Tid1 is a Smad-binding protein that can modulate Smad7 activity in developing embryos

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In a search for binding partners to Smad8, we identified the chicken homologue of the mammalian Tid1 protein (cTid1), which is a regulator of apoptosis related to the *Drosophila* tumour suppressor Tid56. The cTid1 coding sequence is highly conserved with mammalian Tid1, including the DnaJ domain that interacts with Hsp70 (heat-shock protein 70). The cTid1 gene is widely expressed with transcripts enriched in the developing blood islands of the embryonic-yolk sac. We show that cTid1 can bind to other members of the Smad family and that highest binding activity occurs with the negative regulatory Smad7, through the conserved MH2 domain. This interaction can have

functional relevance *in vivo*, since co-expression of Tid1 blocks the dorsalizing and BMP (bone morphogenetic protein)-dependent regulatory activity of Smad7 in developing *Xenopus* embryos. The finding that these proteins can interact suggests the potential for linking two important cell survival/apoptosis pathways.

Key words: bone morphogenetic protein (BMP) signalling, developing embryo, Smad-binding protein, Tid1, transforming growth factor β (TGF- β), *Xenopus*.

INTRODUCTION

The TGF- β (transforming growth factor β) superfamily of peptide signalling molecules regulates a diverse set of developmental and physiological processes, including cell survival, proliferation and differentiation [1,2]. The canonical signalling pathway downstream of receptor complexes is mediated by Smad proteins of three classes: receptor-activated Smad1, Smad2, Smad3, Smad5 and Smad8, the co-Smad4 and the inhibitory Smad6 and Smad7. One mechanism of TGF- β specificity is determined by ligand-dependent activation of specific Smads [3,4]. For example, activation of Smad1, Smad5 or Smad8 is associated primarily with signalling induced by the BMP (bone morphogenetic protein) subclass of peptides, while Smad2 and Smad3 mediate the response to TGF- β , activin and nodal ligands. Given the broad spectrum of phenotypes influenced by TGF- β signalling, it is not surprising that a host of regulatory mechanisms influence the capacity for signalling, including the relative presence of various ligand antagonists and the composition of type I and II receptor complexes in a given cell type or developmental context.

An additional level of control is downstream of the activated receptor complex, based on the composition of cellular Smad-binding proteins (for reviews, see [5,6]). The receptor-regulated Smads interact with 'adapter' proteins that fall into distinct functional classes, for example to control Smad protein stability (Smurfs), cellular localization either at the plasma membrane [SARA (Smad anchor for receptor activation)/Hgs/Hrs] or nuclear membrane (MAN1), or for assembly of higher order multiprotein cytoplasmic complexes (erbin/dv11/par3). In the nucleus, Smad proteins do not bind DNA with high affinity or specificity, and the majority of identified cofactors that associate with Smads are partners that either facilitate active Smad-containing complexes

on responsive target genes (FAST and OAZ), repress Smad activity (Tob, TGIF, PIASy and SANE) or otherwise help recruit more general gene regulatory proteins {p300 (a transcriptional activator protein required to drive p53 expression)/CBP [CREB (cAMP-response-element-binding protein)-binding protein] and HDAC (histone deacetylase)}.

Less is known about factors that mediate the function of Smad-dependent signalling important for cell survival or apoptosis. Both Smad2 and Smad4 are clearly established tumour suppressor proteins [2]. BMP signalling also regulates apoptosis in a variety of developmental contexts including those important for limb formation [7] and the reproductive system [8]. With respect to tumour suppression activity, BMPs induce apoptosis of myeloma cells [9] and the retinoid-induced death of medulloblastoma cells is mediated by BMP signalling [10]. Chromosomal deletions that cause acute myeloid leukaemia are linked to a region that includes Smad5 [11,12]. Therefore Smad-binding proteins are likely to fine-tune the cell survival or apoptotic response to TGF- β and BMP signalling, and these cofactors are also potential tumour suppressors.

Regulation of apoptosis similarly involves modulation of various signalling pathways that sense the cellular environment. The proteins encoded by the Tid1 gene interact with a variety of known signalling pathways and either enhance or suppress an apoptotic response. The human Tid1 protein was initially found by its ability to interact with the herpesvirus E7 oncoprotein [13]. The predominant structural domain of Tid1 classifies it as a member of the DnaJ family of proteins. It is the homologue of the *Drosophila* *l(2)tid* (tumorous imaginal disc) gene, which when mutated causes hyper-proliferation and lethal neoplastic growth of imaginal disc cells. At least one of the protein isoforms expressed from the *l(2)tid* locus is a component of the Hedgehog/Patched signalling

Abbreviations used: BMP, bone morphogenetic protein; EF, elongation factor; GAP, GTPase-activating protein; GST, glutathione S-transferase; JAK, Janus kinase; NP40, Nonidet P40; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TGF- β , transforming growth factor β ; *tid*, tumorous imaginal disc; UAS, upstream activating sequence.

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pathway [14], and alterations in human genes of this pathway (e.g. amplification of the *GLI* gene) cause susceptibility to cancers including gliomas and melanomas.

There are several human Tid1 isoforms that have distinct effects on cell survival when overexpressed in cell culture experiments [15]. The long form mediates increased apoptosis while the shorter isoform suppresses cell death caused by various apoptotic agents. The Tid1 protein has recently been shown to interact with components of several signalling pathways including GAP (GTPase-activating protein) [16], which regulates Ras activity, and JAK (Janus kinase) 2 [17], a key component of interferon signalling. An important developmental role for Tid1 within the immune system is implied by its selective induction in TH2 cells compared with TH1 cells, which may explain the resistance of the former to activation-induced cell death [18]. There are several lines of evidence indicating that Tid1 is a tumour suppressor, in addition to its demonstrated role as a modulator of apoptosis. For example, it is aberrantly expressed in certain tumour cell lines [19] and altered expression interferes with the transformed phenotype of tumour cells [20,21]. RNAi (RNA interference) experiments in cell lines show that loss of Tid1 (much like overexpression of the short isoform) inhibits apoptosis induced by various agents [21]. Recently the Tid1 knockout mouse was shown to die early during embryogenesis [22]. Conditional deletion of the gene in embryonic fibroblasts causes cell death, indicating that Tid1 is an essential cell survival factor.

In a previous work, we reported that Smad8 is an early embryonic cell survival factor during *Xenopus* development [23]. To try and understand the mechanism of Smad8 function, we sought the interacting proteins and identified the chicken homologue of Tid1 (cTid1). Furthermore, we find that cTid1 can interact with additional Smad family members and can regulate the activity of at least one, Smad7. Tid1 is therefore a candidate cellular factor that could influence the cell survival or apoptotic pathway downstream of TGF- β -like signalling.

EXPERIMENTAL

Library and bait construction, and the two-hybrid screen

To generate a two-hybrid target library, poly(A)⁺ (polyadenylated) mRNA was isolated from day 13 chick embryonic blood using an oligo(dT)-cellulose column (Gibco). A directional cDNA library was then constructed using the Superscript Plasmid System for cDNA Synthesis and Plasmid Cloning (Gibco). The cDNA inserts were ligated in separate reactions into the yeast shuttle/expression vectors p500, p501 and p502, which had each been digested with SalI and NotI. These vectors are derived from pGAD424 (Clontech) but with unique polylinker sequences that generate three distinct reading frames for inserts fused to the GAL4 activation domain. The three ligation mixtures were then electroporated into the ElectroMAX DH10B *Escherichia coli* (Gibco) and the transformed colonies were used to generate a pool of target plasmids.

