La Jolla, CA, U.S.A.) and verified by sequence determination (DYEnamic ET Terminator Cycle Sequencing premix kit; Amersham Biosciences, Uppsala, Sweden). Sequences were detected on an ABI Prism 3100 Genetic Analyzer capillary machine (AB Applied Biosystems, Foster City, CA, U.S.A.). Interactions were monitored as described for the yeast two-hybrid screen.

Plasmids

pACT2-Tip60 α and the corresponding deletions were PCR-cloned from IMAGE clone 417933. The pACT-Tip60 β was isolated in the yeast two-hybrid screening and deletions were derived by PCR-aided cloning. The secreted alkaline phosphatase (SEAP) reporter construct directs transcription of a SEAP gene under the control of a β -globin promoter [35]. An oestrogen response element (ERE) and two Gal4-binding sites are positioned upstream of the promoter. The reporter EBS-SEAP (where EBS represents ETS-binding sites) is constructed by inserting three TEL EBS between the Gal4 and ERE sites. The sequence ATAAACAGGAAGTGGTTCGGAAGTGAGGAAGTGAATTC was introduced using the QuickChange Site-Directed Mutagenesis kit (Stratagene) in accordance with the manufacturer's instructions.

Interactions *in vitro*

His-tagged TEL was obtained by cloning TEL into pRSET B (Invitrogen, Carlsbad, CA, U.S.A.) and expressed in *E. coli* BL21(DE3)pRI952. His-TEL was affinity-purified using Ni²⁺-nitrilotriacetate beads (Amersham Biosciences) in accordance with the manufacturer's instructions. Glutathione S-transferase (GST) fusions of Tip60 β and Tip60 α (212–513) were cloned into pGEX-5x-2 (Amersham Biosciences) and expressed in *E. coli* BL21 (DE3) pRI952; 5 μ g of GST-Tip60 β and GST-Tip60 α (212–513), and 20 or 2 μ g of GST (control) were immobilized on glutathione-Sepharose 4B beads (60 μ g of bed volume) by incubation for 2 h under rotation at 4 °C in 1 ml of NETN buffer [2 mM EDTA, 100 mM NaCl, 25 mM Tris (pH 8), 0.5% Nonidet P40, 1 mM PMSF and 1 \times complete proteinase inhibitor cocktail (Roche, Mannheim, GmbH, Germany)]. The beads were washed five times in NETN buffer and 0.5 μ g of affinity-purified His_{6x}-TEL was added and incubated in 1 ml of NETN (1 mM PMSF and 1 \times complete proteinase inhibitor cocktail) overnight to allow binding. The beads were again washed five times in NETN. Finally, bound protein was eluted by boiling in denaturing SDS-loading buffer (50 mM dithiothreitol) and resolved on SDS/PAGE, followed by Western blotting and antibody probing with primary anti-His rabbit polyclonal IgG (DAKO, Glostrup, Denmark) and secondary horseradish peroxidase-conjugated rabbit immunoglobulins (DAKO).

Mammalian two-hybrid analysis

Gal4-tagged TEL constructs were obtained by cloning into the expression vector pM (pPC97; ClonTech Laboratories) modified with a new multiple cloning site [35]. Virus protein 16 (VP16)-tagged Tip60 constructs were obtained by cloning into pTLI-VP16 (A.L. Nielsen, personal communication). The SEAP reporter construct directs transcription of a SEAP gene under the control of a β -globin promoter.

Cells and transfections

Culturing of HT1080 cells was performed in Dulbecco's modified Eagle's medium, containing glutamax, 10% (v/v) foetal calf serum and 2% (v/v) penicillin–streptomycin. HT1080 cells at 70% confluence were transfected using Superfect (Qiagen, Hilden, GmbH, Germany) according to the manufacturer's instructions using 1 μ g of Gal4-ERE-SEAP reporter vector and 1 μ g of pCH110 β -gal internal control vector. Transfections were performed in triplicate in 60 mm dishes. For the repression assay (Figure 6), TEL and Tip60 were expressed from pCMV (ClonTech Laboratories).

Enzyme assays

For SEAP assays, medium samples of 200 μ g were taken 24 and 48 h after transfection. The samples were heated at 65 °C for 10–15 min. Heat incubation was done to inactivate wild-type alkaline phosphatases. The SEAP reporter protein was thermostable. Then 150 μ g of SEAP substrate (Sigma–Aldrich, St. Louis, MO, U.S.A.) was added to each sample and mixed. The plate was incubated at 37 °C and A_{405} measured at different time intervals for a 24 h period. SEAP activity was calculated as follows:

$$\text{SEAP activity/h} = \{ (\Delta A_{405}/\text{time})_{48\text{h sample}} - [\Delta A_{405}/\text{time (media)}]_{48\text{h sample}} \} - \{ (\Delta A_{405}/\text{time})_{24\text{h sample}} - [\Delta A_{405}/\text{time (media)}]_{24\text{h sample}} \}$$

SEAP values were normalized to β -galactosidase values.

