Research paper

The proto-oncogenic protein TAL1 controls TGF-β1 signaling through interaction with SMAD3

Jean-Michel Terme, Sébastien Lemaire, Didier Auboeuf, Vincent Mocquet, Pierre Jalinot*

Univ Lyon, ENS de Lyon, Univ Claude Bernard, CNRS UMR 5239, INSERM U1210, Laboratory of Biology and Modelling of the Cell, 46 Allée d'Italie Site Jacques Monod, F-69007, Lyon, France

Abstract

TGF-β1 is involved in many aspects of tissue development and homeostasis including hematopoiesis. The TAL1 transcription factor is also an important player of this latter process and is expressed very early in the myeloid and erythroid lineages. We previously established a link between TGF-β1 signaling and TAL1 by showing that the cytokine was able to induce its proteolytic degradation by the ubiquitin proteasome pathway. In this manuscript we show that TAL1 interacts with SMAD3 that acts in the pathway downstream of TGF-β1 association with its receptor. TAL1 expression strengthens the positive or negative effect of SMAD3 on various genes. Both transcription factors activate the inhibitory SMAD7 factor through the E box motif present in its transcriptional promoter. DNA precipitation assays showed that TAL1 present in Jurkat or K562 cells binds to this SMAD binding element in a SMAD3 dependent manner. SMAD3 and TAL1 also inhibit several genes including *ID1*, *hTERT* and *TGF-β1* itself. In this latter case TAL1 and SMAD3 can impair the positive effect exerted by E47. Our results indicate that TAL1 expression can modulate TGF-β1 signaling by interacting with SMAD3 and by increasing its transcriptional properties. They also suggest the existence of a negative feedback loop between TAL1 expression and TGF-β1 signaling.
© 2016 The Authors. Published by Elsevier B.V. on behalf of Société Française de Biochimie et Biologie Moléculaire (SFBBM). This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Keywords: TAL1; SMAD3; TGF-β1; SMAD7

1. Introduction

TAL1 (T-cell acute lymphoblastic leukemia protein 1), also known as SCL (stem cell leukemia) is a tissue-specific transcription factor belonging to the basic helix-loop-helix (bHLH) family which dimerizes with other bHLH E factors and binds E-box consensus motifs. By associating with DNA directly or indirectly through interactions with other transcription factors, TAL1 can act either positively or negatively on transcription of specific genes. This has been well-illustrated by systematic approaches characterizing the various TAL1 targets [1–3]. In the case of a positive action it has been shown that TAL1 can intervene in multiprotein complexes by associating with the LIM-only proteins LMO1 and LMO2, as well as the GATA factors [4–8]. TAL1 has also been shown to interact with the p300 coactivator through its basic domain [9]. These molecular interactions, along with others as with SP1 [10], can lead to transcriptional activation by TAL1. However TAL1 can also act negatively [1–3] and it has been reported that it binds to the mSin3A transcriptional repressor, also through its basic domain [11]. Experiments in mice have shown that TAL1 represses the E47/HEB activity in thymocytes by inducing recruitment of mSin3A [12]. The expression of TAL1 is restricted to specific cells and development stages [13]. In particular it is expressed in early hematopoiesis and plays an important role in the generation of the erythroid and myeloid lineages. In the T-cell lineage expression of TAL1 is normally lost early in the differentiation process, but its abnormal maintenance as a consequence of chromosomal rearrangements or epigenetic activation is

---

* Corresponding author. Tel.: +33 472728563; fax: +33 472728080.
E-mail address: pjalinot@ens-lyon.fr (P. Jalinot).

commonly associated with T-cell acute lymphoblastic leukemia (T-ALL) [14−16]. TAL1 expression in this context is likely to play an important role as its suppression by RNA interference in Jurkat cells has been shown to lead to proliferation stop [2]. We have previously shown that TAL1 can be regulated by extracellular cytokines as TGF-β1 which induces its degradation through the ubiquitin proteasome pathway [17]. Association of TGF-β1 with its receptors leads to phosphorylation of the receptor-regulated SMADs SMAD2 and SMAD3 [18,19]. These factors can then associate with SMAD4 and enter the nucleus. These complexes by associating with specific sequence elements regulate either positively or negatively many genes [20,21]. TGF-β1 is important in many aspects of tissue homeostasis and in particular plays an important role in hematopoiesis as TAL1 does [22]. It has a regulatory role on hematopoietic stem cells (HSCs) and it is also important for differentiation of the myeloid and endothelial lineage. These effects involve SMAD3 as shown by gene disruption experiments which have established a role of this transcription factor in hematopoiesis [23].

In this manuscript we show that TAL1 can specifically bind SMAD3 and potentiate its positive or negative effect on specific genes. By activating negative regulator of the pathway as SMAD7 and inhibiting TGF-β1 expression itself, TAL1 and SMAD3 counteracts the TGF-β1 pathway functioning. These data together with previous observations are in favor of negative feedback loops between TGF-β1 and TAL1.

2. Materials and methods

2.1. Constructs

Plasmids used in this study have been previously described: pSGF-TAL1 [24], pSG5-MYC-TAL1 [25] and pSG-HA-Ub [26]. pCMV-Flag-E47 was kindly provided by C. Gallego [27]. pCDNA3-FLAG-SMAD2, -SMAD3 and −SMAD4 were kindly provided by A. Favre-Bonvin.

2.2. Cell culture and transfection

Cell culture and transfection were performed as previously described [24]. When indicated, cells were treated with human platelet TGF-β1 (100 pM). To achieve inhibition of the proteasome, MG132 (Sigma−Aldrich, StLouis, USA) was added at 10 μM for 4 h. Cellular extracts were normalized with respect to protein concentrations which were quantified with the DC protein assay kit (BioRad, Hercules, USA). HeLa cells were stably-transfected with the pCEP-FLAG-TAL or the control pCEP-FLAG-GFP constructs, as previously reported [24].

2.3. Immunoprecipitation and immunoblot

Transfected cells were lysed in Nonidet P-40-desoxycholate buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% Na desoxycholate, 0.5 mM Tris(2-carboxyethyl)phosphine, and 0.5 mM Pefabloc).

Immunoprecipitation were carried out by addition of antibodies diluted 1:250 and incubation at 4 °C for 1h30. Protein A sepharose beads were added and incubated with the mix for 1 h. Beads were collected by centrifugation and washed three times with Nonidet P-40-desoxycholate buffer. For experience in Supplementary Fig. 1 protein G magnetic beads were used. Proteins were eluted in 2× SDS sample buffer at 80 °C for 10 min. After protein gel electrophoresis and transfer to PVDF membrane, detection was carried out by incubation with primary antibodies diluted 1:1000 or as indicated by the manufacturer and revelation was performed by chemiluminescence using ECL, ECL + or ECL Prime kits. Following antibodies were purchased: FLAG (clone M2, Sigma), MYC (clone 9E10, Sigma), HA (clone 12CA5, Roche), SMAD3 (NB600-1258), SMAD2/3 (566412, Calbiochem) and TAL1 (BTL73, Millipore).

2.4. Luciferase assays

HeLa and 293T cells (1.4 10^5 cells) were transfected with 1.5 μg of constructs including the *firefly* luciferase coding sequence under the control of various promoters: TGF-β1 [28], 3Tplux [29], CAGA boxes [30], hTERT [31], ID1 [32], SMAD7-WT and SMAD7-mE [33] together with 15 ng of thymidine kinase *Renilla*-luciferase (pRL-TK) by calcium phosphate precipitation. Reporter gene analysis was performed 48 h after transfection by using the Dual-Luciferase™ Reporter Assay System (Promega, Madison, USA). The luciferase activity associated with each construct was normalized on the basis of pRL-TK activity. The values are those obtained in triplicate, from one representative experiment (out of 3 experiments) and are represented with standard deviation. In figures one, two and three stars indicate Student's T test (two tail, unpaired) p-value less than 0.05, 0.01 and 0.001, respectively.