The Smad8 bait vector was constructed by first ligating the HindIII insert of GBT9 (Clontech), including the GAL4 DNA-binding domain, the multiple-cloning site and the termination sequence of ADH1 (alcohol dehydrogenase 1) into HindIII site of pGAD424 (Clontech), replacing the GAL4 activation domain and multiple cloning site. The resulting plasmid was then digested with EcoRI and BamHI and ligated to the similarly restricted partial Smad8 cDNA (clone 6/2), placing the Smad8 sequence in frame with the GAL4 DNA-binding domain.

The reporter strain PJ69-4A (MATa, trp1-901 leu2-3 and 112 ura3-52 his3-200 gal4 Δ gal80 Δ LYS2::GAL1-HIS3 GAL2-

ADE2 met2::GAL7-lacZ) was first transformed with the bait plasmid GAL4DBD-(6/2)Smad8 by the lithium/caesium acetate method using a commercial transformation kit (Q-biogene) and a strain selected with media lacking leucine. Cells from this strain were subsequently transformed with the target library. Transformants were selected on media lacking leucine and tryptophan. A putative positive interaction was determined by growth after replica-plating colonies on to media lacking leucine, tryptophan and adenosine. The positive clones were isolated from yeast using breaking buffer (2%, v/v, Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris/HCl, pH 8, and 1 mM EDTA) and acid-washed glass beads (Sigma). The specificity of each clone was tested by transforming purified plasmid DNA back into PJ69-4A cells that contained either the original bait, the bait vector (negative control) or a GAL4-GATA1 plasmid (as an additional negative control). For the initial cTid1 clone (clone 4), colonies were also lifted on to filter paper and a strong β -galactosidase signal confirmed the positive interaction with the Smad8 bait.

Isolation of full-length cTid1 cDNA

Attempts to isolate the 5'-end of the cTid cDNA using standard RACE (rapid amplification of cDNA ends) failed, apparently due to strong secondary structure and very high G/C content. Therefore modified RACE was performed using the Generacer kit (Invitrogen). For this purpose, RNA from chick embryo liver was used, since Northern blotting experiments showed that this was enriched for cTid mRNA. Total chicken liver RNA was reverse transcribed at 65°C with gene-specific primer TE678B using thermoscript RT (reverse transcriptase; Invitrogen). Amplification of the 5'-end was achieved using primer TE723 and Advantage polymerase (Clontech) in the presence of 1.5 M betaine (Sigma). Products that represented cTID sequence were identified by Southern-blot analysis using oligomer TE722 as a probe and these fragments were subcloned using the Topo XL PCR Cloning kit (Invitrogen). The subcloned inserts were sequenced, confirmed to encode contiguous cTid1 coding sequence, and a forward primer (TE838) was designed to include the putative translation start site. Full-length cTID cDNA was then amplified using primers TE838 and TE691. The sequences of the relevant oligomers are: TE678B, 5'-CGTTTCCTTTCCCATTCACCGCTCAC; TE723, 5'-GGCGGAGCGCGGCACCCCCAGCACCTC; TE722, 5'-AGTAGTCCTCCTTGCGCGGGC; TE838, 5'-AGTCTCGAGCGCGCGGGCCAAGATG; and TE691, 5'-TCTAGATCAGTTTCCAGTAGAACGTTTCCC.

Northern blotting and *in situ* hybridization

Total RNA was prepared from isolated day 13 chick embryos. Tissues were minced with a razor blade, washed in PBS to remove blood, homogenized using a Tissue-Tearor (Biospec Reagents) in TRI reagent solution (Molecular Research Center) and RNA was processed according to the manufacturer's instructions. For each sample, 10 μ g of total RNA was electrophoresed in a 1% agarose/formaldehyde gel, and transferred passively on to a nylon membrane (Ambion). Blots were hybridized overnight under standard conditions (with 50%, v/v, formamide) [24] at 42°C, with a ³²P-labelled cTid1 probe generated by random priming (Hi-Prime; Roche), followed by washing at 60°C and phosphorimaging. Probes were derived either from a 5'-fragment or a 3'-fragment of the cTid1 cDNA, both of which gave identical results. The blot was then stripped and rehybridized with a probe generated from the chick EF (elongation factor) 1 β cDNA (provided by Dr S. Ghatpande, Yale University, New Haven, CT, U.S.A.). An antisense *in situ* hybridization probe was generated by linearizing the cTid1 clone 4 cDNA (in pBluescript SK) with SacI

followed by transcription using T3 polymerase to incorporate digoxigenin-labelled UTP. For control sense probes, the plasmid was restricted with NotI and RNA generated using T7 polymerase. The *in situ* hybridization method was performed as described in [25]. Embryos were staged by the method of Hamburger and Hamilton [26].

Smad-encoding plasmids

The isolation of the truncated Smad8 cDNA (clone 6/2) used to generate the bait and the isolation of full-length Smad8 cDNA was described in [23]. Other Smad cDNAs were generously provided by Dr G. Thomsen (xSmad1; SUNY, Stonybrook, NY, U.S.A.), Professor D. Melton (xSmad2; Harvard University, Cambridge, MA, U.S.A.), Dr J. Massague (mSmad4; Sloan Kettering Cancer Center, New York, NY, U.S.A.) and Professor J. Christian (xSmad6 and xSmad7; Oregon Health and Sciences University, Portland, OR, U.S.A.). All other truncated isoforms of Smad8 or Smad7 were generated by PCR using the following specific primers and the full-length Smad8 or Smad7 cDNA plasmids as template: N-terminal truncation of Smad8 (TE783 and TE784), C-terminal truncation of Smad8 (TE785 and TE786), Smad7 MH1 (TE1079 and TE1080), Smad7 MH2 (TE1083 and TE1084), N-terminal truncation of Smad7 (TE1079 and TE1111) and the C-terminal truncation of Smad7 (TE1112 and TE1084). Primer sequences are: TE783, 5'-TACGGATCCATGAATAATGAACCACTA; TE784, 5'-TACTCGAGTTACGATACGGAAGAGAT; TE785, 5'-TAAGGATCCATGCACGCCAGCACTCCC; TE786, 5'-TAACTCGAGTTATCCCCATCCTTTAAC; TE1079, 5'-GGGAATTCATGTTCAGGACCAAAAC; TE1080, 5'-TCCTCGAGTCACAGTCTGCTGAG; TE1083, 5'-CAGAATTCAATGGTGGCGTATTGG; TE1084, 5'-TGCTCGAGTCACCGGTTATTAAATA; TE1111, 5'-TACTCGAGTCAGTGGGACGGG; and TE1112, 5'-ATGAAATTCAGAAATCTCCCCACCT.

Each Smad-encoding cDNA was subcloned in-frame with the FLAG epitope of pcDNA3-FLAG. This vector was constructed by ligating oligomers encoding the FLAG epitope into the BamHI- and EcoRI-digested pcDNA3 (Invitrogen). Both full-length cTid1 and Δ JcTid1 were subcloned in-frame with the Myc tag of pCS2MT vector (generated by Dr D. Turner, University of Michigan, Ann Arbor, MI, U.S.A., and Professor R. Rupp, University of Muenchen, Germany). The control bait used in the two-hybrid screen encoded an N-terminal activation domain of cGATA1. The control plasmids for co-immunoprecipitation and GST (glutathione S-transferase)-binding experiments encoded the FLAG-tagged cytoplasmic vesicle-tethering protein N-terminal p115 (provided by Dr D. Shields, Albert Einstein College of Medicine), or FLAG-tagged mGATA4. The oligomer sequences used to generate pcDNA3-FLAG are: TE758, 5'-GATCCACCATGGACTACAAGGACGACGATGACAAG; and TE759, 5'-AATTCTTGTCATCGTCGTCCTTGTTAGTCCATGGTG.