For β -galactosidase assays, media were removed from the 6-well dishes and the cells were washed in 1 ml of PBS. Trypsin (200 μ l) was added to each well and the plate was incubated for 5 min at 20 °C. Then, 1 ml of PBS was added and pipetted up and down to remove all cells from the disc surface, before transferring them to Eppendorf tubes. The cells were resuspended in 100 μ l of 250 mM Tris (pH 7.8) by vortex-mixing and then lysed by freezing in liquid nitrogen for 3 min and thawing in a water bath. This was repeated five times intervened by vigorous vortex-mixing. Finally, the tubes were centrifuged for 10 min at 10000 rev./min and 10 μ l of supernatant/cell extract was mixed with 65 μ l of 4 mg/ml *o*-nitrophenyl β -D-galactopyranoside. The plate was incubated at 37 °C and A_{405} measured at different time intervals. The transformation efficiency was then calculated as $\Delta A_{405} - \Delta A_{405}(\text{media})/\text{time unit}$.

RESULTS

Tip60 β is identified as an interaction partner to TEL

To illuminate the function of TEL, we searched for protein interaction partners using the yeast two-hybrid system [36]. Briefly, the yeast strain L40 was transfected sequentially with full-length TEL fused to LexA (bait) and a human leucocyte cDNA

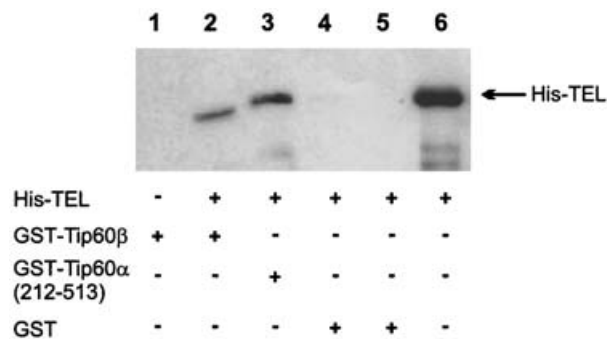


Figure 1 Association of His_{6x}-TEL with GST-Tip60 β and GST-Tip60 α (212–513) *in vitro*

GST-Tip60 β , GST-Tip60 α (212–513) and GST expressed in BL21 (DE3) pRI952 were immobilized on glutathione–Sepharose beads, then incubated with Ni²⁺-nitrilotriacetate affinity-purified His_{6x}-TEL [expressed in BL21 (DE3) pRI952], washed and eluted. The pull-down reactions were resolved by SDS/PAGE [4% (w/v)/15% (w/v)] and analysed by Western blotting and probed with primary anti-His rabbit polyclonal IgG and secondary horseradish peroxidase-conjugated rabbit immunoglobulins. The gels were loaded as follows: lane 1, GST-Tip60 β (5 μ g), negative control; lane 2, GST-Tip60 β (approx. 5 μ g) + His_{6x}-TEL (approx. 0.5 μ g); lane 3, GST-Tip60 α (212–513) (approx. 5 μ g) + His_{6x}-TEL (approx. 0.5 μ g); lane 4, GST (approx. 20 μ g) + His_{6x}-TEL (approx. 0.5 μ g); lane 5, 1/10 GST (approx. 2 μ g) + His_{6x}-TEL (approx. 0.5 μ g); lane 6, 20% of input His_{6x}-TEL (approx. 0.1 μ g).

library (prey). We screened 1.9×10^6 transformants and identified 62 clones that were positive for the expression of the selection markers, histidine residue and β -galactosidase. Sequence analysis showed that more than 50% of the clones were coding for the human small ubiquitin-related modifier-1/ubiquitin-conjugating enzyme UBC9, previously confirmed to have affinity to TEL and mediate TEL sumoylation [37]. Among the residual interacting clones, we found protein inhibitor of signal transducer and activator of transcription 1 (PIAS1) [38], Flice-associated huge protein (FLASH) [39] and death-associated protein (DAXX) [40,41], suggested to be involved in degradation and apoptosis; zinc finger proteins ZNF198 [42] and ZNF237 [43], which are putative transcription factors; peptidyl-prolyl *cis/trans*-isomerase, Nima-interacting (PIN1), which is a peptidyl-prolyl *cis/trans*-isomerase (PPIase) of unknown function [44,45]; and finally a clone of interest for the function of TEL as a transcription regulator, the Tip60 protein, which is a HAT. We identified Tip60 in two independent clones that encoded the C-terminal section and full-length Tip60 β respectively. The C-terminal section is common to the Tip60 α and Tip60 β splice versions (see Figure 3). To analyse for differences between the two Tip60 splice variants, both were cloned and tested in yeast. Tip60 α was similar to Tip60 β in binding TEL, although the interaction seemed a little weaker when estimated from LacZ activation on plates (results not shown). The interaction between TEL and Tip60 is specific, since co-transformation of Tip60 and the empty bait vector or bait vector encoding lamin does not activate the selection markers.