2.5. Real-time quantitative RT-PCR

Total RNAs were extracted from frozen cells using the Rneasy mini-kit (Qiagen, Hilden, Germany). One step RT-PCR reactions were performed using the QuantiTect™ SYBR Green RT-PCR kit (Qiagen) and the LightCycler apparatus (Roche). Sequences of sense and antisense primers were described previously [24].

2.6. Nuclear extracts

Jurkat and K562 cells were grown in RPMI-1640 with 5% fetal calf serum and treated with 781 pM TGF-β1 for 1 h. Cells were collected by centrifugation, washed three times in PBS 1× and incubated for 30 min on ice in 3 pellet volumes of hypotonic buffer (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA pH 8.0, 0.1 mM EGTA, 1 mM DTT, 1× cOmplete™ mini EDTA-free protease inhibitor cocktail, 0.5 mM sodium vanadate). Cells were lysed using Dounce B pestle and nuclei were collected by a 10 min centrifugation at 900 g. These nuclei were incubated for 15 min on ice in

extraction buffer (20 mM Hepes pH 7.9, 360 mM NaCl, 1.5 mM MgCl2, 20% glycerol, 0.1 mM EDTA pH 8.0, 10 µMZnCl2, 1 mM DTT, 1× cOmplete™ mini EDTA-free protease inhibitor cocktail, 0.5 mM sodium vanadate). Nuclear extracts corresponded to the supernatants after a 15 min centrifugation at 12,000 g.

2.7. DNA precipitation assays

The probe corresponding to the SMAD binding element (SBE) of the human *SMAD7* gene was obtained by hybridizing a 5′ biotinylated oligonucleotide (5′- AGCGA-CAGGGTGTCTAGACGGCCACGTGA -3′) with another corresponding to the complementary strand. Probe (0.25 µM) was incubated in 160 µl with 100 µg of nuclear extracts, polydIdC and 15 mM Tris pH 7.9, 3 mM MgCl2 and 0.2% Triton X-100 (final concentration) for 15 min on ice. Streptavidin magnetic beads were added and the mix was further incubated for 15 min on ice. The magnetic beads were washed three times in wash buffer (20 mM Tris pH 7.9, 50 mM KCl, 1.5 mM MgCl2, 10% glycerol, 0.1 mM EDTA pH 8.0, 10 µM ZnCl2, 1 mM DTT, 1× cOmplete™ mini EDTA-free protease inhibitor cocktail, 0.25% Triton X-100). For competition experiments the mix was preincubated for 15 min on ice with double stranded oligonucleotides corresponding to the SMAD7 SBE wild type (5′- CAGGGTGTCTAGACGGCCAC - 3′) or mutated (5′- CAGGGTCATAGCGTGGCCAC - 3′). Proteins associated with DNA were eluted by heating the beads for 10 min at 80 °C in protein loading buffer and analyzed by immunoblot after separation in 9% polyacrylamide SDS gel.

3. Results

3.1. TAL1 interacts with SMAD3

As we have previously shown that the intracellular amount of TAL1 was regulated by TGF-β1 [17], we investigated further whether this transcription factor was able to interfere with the TGF-β1 pathway through association with the SMAD proteins which are tightly regulated by association of the cytokine with its receptor. To test a possible direct binding of TAL1 to these factors, 293 T cells were transfected with vectors expressing SMAD2, SMAD3 and SMAD4 tagged with the FLAG epitope, together with a construct producing TAL1 fused to the MYC epitope. These cells, as HeLa cells do not express endogenous TAL1. These three SMADs were expressed at similar levels as evaluated by an immunoblot analysis of the cellular extracts (Fig. 1A, bottom panel). Coexpression of the SMADs did not modify the level of MYC-tagged TAL1 in the extracts (Fig. 1A, middle panel). Immunoprecipitation using the FLAG antibody showed a clear coprecipitation of TAL1 with SMAD3 (Fig. 1A, top panel, lane 3), but no association with SMAD2 or SMAD4 (Fig. 1A, top panel, lanes 2 and 4). The SMAD3 signal was absent when cells were transfected with the TAL1 or SMAD3 vector alone as controls. To verify this interaction, the experiment was also
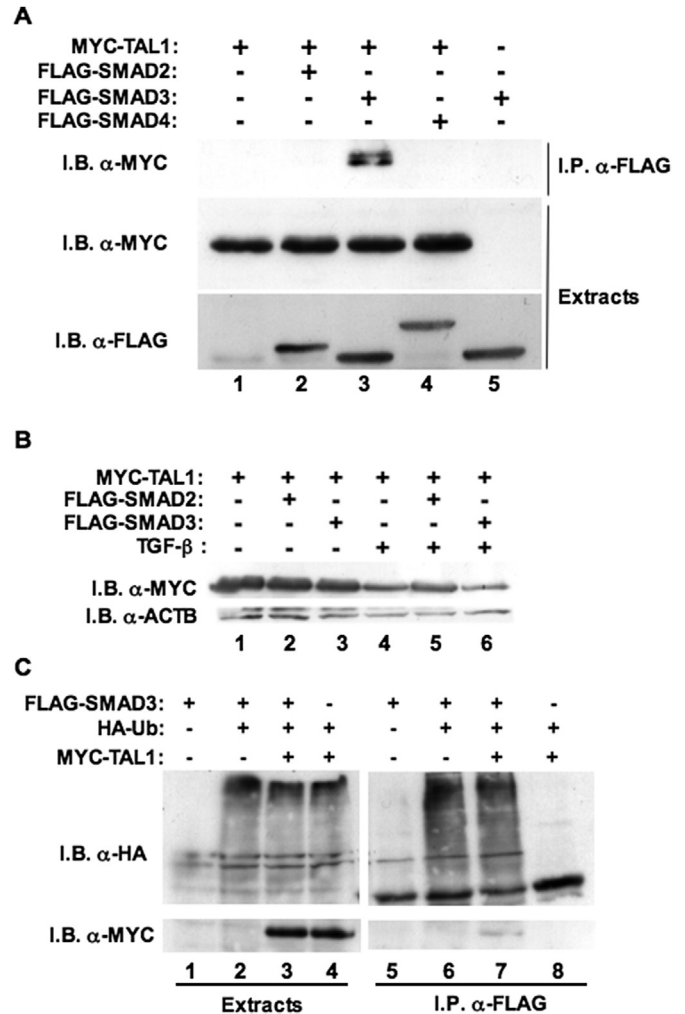

Fig. 1. **Interaction between TAL1 and SMAD3**: (A) 293T cells were transfected with 2 µg of pSG5-MYC-TAL1 and either pCDNA3-FLAG-SMAD2, -SMAD3 or -SMAD4 expression vectors as indicated. To avoid possible TAL1 degradation cells were treated with the MG132 proteasome inhibitor. Cell lysates were used for immunoprecipitation with an antibody to FLAG. Immunoprecipitated proteins were analyzed by immunoblot using antibodies to MYC (top panels) and to FLAG (bottom panel). (B) 293T cells were transfected with pSG5-MYC-TAL1 either alone or in combination with pCDNA3-FLAG-SMAD2 or −SMAD3 and treated with 100 pM of TGF-β1 during 9 h. Cellular extracts were normalized with respect to protein concentration and analyzed by immunoblot using antibodies to MYC (top panel) or to β-actin as control (bottom panel). (C) 293T cells were transfected with pSG5-MYC-TAL1, pCDNA3-FLAG-SMAD3 and pSG-HA-Ub as indicated. Cell lysates were used for immunoprecipitation with an antibody to FLAG. Immunoblot analysis was done using the antibody to HA (top panel) or to MYC (bottom panel).

performed in the reverse way by precipitating an HA-tagged form of TAL1 and detecting SMAD3. Similarly a clear and specific binding of TAL1 to SMAD3 was observed (Supplementary Fig. 1). These observations indicated that TAL1 can bind SMAD3.