Cell culture, transfection and immunoprecipitation

QT6 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 8% (v/v) fetal calf serum (Gibco) and 2% (v/v) chicken serum (Gibco). In a standard transfection, 3×10^5 cells were plated the previous evening into each 35 mm $\times$ 100 mm well of six-well tissue culture plates. Cells were transfected with a total of 5 μ g of FLAG-tagged expression plasmid and Myc-tagged cTID or Myc-tagged Δ JcTID using LipofectamineTM (Invitrogen) according to the manufacturer's instructions. Cells were harvested in 500 μ l of lysis buffer [20 mM Tris, pH 8.0, 1% NP40 (Nonidet P40), 50 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM β -glycerophosphate, 1 mM

Na₃VO₄ and protease inhibitors]. After pelleting, each supernatant was subjected to immunoprecipitation using 5 μ g of either the M2 anti-FLAG antibody (Sigma) or Chrompure mouse IgG (Jackson ImmunoResearch Laboratories) as an isotype-matched control. The reaction mixtures were incubated for 1–2 h at 4°C before addition of 30 μ l of a 50% slurry of Protein G (Amersham Biosciences), followed by a second incubation of 1–2 h at 4°C. After pelleting, precipitates were washed twice in PBS containing 0.5 M NaCl, once in PBS containing 1% NP40 and finally twice in PBS. Pellets were then resuspended in 15 μ l of SDS sample buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2%, w/v, SDS, 0.1%, w/v, Bromophenol Blue and 10%, v/v, glycerol). Samples were electrophoresed through an SDS/12% polyacrylamide gel, transferred on to a PVDF membrane (Bio-Rad) and probed with an anti-Myc, horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology). ECL[®] (enhanced chemiluminescence; Amersham Biosciences) was performed according to the manufacturer's instructions.

To generate a GST-Tid1 fusion protein, the 1.3 kb SalI–NotI cDNA fragment isolated in the two-hybrid screen was subcloned into the SalI–NotI sites of the polylinker in pGEX-4T3 (Pharmacia), which fused 730 bp of C-terminal cTid1 coding sequences in proper reading frame with the GST protein, placed under the regulation of the pTac promoter. This construct (or the vector alone as a control for generating GST protein) was transformed into BL21 *E. coli* cells (Gibco BRL). To generate recombinant proteins, an overnight culture was diluted to 1:100 in a prewarmed 2 $\times$ YTA medium (16%, w/v, bacto-tryptone, 10%, w/v, bacto-yeast extract and 5%, w/v, NaCl), cultured at 37°C with shaking to *D*₆₀₀ (attenuance) of 1.0, induced by addition of isopropyl β -D-thiogalactoside to 0.5 mM, and cultured for an additional 2 h at 30°C with shaking. Bacterial cells were pelleted, resuspended in PBS with lysozyme added at 1 mg/ml, followed by sonication (lysis was monitored by microscopy). Lysates were centrifuged at 12000 *g* for 10 min at 4°C and at this point the GST-Tid protein is found in the insoluble pellet. To resolubilize the protein, the pellet was resuspended in PBS containing 1% Tween 20 and mixed at room temperature (22°C) for 30 min. Soluble protein was recovered by adding to the supernate 100 ml of a 50% washed slurry of glutathione-conjugated Sepharose 4B beads followed by incubation overnight. In some cases, the buffer included addition of 1 mM ZnCl₂. The beads were washed extensively with PBS, followed by addition of rabbit reticulocyte translation lysates (Promega) that had been charged with addition of purified RNA encoding *Xenopus* Smad7 (generated as described below). Beads were washed several times and finally samples were resuspended in 2 $\times$ SDS sample buffer, boiled and proteins electrophoresed through an SDS/12% polyacrylamide gel, which was then fixed and processed for fluorography. Control incubations included equal or excess amounts of GST proteins (in place of GST-Tid1), or substituted radiolabelled p115 for Smad7.

Xenopus embryos and microinjection

Freshly laid *Xenopus* eggs were obtained by gonadotropin induction, fertilized *in vitro* using macerated testes and dejellied in 2% cysteine (pH 7.9). Embryos were staged by the method of Nieuwkoop and Faber [27]. The mRNA used for injection was synthesized using the mMessage mMachine kit (Ambion). Linearized plasmid DNA was treated with 0.5% SDS and 0.6 mg/ml proteinase K (Roche) and, after phenol extraction and ethanol precipitation, the DNA was used as a template to generate capped message. Fertilized eggs were injected with 4.6 nl of mRNA solution into the two presumptive ventro-posterior cells at the

Figure 1 Isolation of a cTid1 cDNA using a Smad8 bait in the yeast two-hybrid assay

A nearly full-length Smad8 cDNA was fused to sequence encoding the GAL4 DNA-binding domain (DBD, amino acids 1–147). The bait clone encoded all but the N-terminal 27 and C-terminal 28 amino acids of xSmad8. The target clone isolated (clone 4) is a Smad8-binding protein that starts at amino acid 190 fused in-frame with the GAL4 activation domain (AD) and encodes the remaining Tid1 sequence including a translational termination codon (*).

four-cell stage. Embryos were cultured in $0.1 \times$ MBS [8.8 mM NaCl, 0.1 mM KCl, 0.04 mM CaCl_2 , 0.03 mM $\text{Ca}(\text{NO}_3)_2$, 0.08 mM MgSO_4 , 0.24 mM NaHCO_3 and 1 mM Hepes, pH 7.4] until stage 31–32 and scored for dorsalization or partial secondary axes. The reporter assays used the full-length Gata2 promoter directing expression of the firefly luciferase reporter gene as described in [28]. Each injection was into the two presumptive ventro-posterior cells at the four-cell stage and included 25 pg of luciferase reporter plasmid and, depending on the sample, 10 pg of Smad7 mRNA and 0, 25, 50 or 100 pg of Tid1 mRNA. RNA encoding lacZ was included as needed to ensure that the total amount of RNA injected was the same for every sample. Each injection also included 0.1 pg of RNA encoding *Renilla* luciferase, as an internal control. Following injection, embryos were cultured in $0.1 \times$ MBS, harvested at stage 10–11, lysates were then prepared using the dual luciferase assay kit (Promega) and analysed for firefly luciferase activity, normalized for *Renilla* luciferase activity. Data was compiled from two independent injection experiments, each of which analysed three to six independent batches of five combined embryo lysates per sample. Activities were then compared relative to that derived from the promoter alone.

RESULTS

cTid1 protein is identified in a two-hybrid screen as a Smad8-binding protein

Smad8 is most closely related to Smad1 and Smad5. However, the activity of Smad8 is clearly distinct. For example, we showed that forced expression of Smad8 does not ventralize embryos equivalent to Smad1 and depletion of maternal Smad8 causes widespread apoptosis of embryonic cells at the mid-gastrula transition, which is not compensated by Smad1 or Smad5 [23]. Compared with Smad1/Smad5, only a few cellular proteins have been shown to interact with Smad8 [5]. Therefore we undertook a two-hybrid screen to search for Smad8-interacting factors. For this purpose, a nearly full-length xSmad8 cDNA (encoding amino acids 28–438) was fused in-frame with sequences encoding the GAL4 DNA-binding domain, to generate a GAL4-Smad8 bait (Figure 1). To generate potential target proteins, a cDNA library was generated from RNA isolated from day 13 chick red blood cells and fused to sequences encoding the GAL4 transactivation domain. The target library was generated from these cells because they provide a convenient source of RNA from transcriptionally active primary embryonic erythroid cells, and embryonic erythroid cells were the original source from which we cloned xSmad8. Our intended goal was to provide a bias for Smad8-interacting proteins that might function in erythroid cells.