TEL interacts directly with Tip60 *in vitro*

To show that the interaction between Tip60 and TEL was direct and not assisted by proteins from yeast, we performed a pull-down assay *in vitro* using proteins produced in *E. coli*. Tip60 β and the N-terminal deletion Tip60 α (212–513), both fused to GST and His-tagged TEL, were expressed in *E. coli* BL21 (DE3) pRI952 and used for GST pull-down assays (see Figure 1). Both Tip60 β and Tip60 α (212–513) bound to TEL (lanes 2 and 3), whereas no binding was detected to GST alone (lanes 4 and 5). The interaction analyses were performed under standard conditions

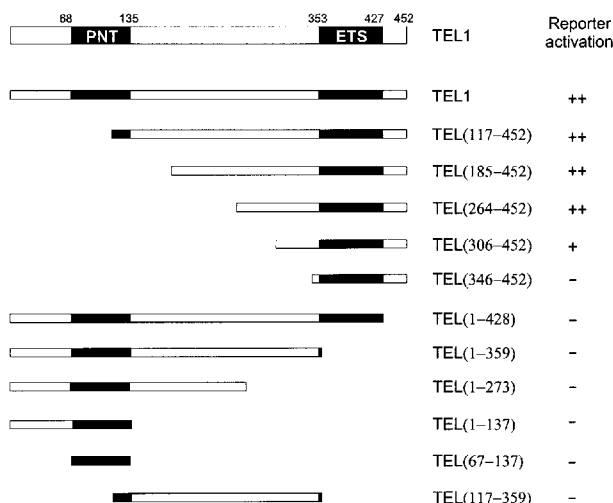


Figure 2 Tip60 interaction region in TEL

Tip60 α and Tip60 β fused to the Gal4 (amino acids 768–881) activation domain and TEL deletions fused to the DBD LexA (amino acids 1–202) were tested for interaction in the yeast two-hybrid system using the yeast strain L40, containing the HIS3 and LacZ reporter genes. Interactions were detected as prototrophic growth on solid media lacking histidine and blue colouring when grown in the presence of X-Gal (see the Materials and methods section). The TEL interaction with Tip60 α and Tip60 β gave identical results. The functional expression of the different TEL deletions was verified in the yeast two-hybrid system using the other interaction partners that we found in the primary screening (protein inhibitor of signal transduction and activators of transcription (PIRS1), Flice-associated huge protein (FLASH) and death-associated protein (DAXX); results not shown). The TEL ETS domain with flanking regions (amino acids 264–345 and 429–452) are required for Tip60 α and Tip60 β interaction with TEL.

at 100 mM NaCl. Furthermore, the His antibody did not detect Tip60 β or any degradation product (lane 1). In conclusion, results confirmed direct interaction between Tip60 and TEL.

The C-terminal third of TEL mediates interaction with Tip60

To map the region of TEL responsible for Tip60 binding, fragments of TEL were tested against full-length Tip60 α and Tip60 β in the yeast two-hybrid system (Figure 2). Deletion of 263 N-terminal amino acids of TEL [clone TEL (264–452)] did not affect the interaction with Tip60. However, removing 42 additional amino acids [clone TEL (306–452)] weakened the Tip60 interaction significantly and the deletion of further 82 amino acids [clone TEL (346–452)] eliminated all interactions. Deletion of only 24 amino acids from the C-terminal, leaving the ETS domain intact [clone TEL (1–428)], discontinued all interactions with Tip60. Results suggest that the ETS domain in TEL is responsible for the interaction with Tip60 and that the flanking regions influence the interaction.

MYST domain in Tip60 mediates TEL binding

The TEL interaction region in Tip60 was mapped by constructing Tip60 deletion mutants that were tested for interaction with TEL in the yeast two-hybrid system (Figure 3). Deletion of the 34 C-terminal amino acids [creating clones Tip60 α (1–479) and Tip60 β (1–427)] eliminated interaction with TEL. This extreme C-terminal region contains the C-terminal part of the MYST domain showing least conservation compared with the closely homologous MYST domains of human MOZ, MOZ-related factor (MORF) and HBO1. Furthermore, this region contains a nuclear receptor box (NR-box); LXXLL is normally present in nuclear hormone receptor-binding proteins. Although NR-binding proteins usually apply more than one NR-box for binding, Tip60 is shown to function as a co-activator for class I NR by directly binding to the Tip60 NR-box, the function being dependent on the functional HAT activity of Tip60 [32–34]. Analysis of the N-terminal deletions showed that the first