As we have previously shown that TGF-β1 was able to induce polyubiquinylation and proteasome degradation of TAL1 [17] we tested if coexpression of SMAD2 or SMAD3 affects this process. By treating cells transfected with the

TAL1 expression vector with TGF-β1, a decrease in the level of TAL1 protein was observed in agreement with our previous observations (Fig. 1B, top panel, compare lanes 1 and 4). This effect was not modified by coexpression of either SMAD2 or SMAD3 (Fig. 1B, top panel, lanes 2, 3, 5 and 6). The SMAD3 protein is also modified post-translationnally by poly-ubiquitinylation [34]. This can be observed by transfecting cells with a SMAD3 expression vector and a construct producing ubiquitin tagged with the HA epitope. Immunoblot analysis using the antibody to HA of immunoprecipitated SMAD3 reveals a smear of polyubiquitinylated forms (Fig. 1C, lane 6). This modification of SMAD3 is not affected by coexpression of TAL1 (Fig. 1C, lanes 6 and 7).

These experiments show an interaction of TAL1 with SMAD3 and that this binding does not interfere with the modification by polyubiquitinylation of both proteins.

3.2. TAL1 and SMAD3 cooperate in activation of TGF-β1-induced genes

To investigate the functional relevance of this interaction between TAL1 and SMAD3 we tested whether it affected the activity of these transcriptional regulators. To this end cells were transfected with constructs placing the luciferase reporter gene under the control of sequence motifs of the human plasminogen activator inhibitor-1 gene mediating transcriptional activation by TGF-β1. These motifs corresponded to a fragment of the *PAI-1* gene (3TPLux) or to CAGA boxes in tandem. Overexpression of SMAD3 transactivated both constructs, as well as TAL1 but in this case to a much lesser extent (Fig. 2A and B compare lanes 1, 2 and 3). Combination of SMAD3 and TAL1 led to a higher transactivation showing that both proteins were able to act synergistically (Fig. 2A and B, lanes 4). To confirm that both proteins were able to cooperatively activate TGF-β1-responsive genes we tested a construct associating luciferase and the SMAD7 transcriptional promoter. SMAD7 expression is known to be induced by TGF-β1 and to downregulate signaling by this cytokine by causing degradation of TGF-β receptor type I (TGFBR1). Both TAL1 and SMAD3 transactivate the SMAD7 promoter and association of both proteins led to a further increase (Fig. 3A compare lanes 1, 2, 3 and 4). Mutation of the E box of the SMAD7 promoter led to a complete loss of the transactivation by the TAL1 SMAD3 combination (Fig. 3A, lane 5). Considering the sensitivity of the effect to a mutation of the SMAD binding element in the SMAD7 transcriptional promoter we tested whether association of both proteins on this DNA sequence could be observed using endogenous proteins. This was done by performing DNA precipitation assays using a biotinylated probe corresponding to the SMAD7 SBE. Nuclear extracts were prepared from TGF-β1 treated Jurkat cells which express endogenous TAL1. In these extracts expression of both SMAD3 and TAL1 as a 34 kD protein can be detected by immunoblot analysis (Fig. 3B, lane 1). Both proteins were also detected in the proteins eluted from the DNA probe (Fig. 3B, lane 4), whereas no signal was obtained when this
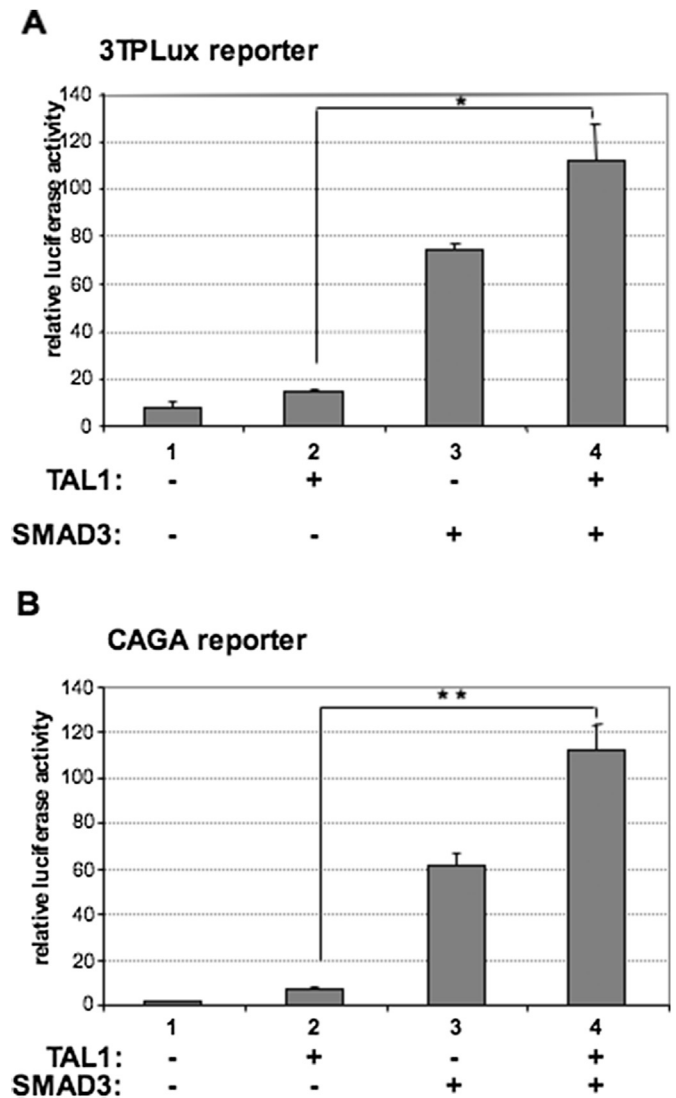

Fig. 2. **TAL1 and SMAD3 transactivate TGF-β responsive promoter sequence elements**: HeLa cells were transfected with 300 ng of pSGF or pSGF-TAL1, and 100 ng of pCDNA3 or pCDNA3-SMAD3, together with 1.5 µg of 3TPLux (A) and CAGA (B). Cell extracts were prepared 48 h after transfection and analyzed for luciferase activity. The graph represents the relative luciferase activity (mean of three points) and error bar corresponds to standard deviation. P-values of a Student's T test are represented as described in materials and methods.