The host yeast strain PJ69-4A was used for the screen, because it contains three different reporter genes, in order to reduce the number of false positives [29]. A yeast strain was generated expressing the Smad8 bait (with leucine selection) and was subsequently transformed with the target library (using tryptophan selection). Yeast colonies were replica-plated to select for expression of adenosine, since the yeast cells contain a UAS (upstream activating sequence)-dependent *ADE2* gene in addition to a UAS-*lacZ* marker. Of approx. 12 initial colonies, one clone (clone 4) was pursued further after meeting the following requirements: strong growth when the target plasmid was isolated and retransformed alone with the bait, no growth when co-transformed only with the GAL4 bait vector and no growth in the presence of an alternative bait encoding a GAL4–GATA1 fusion protein. In addition, the yeast cells co-transformed with the bait and clone 4 showed strong expression of β -galactosidase (results not shown), which was not seen using either the bait or clone 4 alone. The clone 4 cDNA was sequenced and the insert showed strongest homology to hTid1, the human homologue of the *Drosophila* *l(2)tid* gene encoding Tid56.

cTid1 cDNA encodes a protein with strong homology to the short mammalian Tid1 isoform

Clone 4 appeared to encode a partial cDNA, and in particular lacked the predicted sequence of the DnaJ domain, which is the only known functional domain of Tid1 proteins. To obtain additional sequence, we used 5'-RACE with primers that hybridized to internal clone 4 sequences. The cDNA fragments obtained were entirely consistent with Tid1 sequence and included the identical clone 4 sequence intervening the primer and 5'-end of the original cDNA. New primers were generated consistent with the 5'- and 3'-ends of the putative cTid1 coding sequence and were used to amplify by RT-PCR full-length clones from chick mRNA. The sequence of these clones confirmed the contiguous sequence of clone 4 and the 5'-RACE products. The putative cTid1 sequence encodes a protein most similar to the short isoform of hTid1. The sequence is shown in Figure 2, aligned with those for human long and short isoforms and *Drosophila* dTid56.

The putative chicken protein is overall 75% identical with hTid1S. The characteristic DnaJ domain is well conserved near the N-terminal end of the protein. C-terminal to the DnaJ domain, there is also a well-conserved glycine/phenylalanine-rich 'hinge' region, followed by four CXXCXGXG zinc-finger-like repeats (with a single exception of a Gly to Ala alteration in the final residue of the third repeat in the chicken protein). The C-terminal region is also well conserved, including the KRSTGN-stop sequence that is fully consistent with the splice choice encoding a short isoform. The chicken genome presumably encodes a corresponding long isoform, but it would not have been identified from our RT-PCR assay and there is currently no evidence for it in the EST (expressed sequence tag) database. Interestingly, a significant difference in the putative chicken protein is that it lacks the LRP-GV motif, located just N-terminal to the DnaJ domain in the mammalian proteins, proposed to function as a cleavage sequence used to target the mitochondrial matrix.

The cTid1 gene is expressed widely and transcripts are enriched in the developing blood islands

The mammalian Tid proteins have been studied mostly in cell culture systems, and the embryonic expression patterns are not described. To investigate the expression pattern of cTid1, the cDNA clone was used in Northern blotting and *in situ* hybridization experiments. First, RNA was purified from a variety of tissues isolated from day 13 chick embryos. When probed by Northern

Figure 2 Sequence of cTid1 aligned with the mammalian and *Drosophila* homologues

Both the human short (1S) and long (1L) isoforms are shown. The characteristic features highlighted by grey shading include (from the N-terminus to the C-terminus) the putative mitochondrial membrane processing site, the DnaJ domain, the cysteine-rich repeats and the C-terminal sequences that are fully conserved with the human short isoform.

blotting, a predominant transcript of approx. 2.4 kb was detected in all samples, but with highest relative levels found in liver, heart, brain and blood (Figure 3). The same pattern was detected using probes derived from either the 5'- or 3'-end of the cDNA (results not shown). Whole mount *in situ* hybridization experiments were performed to determine if cTid1 is expressed with a specific spatial pattern at early embryonic stages. At the primitive streak (presomitic) stage, we failed to detect any specific transcript patterns (results not shown). However, by around stage 10 (10 somites, ~36 h), the cTid1 probe showed widespread expression and some specificities in the pattern (Figure 4). For example, the gene is expressed at relatively high levels in the developing blood islands, consistent with our initial identification of the cDNA from embryonic erythroid cell samples. Transcripts are also readily detected in the head-fold region, neural tube, heart tube and somites. Therefore cTid1 is expressed widely but there are both developmental and tissue-specific differences in the transcript levels.

Smad8 binds to cTid1 in avian cells and this does not require the DnaJ domain of cTid1

In order to confirm the interaction of Smad8 with Tid1 detected by the yeast two-hybrid assay, we tested whether the two proteins can

Figure 3 cTid1 mRNA is expressed widely in day 13 chick embryo tissues

Shown is the result of a Northern blotting experiment. The arrow indicates the position of the predominant 2.4 kb cTid1 transcript present in RNA isolated from the indicated tissues. The same blot was reprobed for cEF1β mRNA as a control for RNA loading and integrity.

Figure 4 cTid1 mRNA is expressed in numerous embryonic tissues including the blood islands

Upper panel: a representative embryo at approx. stage 10 following *in situ* hybridization with an antisense cTid1 RNA probe. Tissues that are enriched for transcript include the head-fold (hf), neural tube (nt), heart (ht), somites (so) and blood islands (bi). Lower panel: a representative control embryo that was processed similarly but hybridized with a sense-strand probe, and which lacks specific signal.

be co-immunoprecipitated from cell lysates. Currently available antibodies were tested but were inefficient or unable to precipitate either cTid1 or Smad8 alone. Therefore we generated epitope-tagged versions of each and expressed these in the avian QT6 fibroblast cell line. Vectors expressing FLAG-tagged Smad8 and Myc-tagged cTid1 were co-transfected into the cells and lysates were prepared after 48 h. Anti-FLAG antibodies were used to precipitate the tagged Smad8 protein and subsequent Western blotting with an anti-Myc antibody shows that cTid1 is co-immunoprecipitated (Figure 5A). The interaction is specific for

Figure 5 cTid1 protein co-immunoprecipitates with Smad8 and does not require the DnaJ domain for specific binding

(A) Cells were co-transfected with vectors expressing full-length Myc-tagged cTid1 and either full-length xSmad8 (S8), or as controls: mGATA4 (G4), p115 or vector alone (Vc). The lysates were immunoprecipitated (IP) with anti-FLAG antibodies (F) or control isotype-matched IgG antibodies (C), and the pellets analysed by Western blotting (WB) with an anti-Myc antibody (upper panel) to determine if the cTid1 was co-immunoprecipitated, or with an anti-FLAG antibody (lower panel) to confirm the expression of the co-expressed protein. The cTid1 protein does immunoprecipitate with Smad8 and this was specific for the anti-FLAG antibody. Using the full-length cTid1 (but not the isoform with the DnaJ domain deleted, see below), weak interaction with supposed control proteins was sometimes detected, as shown here for GATA4. (B) Comparison of the immunoprecipitation reaction using full-length cTid1 or the protein lacking the DnaJ domain. Reactions were as described in (A), and the isoform of cTid1 (full-length or lacking the DnaJ domain, ΔJ), is indicated above the upper panel, as is the co-expressed FLAG-tagged protein: full-length Smad8 (S8), N-terminal deleted Smad8 (ΔNS8) or C-terminal deleted Smad8 (ΔCS8). The Smad8 isoforms are co-precipitated efficiently and with specificity with the ΔJ cTid1 isoform.