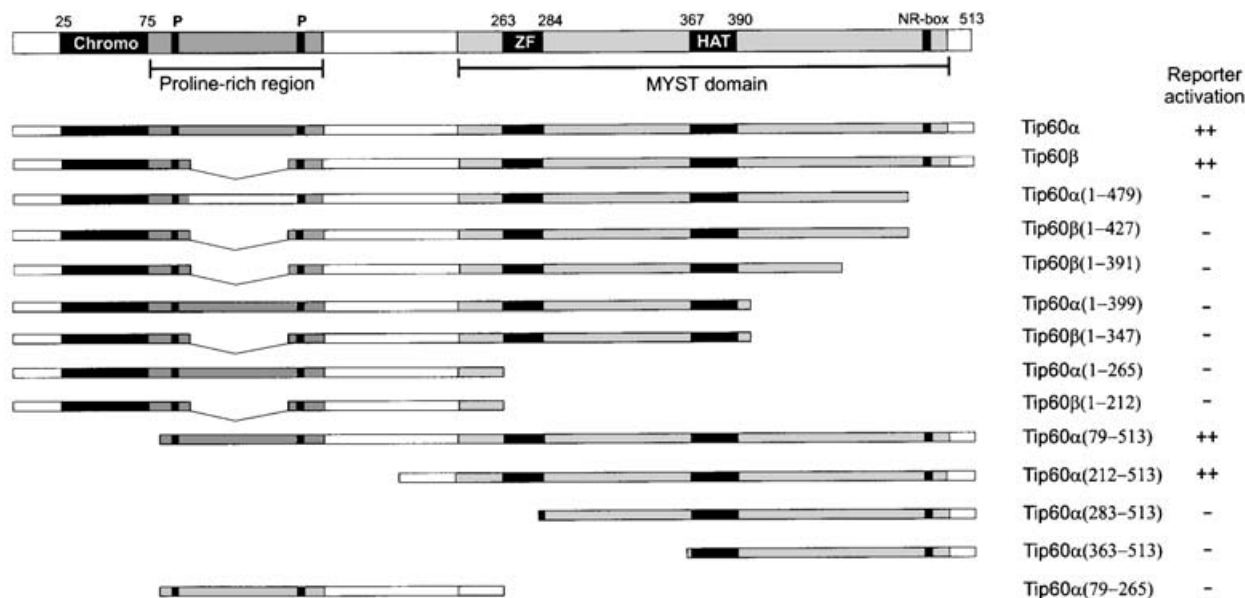


Figure 3 TEL interaction region in Tip60

TEL fused to the DBD LexA (1–202) and Tip60 deletions fused to the Gal4 (768–881) activation domain were tested for interaction in the yeast two-hybrid system using the yeast strain L40, containing the HIS3 and LacZ reporter genes. Interactions were detected as prototrophic growth on solid media lacking histidine and blue colouring when grown in the presence of X-Gal (see the Materials and methods section). The functional expression of the different Tip60 deletions was verified in the yeast two-hybrid system using other Tip60-interaction partners, such as BCL3 (obtained from Christian Langediil Christiansen, Department of Molecular Biology, Aarhus University, Denmark) or internal fragments of TEL (results not shown). C-terminal MYST in Tip60 is essential for TEL binding. P, potential extracellular-signal-regulated kinase 1/2 phosphorylation sites; ZF, zinc fingers.

211 amino acids of Tip60 α [Tip60 α (212–513)], containing CHROMO-like domain and proline-rich region, were not involved in TEL binding. Deletion of the first part of the MYST domain including the C₂HC zinc finger destroyed interaction with TEL [see Tip60 α (283–513) in Figure 3]. This suggests an important role for the zinc finger motif; however, the zinc finger alone is not responsible for TEL interaction, because the C-terminal deletions Tip60 α (1–479), Tip60 β (1–427) and Tip60 β (1–391), all containing both the zinc finger and the HAT domain, did not bind to TEL. The crystal structure of Esa1, the yeast orthologue of Tip60, shows that the β 3-turn- β 4- α 1-loop region corresponding to the Tip60 C₂HC region constitutes an independent subdomain that is not intermingled with the core MYST domain [46]. Accordingly, deletion of the zinc finger in Tip60 α will probably not have a profound impact on the folding of the core MYST domain. This again supports the notion that the C₂HC zinc finger region by itself is critical for TEL binding. Taken together, the mapping analysis showed that the regions with the zinc finger region and the extreme C-terminus harbouring the NR-box were important for TEL binding.