probe was omitted in the incubation, indicating that these two proteins did not bind non specifically to the magnetic beads under these conditions (Fig. 3B, lane 3). These experiments were also performed with an antibody recognizing both SMAD3 and SMAD2. This was done with nuclear extracts of Jurkat and K562 erythroid cells, these latter also expressing endogenous TAL1. In these extracts the signals corresponding to SMAD2 and SMAD3 were clearly detected, the relative abundance of SMAD2 being higher in Jurkat (Fig. 3C, lanes 1 and 5). Interestingly after DNA precipitation only the SMAD3 signal was detected with extracts of both cell types (Fig. 3C, lanes 4 and 6, upper panels). As with Jurkat, the TAL1 protein
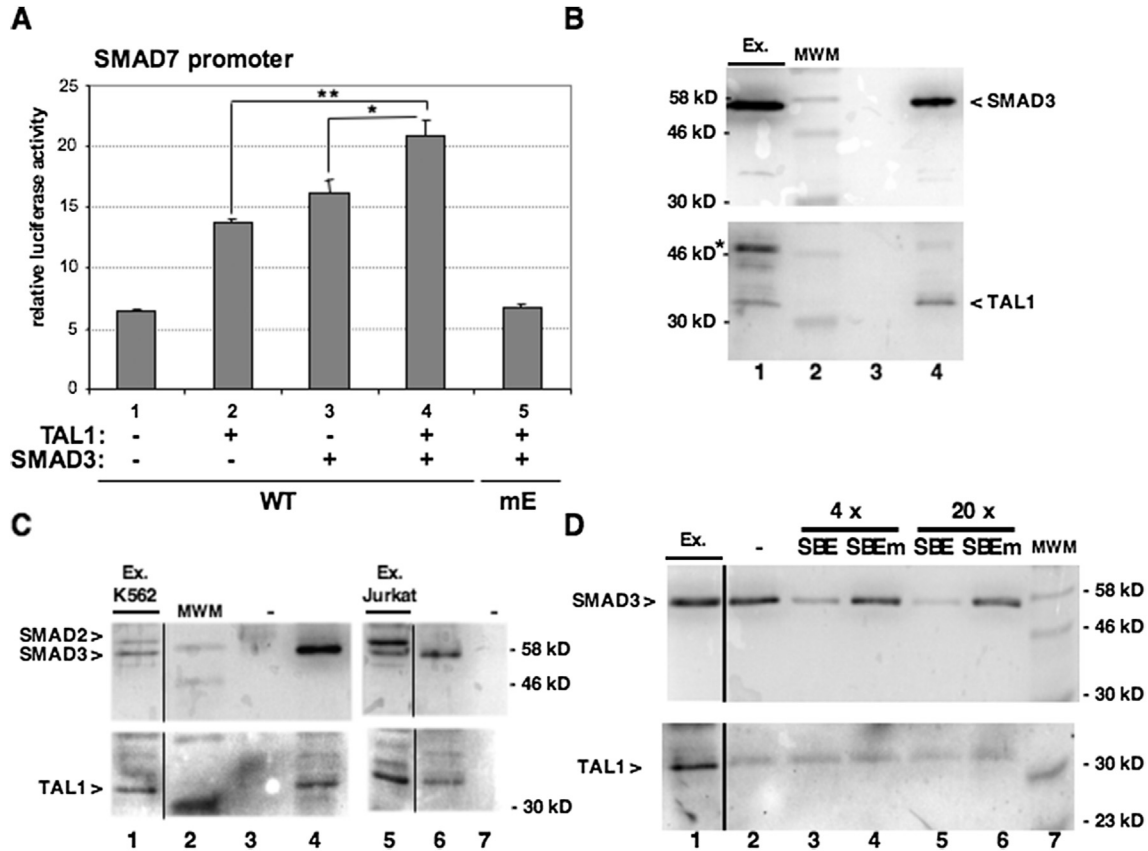
Fig. 3. **TAL1 cooperates with SMAD3 to induce the SMAD7 promoter**: (A) HeLa cells were transfected with 300 ng of pSGF or pSGF-TAL1, and 100 ng of pCDNA3 or pCDNA3-SMAD3, together with 1.5 μg of pSMAD7-WT (WT) or pSMAD7-mE which includes a mutated E-box motif. Analysis of luciferase activity was performed as described in legend to Fig. 2. (B). DNA precipitation assay was carried out using the *SMAD7* SBE probe and a nuclear extract of Jurkat cells treated for 1 h with TGF-β1. Lane 1 corresponds to the direct loading of 10% of the extract and lane 2 to loading of a protein molecular weight marker (MWM). Proteins recovered after DNA precipitation were loaded in lanes 3 and 4 but in lane 3 the DNA probe was omitted as a control of non-specific binding to the beads. The upper and lower panels correspond to immunoblot analyses with the antibodies directed against SMAD3 and TAL1, respectively. The star on the left indicates a non-specific signal. The depicted images correspond to the superposition of colorimetric and chemiluminescence acquisitions with a ChemiDoc Touch Imaging System apparatus (BioRad, Hercules, USA). The molecular weights of the marker bands are indicated on the left. (C). The DNA precipitation experiment was carried out as in B with nuclear extracts of K562 (lanes 1 to 4) or Jurkat cells (lanes 5 to 6) and SMAD revelation was done using an antibody recognizing both SMAD2 (upper band) and SMAD3 (lower band) as indicated. The immunoblots were also revealed with the monoclonal antibody to TAL1 (lower panels). Lanes 1 and 5 correspond to direct loading of the extract. In lane 3 the DNA probe was omitted as control. (D) The DNA precipitation assay was performed with a nuclear extract of TGF-β1-treated K562 cells and the results are shown as in B. Lane 1 corresponds to 10% of this extract. In lanes 3 to 6 the nuclear extract was preincubated with a 4× or 20× excess of double stranded oligonucleotides corresponding to the SBE wild type (SBE) or mutated (SBEm) as indicated. The molecular weight marker was loaded in lane 7 and the molecular weights of the bands are indicated on the right.

of K562 cells bound to the SBE probe together with SMAD3 (Fig. 3C, lanes 4 and 6, lower panels). To check that the TAL1 binding was specific of SMAD3 we performed competition experiment with a 4× and 20× excess of double stranded oligonucleotide corresponding to the SBE. This was done with nuclear extracts of K562 cells and sequences corresponding to wild type or mutated SBE. At 20× excess a clear reduction of the SMAD3 signal was seen with the wild type SBE oligonucleotide but not with the mutated one (Fig. 3D, compare lanes 5 and 6, upper panel). As compared to the mutated one, the wild type SBE oligonucleotide also reduced the TAL1 signal (Fig. 3D, lanes 5 and 6, lower panel). This shows that impairment of SMAD3 binding also affects TAL1 detection indicating that a SMAD3-TAL1 complex binds and activates the SMAD7 SMAD responsive promoter.

3.3. TAL1 and SMAD3 can also cooperate in down-regulation of cellular genes

TAL1 has been described as a transcriptional activator, but also as a transcriptional repressor. Such a negative effect was observed in particular by testing a limited series of genes in a cell line constitutively expressing TAL1 from an episomic EBV derived vector. Using this tool two cell lines were generated expressing either GFP, as a control, or TAL1 as previously reported [24]. In these constructs both proteins were tagged with the FLAG epitope. Quantitative real time PCR analysis of several genes expression level between these two cell lines showed that TAL1 was able to repress expression of *IκBα* and also of the *ID1*, *ID2* and *ID3* genes (Fig. 4A) which are known to be R-SMAD sensitive. By contrast
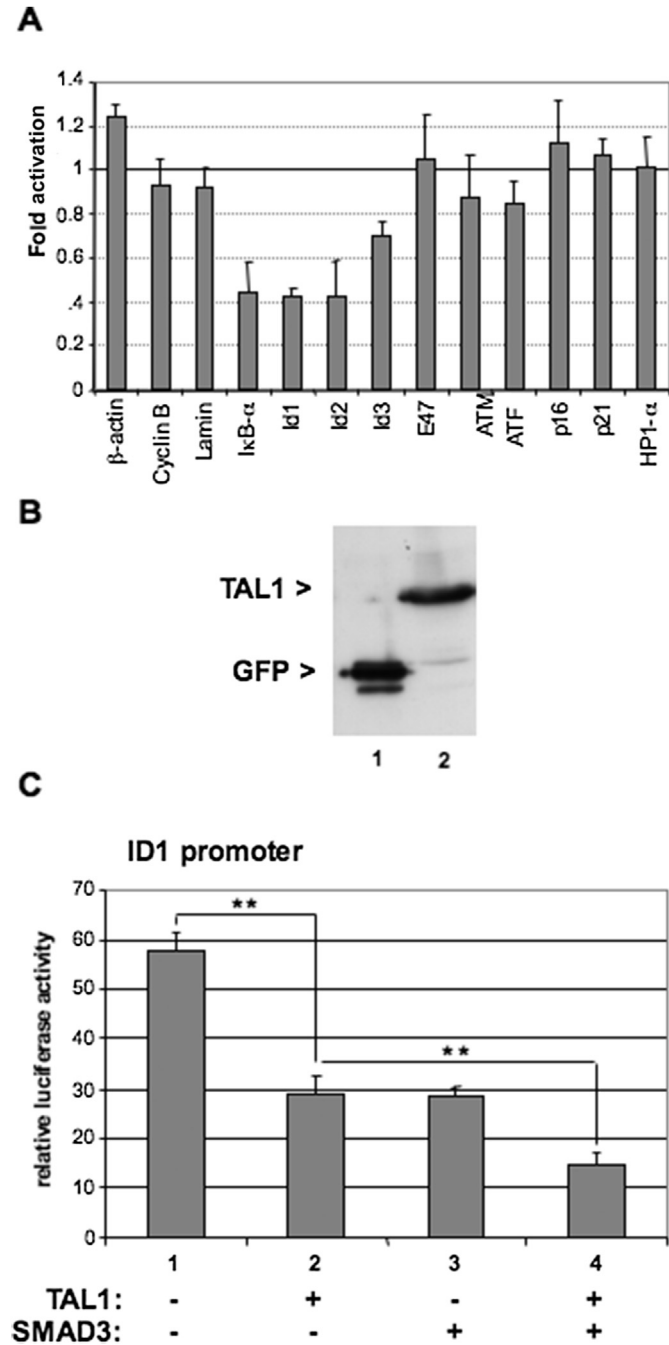
A