Smad8, since transfection of cTid1 alone, control FLAG-tagged proteins, or the use of isotype-matched control antibody fails to precipitate cTid1 as determined by Western blotting (Figure 5A).

Based on analogous isoforms of m (mouse) Smad2 [30] and mSmad8 [31,32], we generated putative dominant negative and constitutively active isoforms of Smad8. The ΔCSmad8 isoform lacks 44 C-terminal amino acids of the MH2 domain and is predicated to function as a dominant-negative protein, while the ΔNSmad8 isoform lacks the initial 165 amino acids of the MH1 domain, and is predicted to function in a constitutively active manner. These mutant isoforms are fully and equally capable of interacting with cTid1 when co-expressed in QT6 cells, as shown by co-immunoprecipitation experiments (Figure 5B). We considered the possibility that the interaction is somehow mediated indirectly through chaperone activity of Tid1. This seems unlikely, since the original isoform of Tid1 isolated in the two-hybrid assay (clone 4) lacked the DnaJ domain. However, we tested directly the ability of FLAG-Smad8 isoforms to co-precipitate Myc-ΔJcTid1 in lysates derived from co-transfection experiments. Indeed, co-expression of full-length (results not shown) or mutant isoforms of Smad8 (Figure 5B) are able to mediate efficient co-precipitation of Myc-ΔJcTid1, as determined by subsequent Western blotting with anti-Myc antibodies. Therefore Tid1 does not require the DnaJ domain for interaction with Smad8 and the removal of the DnaJ domain does not compromise the

specificity. In fact, specificity may be increased by deletion of the DnaJ domain. For example, weak binding to supposed 'control' proteins, which is sometimes seen using the full-length Tid1, is reduced or eliminated when the Tid1 isoform lacking the DnaJ domain is used (Figure 5 and results not shown). In contrast, Smad8 binds equally well to Tid1 containing or lacking the DnaJ domain.

cTid1 protein binds with highest affinity to Smad7, through interaction with the MH2 domain

Because Smad family proteins are structurally related, we tested whether cTid1 binds uniquely to Smad8. To avoid the potential for the DnaJ domain to complicate issues of specificity, we used the Myc-tagged isoform that lacks the DnaJ domain (Myc-ΔJcTid1), and which encodes the sequence sufficient for strong interaction with Smad8. QT6 cells were co-transfected with an expression vector for Myc-ΔJcTid1 and vectors expressing FLAG-tagged versions of Smad1, Smad2, Smad4, Smad6, Smad7, or (as a positive control for comparison) Smad8. Cell lysates were prepared and after precipitation with anti-FLAG antibodies, the samples were probed by Western blotting with anti-Myc antibodies. As shown from a representative experiment in Figure 6(A), each of the Smad proteins is capable of some level of interaction with Tid1, but the efficiency of co-immunoprecipitation varies considerably. Compared with Smad8, Smad1 (in particular) and Smad2 bind weakly, although consistently above the background precipitation in the absence of cTid1 or using an isotype-matched control antibody. The interaction of cTid1 with Smad4 is stronger, while the interaction with Smad6 is still stronger and approximately equivalent to that found with Smad8. The most efficient interaction is found consistently with Smad7, which is the inhibitory Smad that can function to block signalling by multiple TGF-β signalling pathways.

Given that all of the tested Smad proteins can interact at some level with cTid1, it seemed likely that the interaction is mediated by a common structural domain. As shown above, deletion of the 44 C-terminal sequences of the Smad8 MH2 domain, including the serine residues phosphorylated by activated receptor complexes, does not alter the ability of Smad8 to bind to cTid1 (Figure 5B). To map the interacting sequence directly, we used the high-affinity binding protein Smad7 to generate specific isoforms encoding either the MH1 domain, the linker region or the MH2 domain. Expression of the linker region alone (results not shown) or isoforms lacking the MH2 domain fail to consistently co-immunoprecipitate cTid1 (Figure 6B). In contrast, co-immunoprecipitation experiments demonstrated that the presence of the MH2 domain is sufficient to mediate interaction with Smad7 (Figure 6B).

To test if Tid1 is able to interact with Smad7 directly, we expressed and purified from *E. coli* a GST-Tid1 fusion protein, containing GST fused to the Tid1 sequences isolated in the yeast two-hybrid screen (clone 4). The fusion protein expressed in *E. coli* was insoluble, but could be resolubilized in the presence of dilute detergent. For use as a control, the GST protein was similarly purified. RNA encoding Smad7 was generated and purified *in vitro* using a construct with the cDNA placed under the control of a bacteriophage polymerase. Smad7 protein was then expressed and labelled with ³⁵S-methionine by *in vitro* translation using a rabbit reticulocyte lysate. The relative ability of Smad7 to interact with GST or GST-Tid1 was compared by mixing the Smad7-containing lysates with purified GST or GST-Tid1 fusion proteins and isolating complexes using glutathione-conjugated Sepharose beads. The proteins that bound to beads (directly or indirectly) were analysed by SDS/PAGE, followed by autoradiography. As shown in Figure 6(C), a significant amount of Smad7 co-purifies

Figure 6 The cTid1 protein interacts with multiple Smad proteins and, with relatively high affinity to Smad7, via the MH2 domain

(A) Immunoprecipitation reactions were processed as for Figure 5 using the co-expressed FLAG-tagged proteins: Smad1 (S1), Smad2 (S2), Smad4 (S4), Smad6 (S6), Smad7 (S7), Smad8 (S8), p115 or vector alone (Vc). The relative levels of co-precipitated cTid1 were consistent among independent experiments, for example very weak with Smad1, and relatively very strong with Smad7. (B) Immunoprecipitation experiments were performed as described in (A), using the co-expressed Smad7 isoforms indicated above the panel. The MH2 domain of Smad7 is sufficient to co-immunoprecipitate cTid1. (C) *In vitro* transcribed Smad7 binds to a purified bacterially expressed Tid isoform. An equal amount of radiolabelled Smad7 protein was incubated with purified GST (lane 1), GST–Tid1 (lane 2) or GST–Tid1 that had been incubated during renaturation in a buffer supplemented with 1 mM Zn²⁺. Sepharose beads were added to pellet the GST proteins and the resulting sample was resuspended in SDS buffer and analysed by gel electrophoresis, followed by autoradiography of the dried gel. The migration of Smad7, at the predicted molecular mass of 44 kDa is indicated by the arrow, relative to the position of molecular mass standards of 38 and 49 kDa. As a control, *in vitro* translated p115 failed to show any interaction with either GST or GST–Tid1 under any conditions (results not shown).

from an incubation including GST–Tid1 (lane 2), compared with GST alone (lane 1). However, when the denatured GST–Tid1 fusion protein was renatured in the presence of added zinc, the interaction is further significantly enhanced. As a control, *in vitro* translated p115 failed to show any interaction with either GST or GST–Tid1 (results not shown). In summary, the two-hybrid, immunoprecipitation, and purified recombinant protein binding experiments all support the finding that Smad proteins interact