Mutating the Tip60 MYST zinc finger eliminates interaction with TEL in yeast

To assess the roles of the Tip60 C₂HC zinc finger motif and the NR-box in interaction with TEL, we used site-directed mutagenesis to create mutations in either motif. In Tip60 β , we introduced a Cys \rightarrow Ala substitution in the first cysteine residue of the zinc finger creating Tip60 β _{C211A}. In Tip60 β _{L440A,L441A}, we substituted the last two leucine residues of the NR-box (LKRLLL) with alanine residues. Tip60 β _{C211A} and Tip60 β _{L440A,L441A} were tested for interaction with TEL in yeast (Figure 4). Mutation of the zinc finger eliminates interaction with TEL (Figure 4, plate position 2), whereas the NR-box mutation did not influence interaction (Figure 4, plate position 3) when compared with wild-type (wt) Tip60 β (Figure 4, plate position 1). Plate positions 4, 5 and 6 represent co-transformation of Tip60 β _{L440A,L441A}, Tip60 β _{C211A} and Tip60 β respectively with empty bait vector and serve as negative controls. This analysis shows that the Tip60 zinc finger motif is critical for the TEL interaction.

Interaction of TEL with Tip60 *in vivo*

To confirm interaction between TEL and Tip60 *in vivo*, a mammalian two-hybrid assay was applied. TEL and the N-terminal deletion TEL (117–452) (see Figure 2) were fused to the Gal4 DBD, and Tip60 β was fused to the activation domain of the herpes simplex virus transcription activator VP16, all in relevant mammalian expression vectors. Pairs of TEL and Tip60 constructs were transfected into HT1080 cells along with the SEAP reporter (Figure 5A) and interactions were monitored by measuring SEAP activity (Figure 5B). By installing Gal4 and VP16 at the N-terminal ends of TEL and Tip60 respectively, the risk of interfering with the interaction between TEL and Tip60 should be minimized, because the yeast interaction mapping identified the C-terminal segments of both TEL and Tip60 as being responsible for the interaction. In Gal4-TEL, TEL may function as a transcription repressor and thus obscure the analysis. We therefore included the TEL (117–452) construct in the analysis. In independent studies, TEL represses two times more than TEL (117–452) (results not shown). Figure 5(B) shows that the presence of Gal4-TEL and Gal4-TEL (117–452) recruited Tip60-VP16 to the promoter of the reporter and activated SEAP expression, thus demonstrating *in vivo* interaction between TEL and Tip60.

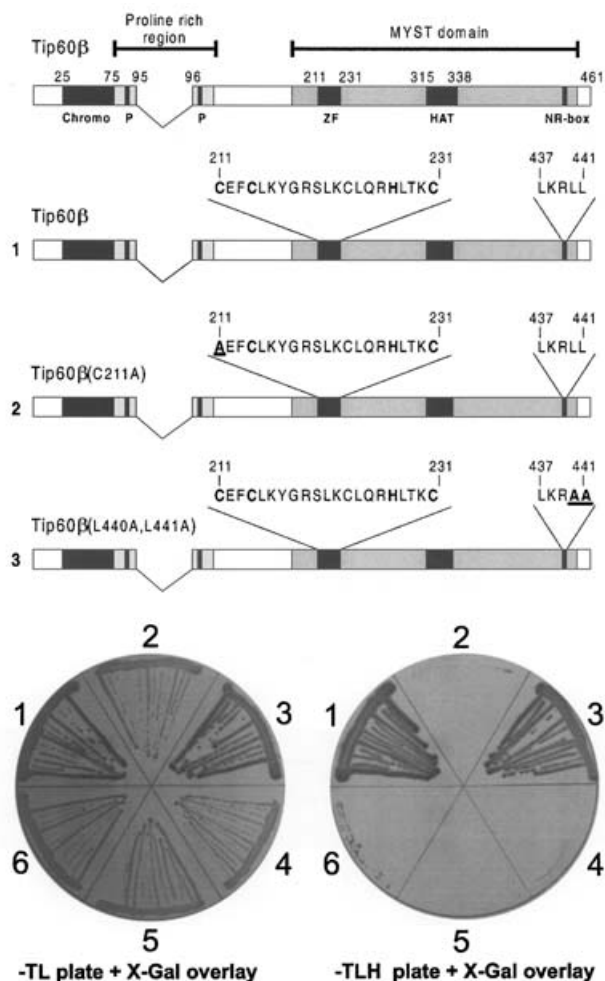


Figure 4 Zinc finger in the Tip60 MYST domain is essential for TEL interaction

TEL, fused to the DBD LexA (1–202), and Tip60 β (1), Tip60 β _{C211A} (2) and Tip60 β _{L440A,L441A} (3), fused to the Gal4 (768–881) activation domain were tested for interaction in the yeast strain L40, containing the HIS3 and LacZ reporter genes. Interactions were detected as prototrophic growth on solid media lacking histidine residue and blue colouring when grown in the presence of X-Gal (see the Materials and methods section). -TL and -TLH media plate positions 4, 5 and 6 are negative controls for Tip60 β _{L440A,L441A}, Tip60 β _{C211A} and Tip60 β respectively, co-transformed with the empty bait vector pBTM116. Yeast colonies streaked on -TL plates were allowed to grow for 3 days and then transferred to -TLH plates and again grown for 3 days. -TL and -TLH media plates were then covered with X-Gal-containing agarose (see the Materials and methods section) and incubated for 24 h before pictures were taken.