B

C

ID1 promoter

Fig. 4. **ID1 promoter repression by TAL1 and SMAD3**: (A) HeLa cells were stably-transfected with the pCEP-FLAG-TAL1 or the control pCEP-FLAG-GFP constructs. After selection of stably-transfected cells, total RNAs were prepared and analyzed by real-time quantitative RT-PCR for expression of various genes as indicated. The amount of mRNAs are expressed as a ratio FLAG-TAL1 cells versus FLAG-GFP cells. (B) Immunoblot analyses of cells transfected with pCEP-FLAG-GFP (lane 1) or pCEP-FLAG-TAL1 (lane 2) using antibodies to FLAG. (C) HeLa cells were transfected with 150 ng of pSGF (lanes 1 and 3) or pSGF-TAL1 (lanes 2 and 4), and 100 ng of pCDNA3 (lanes 1 and 2) or pCDNA3-SMAD3 (lanes 3 and 4), together with 1.5 μg of pID1-Luc. Analysis of luciferase activity was performed as described in legend to Fig. 2.

expression of the *β-actin, cyclin B1, lamin, E47, ATM, ATF, p16, p21 and HPI-α* genes which have not been described as responding to TGF-β was not affected by the presence of TAL1. Immunoblot analysis using the antibody to FLAG

showed that both proteins were expressed at a similar level (Fig. 4B, compare lanes 1 and 2). The TAL1 negative effect was also observed in transient expression studies using a construct including the luciferase reporter gene under the control of the ID1 transcriptional promoter (Fig. 4C, compare lanes 1 and 2). Interestingly SMAD3 was also able to repress the ID1 promoter and coexpression of both TAL1 and SMAD3 led to a stronger effect (Fig. 4C, lanes 3 and 4). We previously reported that TAL1 was able to negatively regulate the promoter of the protein subunit of the telomerase enzyme [25]. By testing the effect of SMAD3 on the hTERT promoter we also observed a dose dependent negative effect (Fig. 5, compare lanes 1, 2 and 3). Coexpression of TAL1 also reinforced this negative effect of SMAD3 (Fig. 5, lanes 5, 6).

Finally as SMAD3 is known to be a negative regulator of the TGF-β1 promoter we also investigated the TAL1 effect on this transcriptional regulatory element. TAL1 was observed to down regulate the TGF-β1 promoter (Fig. 6A, compare lanes 1, 2 and 3) and also to counteract its activation by the type I bHLH protein E47 (Fig. 6B). Indeed overexpression of E47 markedly increased the activity of this promoter, but coexpression of TAL1 impeded this effect in a dose dependent manner (Fig. 6B, lanes 4, 5 and 6). By expressing a low amount of TAL1 this negative effect on E47 transactivation was not observed (Fig. 6C, lanes 2 and 3) but coexpression of SMAD3 led to a clear impairment of the E47 effect (Fig. 6C, lane 5). This clearly showed that the TAL1 SMAD3 complex can negatively downregulate several genes, including *TGF-β1* itself.

By combining these different observations it appears that TAL1 and SMAD3 might play a clear role in a negative feedback loop downregulating TGF-β1 signaling. Indeed by activating SMAD7 and inhibiting TGF-β1 they are likely to act negatively on this pathway. In addition we have previously
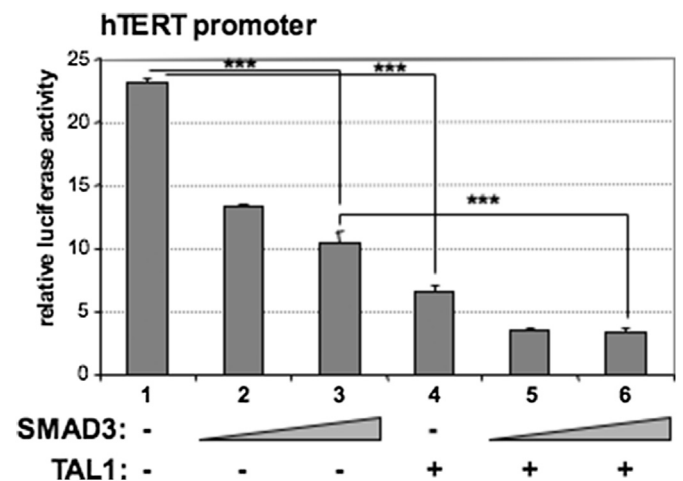