Figure 7 Co-expression of cTid1 modulates the dorsalizing activity of Smad7 in developing embryos

(A–C) Shown are representative stage 31–32 embryos that had been injected at the four cell stage into the two presumptive ventro-posterior blastomeres with RNA encoding Smad7 (20 pg) and 100 pg of RNA encoding EF1α as a control (A) or cTid1 (B). A third set of embryos was injected with 120 pg of EF1α alone (C). Arrows indicate representative protuberances that define a secondary axis induced by expressing Smad7 in the ventral blastomeres. The co-expression of cTid1 largely restored normal development to Smad7 expressing embryos. The full data set from four independent experiments is shown in Table 1. (D) Likewise, cTid1 is able to relieve the suppression by Smad7 of a BMP responsive promoter. Embryos were injected as described above, but with 25 pg of a luciferase reporter gene under the control of the Gata2 promoter. The promoter is approx. 2-fold more active when injected into blastomeres that contribute to the ventral side of the embryo compared with injection on the dorsal side (see [28]). These samples were normalized to represent a value of 1 (sample 1). This activity is inhibited by co-injection of 10 pg of Smad7 mRNA (lane 2), but this inhibition is suppressed by co-injection of increasing amounts of cTid1 mRNA (25, 50 or 100 pg; lanes 3–5 respectively). The suppression by Smad7 is statistically significant, comparing the data for sample 2 with sample 1 (the asterisk over the bar for sample 2 represents $P < 0.002$), and also comparing the data for sample 4 or 5 with sample 2 (the double asterisks over the bars for sample 4 or 5 represent $P < 0.003$ and $P < 0.002$ respectively). Bars represent the mean luciferase activities (normalized to control *Renilla* luciferase activity) from nine independent batches of injected embryos, derived from two independent injection experiments, and error bars are the S.E.M. based on a two-tailed Student's *t* test.

with Tid1. The interaction occurs not through the Tid1 DnaJ domain (which is lacking in the GST–Tid1 fusion protein), but rather with the cysteine-rich region (which would be expected to require zinc for full activity).

Tid1 can regulate the activity of Smad7 in developing embryos

We next tested if the interaction between Smad proteins and Tid1 can have functional consequences *in vivo*. *Xenopus* embryos provide an excellent developmental system to test the function of proteins by expression from injected mRNA. Overexpression of Smad8 does not generate an overt phenotype [23]. Likewise, embryos injected with mRNA encoding cTid1 appear normal (results not shown). However, embryos injected with Smad7 mRNA show a very pronounced phenotype, because this protein strongly inhibits Smad-dependent signalling [33,34]. Injection of 20 pg of Smad7 mRNA into the two ventro-posterior blastomeres of a four-cell embryo results in a high percentage of embryos that are dorsalized or generate a secondary dorsal axis, as shown in Figure 7(A). Co-injection of 100 pg of mRNA encoding cTid1 results in a significant rescue of this Smad7-induced phenotype

Table 1 *Xenopus* embryos were injected at the four-cell stage into the two presumptive ventral blastomeres with mRNA encoding xSmad7 (20 pg) and mRNA encoding either xEF1 α as a negative control (100 pg) or cTid1 (100 pg)

Embryos were allowed to develop until stage 31–32 at which time they were scored as either appearing normal or dorsalized. Dorsalization was characterized either as the presence of an additional partial axis, or the lack of normal ventro-posterior structures, as described previously for Smad7 injections [33]. The number of embryos injected for each experiment is indicated (*n*). The remaining embryos of a batch (to total 100%) either died during the culture or were otherwise morphologically abnormal but could not be clearly scored as dorsalized. On averaging over the four experiments, the number of dorsalized embryos was reduced from 65 to 8% by the co-expression of cTid1.

Experiment ... Smad7+ (<i>n</i>) ...	Morphology (%)							
	1 EF1 α (34)	cTid1 (38)	2 EF1 α (38)	cTid1 (36)	3 EF1 α (29)	cTid1 (25)	4 EF1 α (24)	cTid1 (21)
Normal	3	58	26	78	14	64	37	86
Dorsalized	85	3	58	0	55	20	63	10

(Figure 7B), in contrast with the lack of any rescue when Smad7 is injected with the control mRNA encoding EF1 α (Figure 7A). The data from multiple injection experiments are summarized in Table 1. Over the course of four independent experiments, 65% of the embryos injected with only Smad7 RNA showed an obvious dorsalization or partial secondary axis. For the embryos co-injected with cTid1 mRNA, these phenotypes were observed on an average in only 8% of the embryos. In other experiments, we could show a dose–response effect, in that increasing levels of Tid generated an increasingly higher percentage of normal embryos (results not shown). These results demonstrate that the Smad-binding protein Tid1 can antagonize the Smad-inhibitory action of Smad7.

We further analysed if the ability of Tid1 to rescue the Smad7-dependent phenotype was in fact due to modulation of Smad7 activity, rather than an indirect effect unrelated to Smad7 activity. For this purpose we tested if Tid1 can alter Smad7-dependent regulation of a BMP-responsive promoter. We and others have shown that the Gata2 gene is activated by BMP signalling, and recently we mapped the responsive sequences to a specific set of elements of the Gata2 promoter region [28]. This promoter is approx. 2-fold more active when injected into the two ventro-posterior blastomeres at the four-cell stage, compared with when injected into the two dorso-anterior blastomeres, consistent with the gradient of BMP signalling across this presumptive axis. We confirmed that the Gata2 promoter activity on the ventral side of the embryo is inhibited by co-expression of Smad7, which as expected reduces the activity of the promoter 2-fold (Figure 7D, lane 2, $P < 0.002$). Co-expression of Tid1 relieves this suppression (Figure 7D, lanes 3–5, $P < 0.002$). Therefore Tid1 can modulate the transcriptional activity of Smad7 on a bonafide BMP-responsive promoter and this may explain its ability to rescue the Smad7-dependent embryonic phenotype. We note however, that not all Smad-dependent genes may be modulated by Tid1. For example, we found that an artificial promoter that uses sequences derived from the BMP-responsive Vent2 promoter was suppressed by Smad7 but this suppression was not significantly relieved by co-expression of Tid1 (results not shown). Therefore the ability of Tid1 to modulate Smad7 activity may depend on promoter context or the nature of the Smad-containing complexes.

DISCUSSION

We report the isolation of the chicken homologue of Tid1 encoding a protein implicated in the control of cell proliferation and apoptosis in several cellular contexts. This is the first description of a non-mammalian vertebrate Tid1 gene. We find that the

sequence is well conserved in vertebrates, including the DnaJ domain that mediates binding with Hsp70 (heat-shock protein 70) chaperone proteins and regulates their interaction with specific substrate proteins. Although this is the only region of Tid1 previously ascribed a function, the rest of the protein is equally well conserved among the vertebrate genes. The least conserved region is the N-terminal sequence, including the putative consensus site in the mammalian proteins for mitochondrial processing. The lack of strong sequence conservation at the N-terminus is consistent with this being a cleavable signal sequence. Indeed, the sequence is strongly predicted to form a positively charged amphiphilic α -helix, and there is a sequence present at the same location of the putative chicken protein [RVPA (Arg-Val-Pro-Ala)] consistent with a proposed cleavage consensus site RXY↓S/A [35].