It was shown in Figure 4 that the Tip60 zinc finger was critical for TEL interaction in yeast. To confirm this interaction in mammalian cells, we constructed VP16 fusions with Tip60 β _{C211A} and wt Tip60 β . These were tested against Gal4-TEL (117–452) using a SEAP reporter to measure the interaction (see Figure 5C). Co-transfection of Gal4-TEL (117–452) and VP16-Tip60 β gave a 5-fold activation (lane 3). In contrast, co-transfection of Gal4-TEL (117–452) and VP16-Tip60 β _{C211A} gave no activation (lane 4). Results showed unambiguously that the Tip60 C₂HC zinc finger motif was essential for TEL binding in mammalian cells.

Tip60 is a co-repressor in TEL-mediated transcription repression

To characterize the effect of Tip60 binding to TEL on the transcription-regulatory properties, we constructed a TEL-responsive

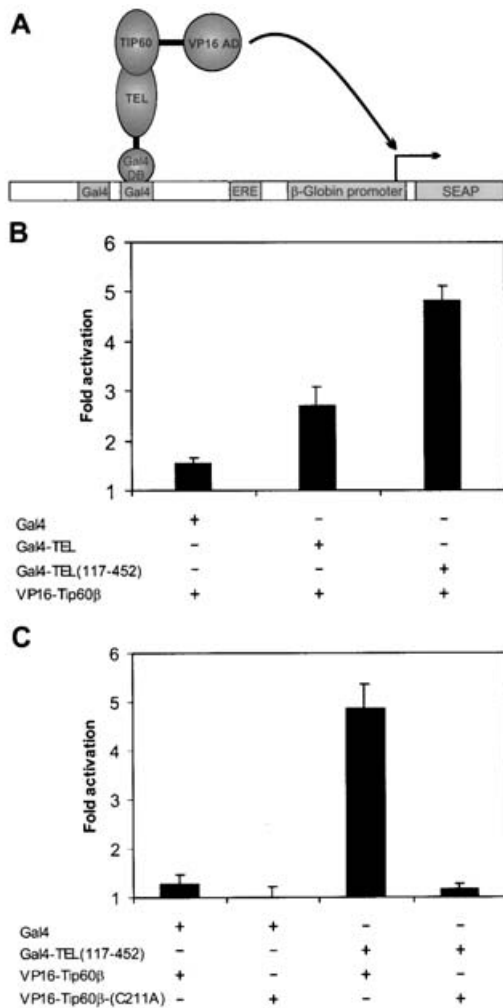


Figure 5 TEL and Tip60 interactions *in vivo*

(A) The mammalian two-hybrid system used for detection of the interactions between TEL and Tip60. The SEAP reporter is depicted. (B, C) Human fibroblast HT1080 cells were transfected with 1 μ g of SEAP reporter plasmid and 1 μ g of pCH110 β -gal plasmid (internal control of transfection), together with 1 μ g of each construct listed under the columns. Gal4, Gal4 alone; Gal4-TEL, Gal4 fused to full-length TEL; Gal4-TEL (117–452), Gal4 fused to TEL (amino acids 117–452); VP16-Tip60 β , VP16 fused to full-length Tip60 β ; VP16-Tip60 β (C211A), VP16 fused to Tip60 β carrying a point mutation at position 211. Fold activation is calculated as VP16-Tip60 β /VP16 and VP16-Tip60 β _{C211A}/VP16 after normalization to the internal β -gal reporter. All transfections were performed in triplicate and SEAP activity/h was normalized to the value of the empty plasmid that is set to 1 (= no activation).

reporter. The ETS domain of the ETS transcription factors recognizes specific DNA sequences termed as EBS. These are composed of a core GGA trinucleotide sequence and their ETS-binding specificity is governed by short sequences (nt 3–5) flanking the GGA sequence. The TEL EBS consensus sequence is reported to be TG/TA/CGGAAGT [13] and the sequence ATAAACAGGAAGTGG is also shown to bind TEL [2]. Three different TEL EBS sequences (TTCGGAAGT, TGAGGAAGT and ATAAACAGGAAGTGG) were inserted side by side into the SEAP reporter at the enhancer position next to the oestrogen receptor (ER)-binding site, creating the TEL-EBS reporter (Figure 6A). HT1080 cells were transfected with TEL and Tip60 expression vectors, the TEL-EBS reporter and finally plasmids expressing transcription activators Gal4-E1A (Figure 6B) or ER-VP16 (Figure 6C). The effect on transcription was estimated by