Fig. 5. **Repression of the hTERT promoter by TAL1 and SMAD3**: HeLa cells were transfected with 300 ng of pSGF (lanes 1 to 3) or pSGF-TAL1 (lanes 4 to 6), 100 and 50 ng of pCDNA3 (lanes 1, 4 and 2, 5) and 50 or 100 ng of pCDNA3-SMAD3 (lanes 2, 5 and 3, 6), together with 1.5 μg of pGL3-hTERT-3300. Analysis of luciferase activity was performed as described in legend to Fig. 2.
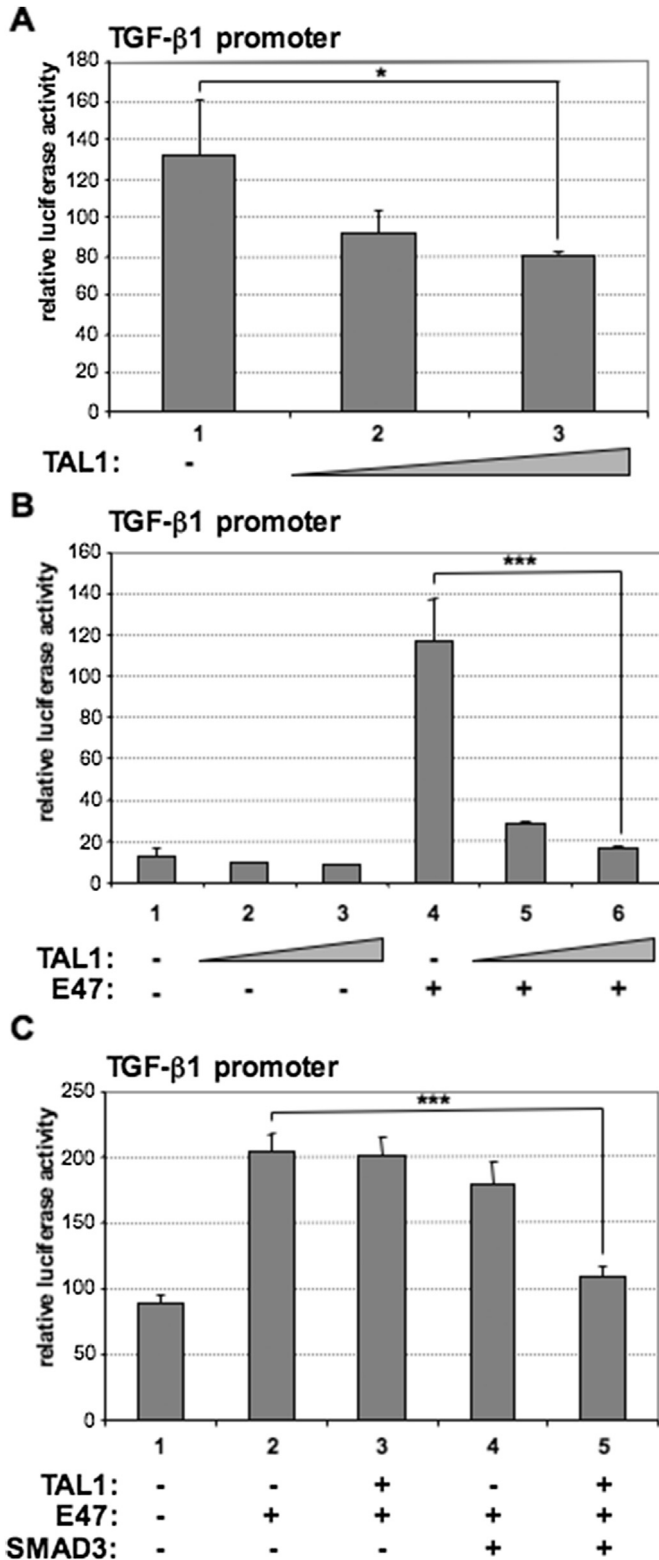
Fig. 6. **TAL1 represses TGF-β1 promoter**: (A) HeLa cells were transfected with pSGF (lanes 1) or 300 ng and 600 ng of pSGF-TAL1 (lanes 2 and 3), together with 1.5 μg of pTGFβ1-1132-luc (B) HeLa cells were transfected with pSGF (lanes 1 and 4) or 300 ng and 600 ng of pSGF-TAL1 (lanes 2 and 3, 5 and 6), together with 150 ng of pCMV (lanes 1 to 3) or pCMV-E47 (lanes 4 to 6) and 1.5 μg of pTGFβ1-1132-luc. (C) HeLa cells were transfected with 50 ng of pSGF or pSGF-TAL1, 50 ng of pCMV or pCMV-E47, 75 ng of pCDNA3 or pCDNA3-SMAD3 as indicated, together with 1.5 μg of pTGFβ1-1132-luc. For all three panels analyses of luciferase activity were performed as described in legend to Fig. 2.

shown that TGF-β1 was able to induce TAL1 degradation through AKT activation. This shows that TAL1 in combination with SMAD3 could act negatively on key factors of the TGF-β1 pathway (Fig. 7).

4. Discussion

Both TAL1 and TGF-β1 have been established as important regulators of embryonic hematopoiesis. This is in particular illustrated by knock-out experiments in mice. TAL1-deficient mice indeed die around E9.5, with defects of hematopoietic and endothelial cell lineages [35,36]. Targeted disruption of the *TGF-β1* gene also results in lethality at E10.5 in about 50% of mice with defective hematopoiesis and yolk sac vasculogenesis, transient survival of other mice being interpreted as a consequence of maternal transfer of the cytokine [22,37]. Unexpectedly our results indicate a link between the events downstream of TGF-β1 binding to its receptor and TAL1.

Indeed analysis of the interaction of TAL1 with the SMADs mediating the TGF-β1 effect showed a specific binding of TAL1 to SMAD3. Both TAL1 and SMAD3 have been described to be regulated by polyubiquitinylation and proteasome degradation [17,34]. From our observations this post translational modification does not seem to be affected by interaction between both proteins. In particular the overexpression of SMAD3 does not modify the TGF-β1-induced degradation of TAL1 that we previously reported [17]. From a functional point of view, association between TAL1 and SMAD3 results in an increase of their effect on specific transcriptional targets, both in the case of a positive or a negative action. Analysis of the sequences mediating TGF-β1-activation of the human *PAI-1* gene has shown the importance of three CAGA boxes present in the promoter [38]. SMAD3 overexpression leads to activation of reporter constructs bearing a *PAI-1* TGF-β1 responsive element including this motif [29] or multiple repeats of these CAGAs boxes [30] and coexpression of TAL1 clearly potentiates this effect. Conversely SMAD3 can downregulate several genes, as *hTERT* or *ID1*, and TAL1 reinforces this effect. Such an inhibitory effect of SMAD3 on ID1 has already been reported [39], but appears to be cell-type dependent [40]. From the limited number of genes tested in this study TAL1 and SMAD3 seem to act in the same way and to potentiate their mutual action on specific targets. Future systematic studies performed in specific cell types should help to test more extensively this overlap between TAL1 and SMAD3 targets. As a first clue to this question we looked at the available chromatin immunoprecipitation sequencing (ChIP-seq) data. If many experiments have been performed with TAL1 and SMAD3 we did not find ChIP-seq results performed with both TAL1 and SMAD3 in the same cells in the presence of TGF-β1. However Micrococcal Nuclease (MNase) ChIP-seq data with both TAL1 and SMAD2/3 are reported in human embryonic stem cells [41]. Hence we analyzed these data and observed that several TAL1 and SMAD2/3 peaks overlap in a number of genes (Supplementary Fig. 2). This can be observed in the promoter region and also in the transcribed sequence.
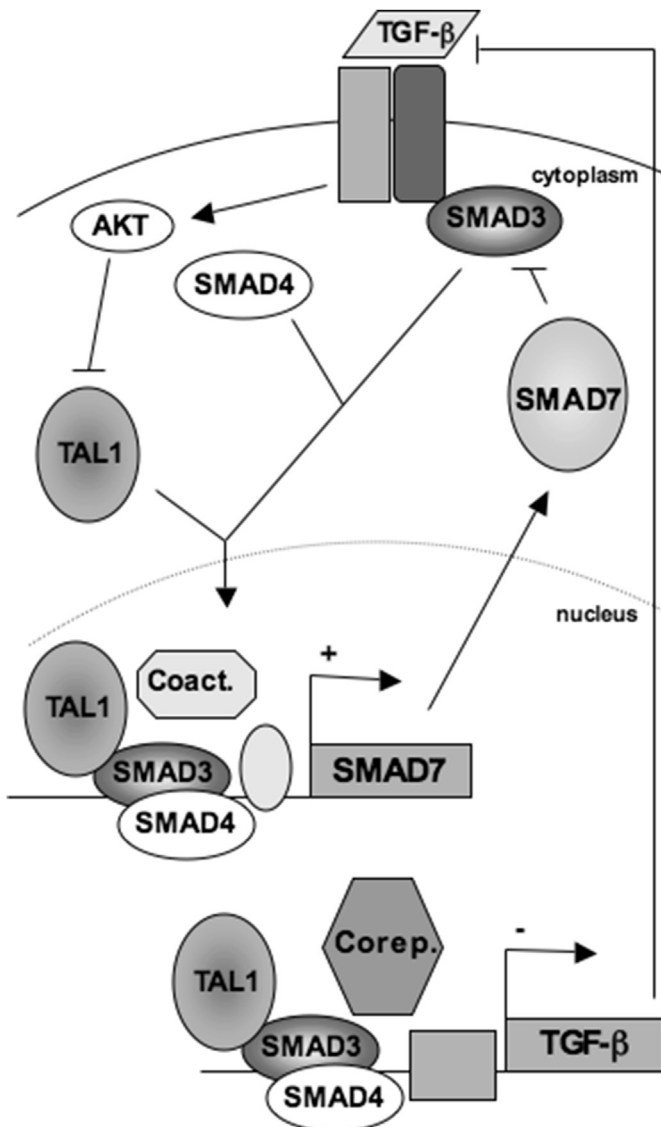
Fig. 7. **Crosstalk between TGF-β signaling pathways and TAL1**: This scheme recapitulates how TAL1 and SMAD3 act positively or negatively on TGF-β1 target genes, this leading to a downregulation of TGF-β1 signaling through SMAD7 and reduction of the cytokine expression. Conversely TGF-β1 can lead to TAL1 degradation through AKT activation.