However, it is not known if mitochondrial processing is relevant to all Tid1 functions. An early report on the human Tid1 protein showed that it is localized to the mitochondrial matrix [15], but subsequent studies revealed that Tid1 is also found in the cytoplasm and the nucleus [16], so that it has the potential to interact with signalling pathways in various cellular compartments. Here we show that Tid1 has the potential to interact with components of TGF- β signalling. Smad signalling represents the third major pathway Tid1 is capable of regulating, following the previous demonstrations that Tid1 can modulate Ras- [16] and interferon-dependent [17] signalling, in addition to apoptotic pathways. Therefore Tid1 is a multifunctional protein that could potentially act in several cellular compartments and affect multiple pathways. Smad7 is also known to act either at the cell membrane or the nucleus [34] and so could interact with Tid1 in several contexts.

The identification of Smad proteins as potential binding partners for Tid1 is intriguing, since it provides a mechanism to bridge two independent sets of putative tumour suppressor proteins. We showed previously that Smad8 regulates early embryonic cell survival by inhibiting a caspase 3-mediated cell death programme [23]. Tid1 is also proposed to resist activation-induced cell death in TH2 cells also at the level of caspase 3 [18]. Using Tid1 as a bait in a two-hybrid screen, a ring finger protein homologous to IAP (inhibitor of apoptosis protein) was identified [36], although direct interaction could not be verified. Given our data, one possibility is that Tid1 interacts with Smad8 or other Smads to modulate their ability to respond to apoptotic stimuli, or that Smad proteins determine the ability of Tid1 to mediate apoptotic pathways. Several recent reports provide strong support for Tid1 as a regulator of oncogenic and/or proliferative pathways, as predicted by the original characterization of the *Drosophila* mutant [20,37,38]. For example, Tid1 can suppress ErbB2-dependent tumour progression in mammary carcinoma cells [37].

Expression of Tid1 can also suppress spontaneous immortalization of rat embryo fibroblasts, acting as a repressor of NF- κ B (nuclear factor κ B) signalling [38], capable of acting by direct interaction with and suppression of cytoplasmic IKK (inhibitor of κ B kinase) activity [20]. These tumour suppressor functions all appear to be dependent on the DnaJ domain, whereas the interaction we describe with Smad proteins is independent of this domain. Therefore Smad proteins, which are known tumour suppressors, could co-operate with independent pathways that function through DnaJ.

In summary, we have shown that the chick homologue of mammalian Tid1 is highly conserved, widely expressed during embryogenesis with some preferential accumulation and with the capability to interact and modulate Smad signalling. There are several open questions to be addressed based on these initial findings. First, it will be important to determine the normal cellular contexts in which Smad–Tid interactions occur during development and in specific cell types. Secondly, it will be interesting to test if interactions are enhanced or inhibited under conditions of stimulated apoptosis. This is complicated by the fact that neither Tid1 nor Smad proteins are strictly localized to a defined compartment, and both Tid1 and Smad proteins have multiple identified partners. Thirdly, it will be interesting to know whether TGF- β signalling influences the interaction of Smad proteins with Tid1. The high affinity to Smad7 is interesting, since this protein has broad regulatory potential for modulating Smad-dependent signalling downstream of multiple TGF- β -like ligands, and Smad7 expression can induce, mediate or sensitize cells to apoptosis in a number of cellular contexts [39–43]. Interaction of Tid1 with Smad7 could also indirectly modulate the function of Smad2 or Smad4, proteins that are both demonstrated tumour suppressors. This type of mechanism was demonstrated *in vivo* with our *Xenopus* model, whereby Tid1 blocks the dorsalizing activity of Smad7 in developing embryos. Smad7 presumably functions in this context to sequester or block activation of the BMP-dependent Smads (Smad1/Smad5/Smad8), which leads to a relative imbalance of dorsalizing Smad2/Smad3. It is equally plausible that Smad proteins could modulate other functions of Tid1, by regulating the accessibility of Tid1 to mitochondria, or interaction with its other defined signalling partners, such as GAP, JAK2 or oncoproteins. Defining the physiological significance of this interaction with respect to sensing apoptotic stimuli provides an important area for further investigation.