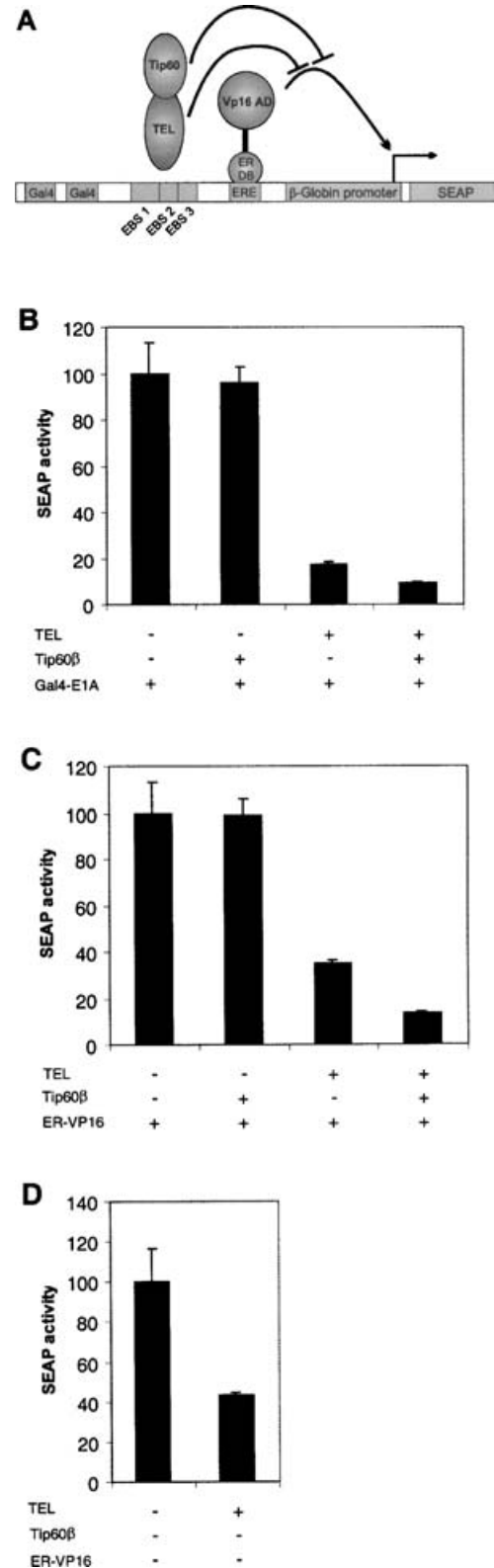


Figure 6 Tip60 is a co-repressor for TEL

(A) Modified SEAP reporter system. The EBS-SEAP reporter carries binding sites for TEL. (B–D) HT1080 cells were transfected with 0.5 μ g of each of the construct listed under the columns (+, with insert; –, corresponding plasmid without insert), 1 μ g of EBS-SEAP reporter plasmid and 25 ng of ER-VP16 or ER-E1A to enhance transcription. All transfections were performed in triplicate and SEAP activity/h was normalized to the value of the empty plasmids that is set to 100.

measuring SEAP activity. Tip60 had no effect on the reporter (columns 2) and TEL represses the reporter transcription by approx. 3-fold (columns 3), which is comparable with Gal4-TEL repression of the SEAP reporter without the TEL-binding sites (results not shown). Co-expression of Tip60 further represses the transcription in a TEL-dependent manner (columns 4). Figure 6(D) shows that expression of TEL without overexpression of transcription activators still gave a significant repression, confirming that TEL mediates active repression and not passive repression by blocking ER-VP16 or Gal4-E1A binding. We conclude that Tip60 functionally attenuates TEL-EBS reporter expression in a TEL-dependent manner.

DISCUSSION

The first interest in TEL arose from its involvement in chromosomal translocations associated with haematological malignancies. At present, TEL is known to be involved in more than 40 translocations and the most frequent, the t(12;21)(p13;q22), fuses the TEL gene to the AML1 transcription activator. This translocation is associated with B-cell ALL, present in 25–30 % of paediatric and 3 % of adult B-cell ALL [14,47]. TEL belongs to the large and rapidly growing family of ETS transcription factors. The ETS protein family encompasses more than 45 members, each containing the highly conserved ETS DBD of approx. 85 amino acids. The ETS domain binds to purine-rich DNA sequences containing the core-binding motif GGAA/T, which is flanked on both sides by 2–4 nucleotides giving specificity [13]. ETS proteins bind to unique EBS either alone or in combination with other assisting proteins. ETS transcription-factor-regulated genes are involved in numerous cellular processes, including proliferation, differentiation, migration/tissue remodelling, haematopoiesis, angiogenesis, apoptosis and cell transformation [48–52]. TEL is essential for yolk sac angiogenesis and for survival of neural and mesenchymal cells [3]. Further analysis of haematopoietic progenitor cells of all lineages from chimaeric mice generated by transfer of TEL^{-/-} ES cells to blastocysts revealed that TEL was dispensable for adult-type haematopoietic lineages in the yolk sac and foetal liver, but is required specifically for establishment of bone-marrow haematopoiesis of all lineages [53].