These sequences represent potential regulatory elements involving both TAL1 and SMAD3. The list of the genes showing combined TAL1-SMAD2/3 peaks is given in Supplementary Table 1. Interestingly one the gene exhibiting a combined TAL1/SMAD3 peak in its promoter, SKIL, codes for a component of the SMAD pathway which binds to promoters of TGF-β responsive genes and recruits a repressor complex [42]. Two other genes belonging to this category code for proteins also related to the SMAD pathway, ZCCHC14 is indeed reported to interact with SMAD3 and ZZEF1 with SKIL [43,44]. These data support the notion that TAL1 and SMAD3 can co-regulate various genes. However this ChIP-seq approach will have to be carried out in various conditions of TGF-β1 treatment to increase the number of SMAD3 binding

peaks to promoters which is relatively low in these HUES64 cells. Depending on the cellular context as well as on the presence of other partner factors in relation with the effect of TGF-β1, the regulatory effects reported in this manuscript might vary. However, as also supported by the MNase ChIP-seq data mentioned, our experiments show that co-binding of both TAL1 and SMAD3 to promoter sequences can be clearly observed with the endogenous proteins of both erythroid and leukemic lymphoid cells. Future detailed analyses will help to determine the exact contribution of the SMAD3-TAL1 protein–protein interaction and of DNA binding of both transcription factors to promoter sequences. Interestingly Dogan et al. have recently reported that binding of both TAL1 and SMAD1 faithfully predicts the existence of an enhancer element, in a better way that the presence of specific epigenetic marks. Hence co-binding of both TAL1 and SMAD3 is likely to be a good predictor of an active regulatory element [45].

Our analysis of the transcriptional targets of SMAD3 and TAL1 interestingly showed that both factors are likely to retroact negatively on TGF-β1 signaling. SMAD7 is indeed well established as a negative regulator of the TGF-β1 signaling by impairing proper activation of the receptor-regulated SMAD2 and SMAD3 [46] or by triggering degradation of activated TGFBR-I [47]. TAL1 and SMAD3 synergistically activate expression of this inhibitory SMAD. Also analysis of the TGF-β1 promoter itself indicates a negative effect of both factors. In agreement with previous description of the negative effect of TAL1 on E47 [12], expression of the former was observed to impede the strong activation exerted by the latter on this promoter and SMAD3 strengthened this effect. These observations indicate that both factors are likely to facilitate termination of TGF-β1 signaling through these specific actions. As we previously showed that TGF-β1 causes degradation of TAL1 by the ubiquitin-proteasome pathway through AKT phosphorylation it appears that a negative feedback loop is likely to exist between TGF-β1 and TAL1.

Hence in TAL1 expressing cells, in particular the precursors of the hematopoietic and endothelial lineages, the action of TGF-β1 is probably restricted by the presence of this tissue-specific bHLH factor. In this line we have observed the SMAD3-TAL1 interaction on the SMAD7 SBE with K562 cells extracts. Accordingly the well-established negative effect of TGF-β1 on proliferation of HSC [22] might be related to a downregulation of TAL1. It is interesting to mention that SMAD3 gene targeted disruption mimics at least to some extent the TGF-β1 effects on hematopoiesis [23], showing a specific effect of this particular SMAD in these cells. TGF-β1 restricts HSC proliferation, but is also important for differentiation of these cells [22]. Our observations indicate that this process might involve a fine tuning of the TGF-β1 signaling by the level of TAL1 expression. It will be also interesting to study this connection between TGF-β1 signaling and TAL1 in the case of T-ALL as we have also observed its binding to SMAD3 in such cells. TAL1 is an important factor in many pediatric and adult T-ALL and mutations in the

TGFBR1 receptor have been reported in some cases of T-ALL [48]. Hence it will be interesting to decipher how both TAL1 and TGF-β1 intervene in various types of T-ALL cells.

In conclusion our observations establish a new and unanticipated link between TAL1 and TGF-β1 through SMAD3. These data along with the current knowledge on these proteins incite to develop future studies to assess the importance of this connection in hematopoiesis, as well as in T-cell leukemia onset.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biopen.2016.05.001.

References

[1] M.T. Kassouf, J.R. Hughes, S. Taylor, S.J. McGowan, S. Soneji, et al., Genome-wide identification of TAL1's functional targets: insights into its mechanisms of action in primary erythroid cells, Genome Res. 20 (2010) 1064−1083.

[2] T. Palomero, D.T. Odom, J. O'Neil, A.A. Ferrando, A. Margolin, et al., Transcriptional regulatory networks downstream of TAL1/SCL in T-cell acute lymphoblastic leukemia, Blood 108 (2006) 986−992.

[3] N.K. Wilson, D. Miranda-Saavedra, S. Kinston, N. Bonadies, S.D. Foster, et al., The transcriptional program controlled by the stem cell leukemia gene Scl/Tal1 during early embryonic hematopoietic development, Blood 113 (2009) 5456−5465.

[4] V. Deleuze, E. Chalhoub, R. El-Hajj, C. Dohet, M. Le Clech, et al., TAL-1/SCL and its partners E47 and LMO2 up-regulate VE-cadherin expression in endothelial cells, Mol. Cell Biol. 27 (2007) 2687−2697.

[5] R. Lahlil, E. Lecuyer, S. Herblot, T. Hoang, SCL assembles a multifactorial complex that determines glycophorin A expression, Mol. Cell Biol. 24 (2004) 1439−1452.

[6] H. Osada, G. Grutz, H. Axelson, A. Forster, T.H. Rabbitts, Association of erythroid transcription factors: complexes involving the LIM protein RBTN2 and the zinc-finger protein GATA1, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 9585−9589.

[7] V.E. Valge-Archer, H. Osada, A.J. Warren, A. Forster, J. Li, et al., The LIM protein RBTN2 and the basic helix-loop-helix protein TAL1 are present in a complex in erythroid cells, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 8617−8621.

[8] M. Yu, L. Riva, H. Xie, Y. Schindler, T.B. Moran, et al., Insights into GATA-1-mediated gene activation versus repression via genome-wide chromatin occupancy analysis, Mol. Cell 36 (2009) 682−695.

[9] S. Huang, Y. Qiu, R.W. Stein, S.J. Brandt, p300 functions as a transcriptional coactivator for the TAL1/SCL oncoprotein, Oncogene 18 (1999) 4958−4967.

[10] E. Lecuyer, S. Herblot, M. Saint-Denis, R. Martin, C.G. Begley, et al., The SCL complex regulates c-kit expression in hematopoietic cells through functional interaction with Sp1, Blood 100 (2002) 2430−2440.

[11] S. Huang, S.J. Brandt, mSin3A regulates murine erythroleukemia cell differentiation through association with the TAL1 (or SCL) transcription factor, Mol. Cell Biol. 20 (2000) 2248−2259.

[12] J. O'Neil, J. Shank, N. Cusson, C. Murre, M. Kelliher, TAL1/SCL induces leukemia by inhibiting the transcriptional activity of E47/HEB, Cancer Cell 5 (2004) 587−596.

[13] K. Pulford, N. Lecointe, K. Leroy-Viard, M. Jones, D. Mathieu-Mahul, et al., Expression of TAL-1 proteins in human tissues, Blood 85 (1995) 675−684.