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REFERENCES

- Ten Dijke, P., Goumans, M. J., Itoh, F. and Itoh, S. (2002) Regulation of cell proliferation by Smad proteins. *J. Cell. Physiol.* **191**, 1–16
- Massague, J., Blain, S. W. and Lo, R. S. (2000) TGF- β signaling in growth control, cancer and heritable disorders. *Cell (Cambridge, Mass.)* **103**, 295–309
- Massague, J. (1998) TGF β signal transduction. *Annu. Rev. Biochem.* **67**, 753–791
- Massague, J. (2000) How cells read TGF- β signals. *Nat. Rev. Mol. Cell Biol.* **1**, 169–178
- Zwijsen, A., Verschuuren, K. and Huybrechts, D. (2003) New intracellular components of bone morphogenetic protein/Smad signaling cascades. *FEBS Lett.* **546**, 133–139
- Chen, Y. G., Hata, A., Lo, R. S., Wotton, D., Shi, Y., Pavletich, N. and Massague, J. (1998) Determinants of specificity in TGF- β signal transduction. *Genes Dev.* **12**, 2144–2152
- Rodriguez-Leon, J., Merino, R., Macias, D., Ganan, Y., Santesteban, E. and Hurler, J. M. (1999) Retinoic acid regulates programmed cell death through BMP signalling. *Nat. Cell Biol.* **1**, 125–126
- Shimasaki, S., Moore, R. K., Otsuka, F. and Erickson, G. F. (2004) The bone morphogenetic protein system in mammalian reproduction. *Endocr. Rev.* **25**, 72–101
- Baade Ro, T., Utne Holt, R., Brenne, A.-T., Hjorth-Hansen, H., Waage, A., Hjertner, O., Sundan, A. and Borset, M. (2004) Bone morphogenetic protein-5, -6, and -7 inhibit growth and induce apoptosis in human myeloma cells. *Oncogene* **23**, 3024–3032
- Hallahan, A. R., Pritchard, J. I., Chandraratna, R. A., Ellenbogen, R. G., Geyer, J. R., Overland, R. P., Strand, A. D., Tapscott, S. J. and Olson, J. M. (2003) BMP-2 mediates retinoid-induced apoptosis in medulloblastoma cells through a paracrine effect. *Nat. Med.* **9**, 1033–1038
- Hejlik, D. P., Kottickal, L. V., Liang, H., Fairman, J., Davis, T., Janneck, T., Sexton, D., Perry, III, W., Tavtigian, S. V., Teng, D. H. et al. (1997) Localization of SMAD5 and its evaluation as a candidate myeloid tumor suppressor. *Cancer Res.* **57**, 3779–3783
- Zavadil, J., Brezina, J., Svoboda, P., Zemanova, Z. and Michalova, K. (1997) Smad5, a tumor suppressor candidate at 5q31.1, is hemizygously lost and not mutated in the retained allele in human leukemia cell line HL60. *Leukemia* **11**, 1187–1192
- Schilling, B., De-Medina, T., Syken, J., Vidal, M. and Munger, K. (1998) A novel human DnaJ protein, hTid-1, a homolog of the Drosophila tumor suppressor protein Tid56, can interact with the human papillomavirus type 16 E7 oncoprotein. *Virology* **247**, 74–85
- Canamasas, I., Debes, A., Natali, P. G. and Kurzik-Dumke, U. (2003) Understanding human cancer using Drosophila: Tid47, a cytosolic product of the DnaJ-like tumor suppressor gene l2Tid, is a novel molecular partner of patched related to skin cancer. *J. Biol. Chem.* **278**, 30952–30960
- Syken, J., De-Medina, T. and Munger, K. (1999) TID1, a human homolog of the Drosophila tumor suppressor l(2)tid, encodes two mitochondrial modulators of apoptosis with opposing functions. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 8499–8504
- Trentin, G. A., Yin, X., Tahir, S., Lhotak, S., Farhang-Fallah, J., Li, Y. and Rozakis-Adcock, M. (2001) A mouse homologue of the Drosophila tumor suppressor l(2)tid gene defines a novel RasGAP binding protein. *J. Biol. Chem.* **276**, 13087–13095
- Sarkar, S., Pollack, B. P., Lin, K. T., Kotenko, S. V., Cook, J. R., Lewis, A. and Pestka, S. (2001) hTid-1, a human DnaJ protein, modulates the interferon signaling pathway. *J. Biol. Chem.* **276**, 49034–49042
- Syken, J., Macian, F., Agarwal, S., Rao, A. and Munger, K. (2003) TID1, a mammalian homologue of the drosophila tumor suppressor lethal(2) tumorous imaginal discs, regulates activation-induced cell death in Th2 cells. *Oncogene* **22**, 4636–4641
- Yin, X. and Rozakis-Adcock, M. (2001) Genomic organization and expression of the human tumorous imaginal disc (TID1) gene. *Gene* **278**, 201–210
- Cheng, H., Cenciarelli, C., Nelkin, G., Tsan, R., Fan, D., Cheng-Mayer, C. and Fidler, I. J. (2005) Molecular mechanism of hTid-1, the human homolog of Drosophila tumor suppressor l(2)Tid, in the regulation of NF- κ B activity and suppression of tumor growth. *Mol. Cell. Biol.* **25**, 44–59
- Edwards, K. M. and Munger, K. (2004) Depletion of physiological levels of the human TID1 protein renders cancer cell lines resistant to apoptosis mediated by multiple exogenous stimuli. *Oncogene* **23**, 8419–8431
- Lo, J. F., Hayashi, M., Woo-Kim, S., Tian, B., Huang, J. F., Fearn, C., Takayama, S., Zapata, J. M., Yang, Y. and Lee, J. D. (2004) Tid1, a cochaperone of the heat shock 70 protein and the mammalian counterpart of the Drosophila tumor suppressor l(2)tid, is critical for early embryonic development and cell survival. *Mol. Cell. Biol.* **24**, 2226–2236
- Miyayama, Y., Torregroza, I. and Evans, T. (2002) A maternal Smad protein regulates early embryonic apoptosis in *Xenopus laevis*. *Mol. Cell. Biol.* **22**, 1317–1328
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Plainview, NY
- Jiang, Y., Tarzami, S., Burch, J. B. E. and Evans, T. (1998) Common role for each of the cGATA-4/5/6 genes in the regulation of cardiac morphogenesis. *Dev. Genet.* **22**, 263–277
- Hamburger, V. and Hamilton, H. L. (1951) A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92
- Nieuwkoop, P. D. and Faber, J. (1967) *Normal Table of Xenopus laevis* (Daudin), North-Holland Publishing Co., Amsterdam
- Oren, T., Torregroza, I. and Evans, T. (2005) An Oct-1 binding site mediates activation of the gata2 promoter by BMP signaling. *Nucleic Acids Res.* **33**, 4357–4367
- James, P., Halladay, J. and Craig, E. A. (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144**, 1425–1436
- Hata, A., Lo, R. S., Wotton, D., Lagna, G. and Massague, J. (1997) Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. *Nature (London)* **388**, 82–87
- Nishita, M., Ueno, N. and Shibuya, H. (1999) Smad8B, a Smad8 splice variant lacking the SSXS site that inhibits Smad8-mediated signalling. *Genes Cells* **4**, 583–591
- Kawai, S., Faucheu, C., Gallea, S., Spinella-Jaegle, S., Atfi, A., Baron, R. and Roman, S. R. (2000) Mouse smad8 phosphorylation downstream of BMP receptors ALK-2, ALK-3, and ALK-6 induces its association with Smad4 and transcriptional activity. *Biochem. Biophys. Res. Commun.* **271**, 682–687

- 33 Nakao, A., Afrakhte, M., Moren, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N. E., Heldin, C. H. et al. (1997) Identification of Smad7, a TGF β -inducible antagonist of TGF- β signalling. *Nature (London)* **389**, 631–635
- 34 Christian, J. L. and Nakayama, T. (1999) Can't get no SMADisfaction: Smad proteins as positive and negative regulators of TGF- β family signals. *BioEssays* **21**, 382–390
- 35 Gavel, Y. and von Heijne, G. (1990) Cleavage-site motifs in mitochondrial targeting peptides. *Protein Eng.* **4**, 33–37
- 36 Sasaki, S., Nakamura, T., Arakawa, H., Mori, M., Watanabe, T., Nagawa, H. and Croce, C. M. (2002) Isolation and characterization of a novel gene, hRF1, preferentially expressed in esophageal cancer. *Oncogene* **21**, 5024–5030
- 37 Kim, S. W., Chao, T. H., Xiang, R., Lo, J. F., Campbell, M. J., Fearn, C. and Lee, J. D. (2004) Tid1, the human homologue of a Drosophila tumor suppressor, reduces the malignant activity of ErbB-2 in carcinoma cells. *Cancer Res.* **64**, 7732–7739
- 38 Tarunina, M., Alger, L., Chu, G., Munger, K., Gudkov, A. and Jat, P. S. (2004) Functional genetic screen for genes involved in senescence: role of Tid1, a homologue of the Drosophila tumor suppressor l(2)tid, in senescence and cell survival. *Mol. Cell. Biol.* **24**, 10792–10801
- 39 Mazars, A., Lallemand, F., Prunier, C., Marais, J., Ferrand, N., Pessah, M., Cherqui, G. and Atfi, A. (2001) Evidence for a role of the JNK cascade in Smad7-mediated apoptosis. *J. Biol. Chem.* **276**, 36797–36803
- 40 Yamamura, Y., Hua, X., Bergelson, S. and Lodish, H. F. (2000) Critical role of Smads and AP-1 complex in transforming growth factor- β -dependent apoptosis. *J. Biol. Chem.* **275**, 36295–36302
- 41 Edlund, S., Bu, S., Schuster, N., Aspenstrom, P., Heuchel, R., Heldin, N. E., ten Dijke, P., Heldin, C. H. and Landstrom, M. (2003) Transforming growth factor- β 1 (TGF- β)-induced apoptosis of prostate cancer cells involves Smad7-dependent activation of p38 by TGF- β -activated kinase 1 and mitogen-activated protein kinase kinase 3. *Mol. Biol. Cell* **14**, 529–544
- 42 Landstrom, M., Heldin, N. E., Bu, S., Hermansson, A., Itoh, S., ten Dijke, P. and Heldin, C. H. (2000) Smad7 mediates apoptosis induced by transforming growth factor β in prostatic carcinoma cells. *Curr. Biol.* **10**, 535–538
- 43 Lallemand, F., Mazars, A., Prunier, C., Bertrand, F., Kornprost, M., Gallea, S., Roman-Roman, S., Cherqui, G. and Atfi, A. (2001) Smad7 inhibits the survival nuclear factor kappaB and potentiates apoptosis in epithelial cells. *Oncogene* **20**, 879–884

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