In the present study, we have shown that TEL interacts directly with the MYST HAT protein Tip60. The interaction region of TEL is located within the 200 C-terminal amino acids that include the ETS domain. We note that regions outside the classical ETS domain seem to be involved in Tip60 binding, but we cannot exclude that these regions may affect the overall conformation of TEL. The TEL interaction domain of Tip60 was mapped to a region including the 300 C-terminal amino acids with the MYST domain. From analysis of the C-terminal deletions, we observed that deletion of the amino acids 212–283 abrogated interactions with TEL. This segment includes the C-terminal end of the MYST domain and, thus, we cannot exclude that it may affect the overall structure of the MYST domain. The MYST domain includes three distinct structures: a zinc finger, a HAT domain and an NR-binding domain (see Figure 3). The crystal structure of the yeast MYST protein, Esa1, shows that both the zinc finger and the NR corresponding domain are located at the surface of the protein. As Tip60 is homologous with Esa1, we tested whether these two domains were involved in TEL interactions, and we found in yeast and mammalian two-hybrid experiments that the C₂HC zinc finger motif is essential for TEL interaction, whereas the C-terminal NR-box was found to be dispensable. The observation that the DBD in TEL is involved in the Tip60 interaction raised

the question of whether DNA-binding ability of TEL was affected by Tip60.

We found that Tip60 attenuated transcription in a TEL-dependent manner and, thus, performed functionally as a co-repressor. A co-repressor function may be achieved by recruiting other repressors as histone-modifying enzymes. Alternatively, repression may be completed by stabilization of the repressor or its binding to DNA. Hence, we have shown that Tip60 and TEL interacted in yeast and mammalian cells *in vivo*. The most probable explanation is that the Tip60 effect may be effected by a direct interaction with TEL, presumably in a complex of proteins instituted at the promoter. However, we cannot exclude an indirect effect of Tip60 that stimulates the expression or the function of another factor that supports Tel repression. Tip60 is a co-activator of the androgen receptor (AR). Tip60 acetylates AR, and HAT mutations eliminate Tip60-mediated co-activation of AR [34]. Thus we have analysed the acetylation potential of recombinant Tip60 *in vitro* and found that Tip60, in addition to being autoacetylated, acetylated histone H3 and BSA, but recombinant TEL was not a substrate for Tip60 acetylation (results not shown) suggesting that DNA-binding ability of TEL is not directly modified by acetylation. However, as noted, Tip60 may stimulate the interaction between TEL and its co-repressors mSin3A, silencing mediator for retinoid and thyroid receptor (SMRT)/nuclear co-repressor (NCoR) and HDAC-3 [19–21,54]. Bandshift and *in vivo* functional analysis of TEL indicate that in full-length TEL, the ETS region may be masked/inhibited intramolecularly [18]. We also note that, in TEL, the C-terminal ETS-flanking region is composed of acidic amino acid that may interfere with DNA binding. On Tip60 binding, a conformational change might expose the ETS domain or inhibitory regions may be shielded. If the weak TEL DNA-binding affinity is caused by such intramolecular inhibition of the ETS domain, Tip60 binding might remove this inhibition and expose the ETS motif. Studies of the eukaryotic ETS transcription factor, Ets1, showed that the DNA binding is regulated negatively by intermolecular inhibition up to 23-fold and that two regions flanking the ETS domain mediate the inhibition by promoting the dissociation from DNA [55,56]. Interestingly, in the transcription factor Ets1, the N- and C-flanking inhibitory regions function in an inter-dependent mode. Concerning TEL, it is probable that N- and C-terminal regions of the ETS domain interact, mask the ETS domain and form an interaction interface to Tip60. The *Drosophila* MYST protein MOF (Tip60 homologue) uses its C₂HC zinc finger to bind the globular part of the nucleosome and the tail of histone H4 [57]. Accordingly, it is possible that the Tip60 C₂HC zinc finger binds TEL and assists in DNA-binding/complex formation.

As noted, Tip60 is important for DNA repair and apoptotic signalling. If the Tip60 complex requires interaction with TEL for these processes, this could in association with the observed loss of the non-translocated TEL allele in t(12;21)(p13;q22)-associated ALL explain progression in the malignancy. The B- and T-cells are vulnerable especially to genetic lesions as B- and T-cells exhibit high activity of immunoglobulin and T-cell receptor gene rearrangements. As TEL is expressed highly in the haematopoietic cells, it may, in complex with Tip60, play a pivotal role in protecting the cell from mutations; in contrast, when absent from ALL cells, TEL may lead to accumulation of new putative oncogenic mutations.

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