[14] P.D. Aplan, D.P. Lombardi, G.H. Reaman, H.N. Sather, G.D. Hammond, et al., Involvement of the putative hematopoietic transcription factor SCL in T-cell acute lymphoblastic leukemia, Blood 79 (1992) 1327−1333.

[15] E. Lecuyer, T. Hoang, SCL: from the origin of hematopoiesis to stem cells and leukemia, Exp. Hematol. 32 (2004) 11−24.

[16] E.A. Macintyre, L. Smit, J. Ritz, I.R. Kirsch, J.L. Strominger, Disruption of the SCL locus in T-lymphoid malignancies correlates with commitment to the T-cell receptor alpha beta lineage, Blood 80 (1992) 1511−1520.

[17] J.M. Terme, L. Lhermitte, V. Asnafi, P. Jalinot, TGF-beta induces degradation of TAL1/SCL by the ubiquitin-proteasome pathway through AKT-mediated phosphorylation, Blood 113 (2009) 6695−6698.

[18] J. Massague, J. Seoane, D. Wotton, Smad transcription factors, Genes Dev. 19 (2005) 2783−2810.

[19] Y. Shi, J. Massague, Mechanisms of TGF-beta signalling from cell membrane to the nucleus, Cell 113 (2003) 685−700.

[20] D. Koinuma, S. Tsutsumi, N. Kamimura, T. Imamura, H. Aburatani, et al., Promoter-wide analysis of Smad4 binding sites in human epithelial cells, Cancer Sci. 100 (2009) 2133−2142.

[21] H. Qin, M.W. Chan, S. Liyanarachchi, C. Balch, D. Potter, et al., An integrative ChIP-chip and gene expression profiling to model SMAD regulatory modules, BMC Syst. Biol. 3 (2009) 73.

[22] S.S. Soderberg, G. Karlsson, S. Karlsson, Complex and context dependent regulation of hematopoiesis by TGF-beta superfamily signalling, Ann. N. Y. Acad. Sci. 1176 (2009) 55−69.

[23] M.W. Epperly, S. Cao, J. Goff, D. Shields, S. Zhou, et al., Increased longevity of hematopoiesis in continuous bone marrow cultures and adipocytogenesis in marrow stromal cells derived from Smad3(-/-) mice, Exp. Hematol. 33 (2005) 353−362.

[24] J.M. Terme, M. Wencker, A. Favre-Bonvin, F. Bex, L. Gazzolo, et al., Crosstalk between expression of the HTLV-1 Tax transactivator and the oncogenic bHLH transcription factor TAL1, J. Virol. 82 (2008) 7913−7922.

[25] J.M. Terme, V. Mocquet, A.S. Kuhlmann, L. Zane, F. Mortreux, et al., Inhibition of the hTERT promoter by the proto-oncogenic protein TAL1, Leukemia 23 (2009) 2081−2089.

[26] S. Buchsbaum, C. Morris, V. Bochard, P. Jalinot, Human INT6 interacts with MCM7 and regulates its stability during S phase of the cell cycle, Oncogene 26 (2007) 5132−5144.

[27] Y. Liu, M. Encinas, J.X. Comella, M. Aldea, C. Gallego, Basic helix-loop-helix proteins bind to TrkB and p21(Cip1) promoters linking differentiation and cell cycle arrest in neuroblastoma cells, Mol. Cell Biol. 24 (2004) 2662−2672.

[28] S.J. Lee, E.K. Yang, S.G. Kim, Peroxisome proliferator-activated receptor-gamma and retinoic acid X receptor alpha represses the TGFbeta1 gene via PTEN-mediated p70 ribosomal S6 kinase-1 inhibition: role for Zf9 dephosphorylation, Mol. Pharmacol. 70 (2006) 415−425.

[29] H. Li, A. Seth, An RNF11: Smurf2 complex mediates ubiquitination of the AMSH protein, Oncogene 23 (2004) 1801−1808.

[30] H. Xin, X. Xu, L. Li, H. Ning, Y. Rong, et al., CHIP controls the sensitivity of transforming growth factor-beta signalling by modulating

the basal level of Smad3 through ubiquitin-mediated degradation, J. Biol. Chem. 280 (2005) 20842—20850.

[31] S. Kyo, M. Takakura, T. Taira, T. Kanaya, H. Itoh, et al., Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT), Nucleic Acids Res. 28 (2000) 669—677.

[32] J.O. Nehlin, E. Hara, W.L. Kuo, C. Collins, J. Campisi, Genomic organization, sequence, and chromosomal localization of the human helix-loop-helix Id1 gene, Biochem. Biophys. Res. Commun. 231 (1997) 628—634.

[33] X. Hua, Z.A. Miller, H. Benchabane, J.L. Wrana, H.F. Lodish, Synergism between transcription factors TFE3 and Smad3 in transforming growth factor-beta-induced transcription of the Smad7 gene, J. Biol. Chem. 275 (2000) 33205—33208.

[34] S. Gao, C. Alarcon, G. Sapkota, S. Rahman, P.Y. Chen, et al., Ubiquitin ligase Nedd4L targets activated Smad2/3 to limit TGF-beta signalling, Mol. Cell 36 (2009) 457—468.

[35] L. Robb, I. Lyons, R. Li, L. Hartley, F. Kontgen, et al., Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 7075—7079.

[36] R.A. Shivdasani, E.L. Mayer, S.H. Orkin, Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL, Nature 373 (1995) 432—434.

[37] M.C. Dickson, J.S. Martin, F.M. Cousins, A.B. Kulkarni, S. Karlsson, et al., Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice, Development 121 (1995) 1845—1854.

[38] S. Dennler, S. Itoh, D. Vivien, P. ten Dijke, S. Huet, et al., Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene, Embo J. 17 (1998) 3091—3100.

[39] H. Song, B. Guo, J. Zhang, C. Song, Transforming growth factor-beta suppressed Id-1 Expression in a smad3-dependent manner in LoVo cells, Anat. Rec. (Hoboken) 293 (2010) 42—47.

[40] Y.Y. Liang, F.C. Brunicardi, X. Lin, Smad3 mediates immediate early induction of Id1 by TGF-beta, Cell Res. 19 (2009) 140—148.

[41] A.M. Tsankov, H. Gu, V. Akopian, M.J. Ziller, J. Donaghey, et al., Transcription factor binding dynamics during human ES cell differentiation, Nature 518 (2015) 344—349.

[42] S.L. Stroschein, W. Wang, S. Zhou, Q. Zhou, K. Luo, Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein, Science 286 (1999) 771—774.

[43] J. Wang, K. Huo, L. Ma, L. Tang, D. Li, et al., Toward an understanding of the protein interaction network of the human liver, Mol. Syst. Biol. 7 (2011) 536.

[44] F. Colland, X. Jacq, V. Trouplin, C. Mougin, C. Groizeleau, et al., Functional proteomics mapping of a human signaling pathway, Genome Res. 14 (2004) 1324—1332.

[45] N. Dogan, W. Wu, C.S. Morrissey, K.B. Chen, A. Stonestrom, et al., Occupancy by key transcription factors is a more accurate predictor of enhancer activity than histone modifications or chromatin accessibility, Epigenetics Chromatin 8 (2015) 16.

[46] H. Hayashi, S. Abdollah, Y. Qiu, J. Cai, Y.Y. Xu, et al., The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signalling, Cell 89 (1997) 1165—1173.

[47] X. Yan, Z. Liu, Y. Chen, Regulation of TGF-beta signalling by Smad7, Acta Biochim. Biophys. Sin. (Shanghai) 41 (2009) 263—272.

[48] S.J. Kim, J. Letterio, Transforming growth factor-beta signalling in normal and malignant hematopoiesis, Leukemia 17 (2003) 1731—1737.