Smad regulation in TGF-β signal transduction

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Summary

Smad proteins transduce signals from transforming growth factor-β (TGF-β) superfamily ligands that regulate cell proliferation, differentiation and death through activation of receptor serine/threonine kinases. Phosphorylation of receptor-activated Smads (R-Smads) leads to formation of complexes with the common mediator Smad (Co-Smad), which are imported to the nucleus. Nuclear Smad oligomers bind to DNA and associate with transcription factors to regulate expression of target genes. Alternatively, nuclear R-Smads associate with ubiquitin ligases and promote degradation of transcriptional repressors, thus facilitating target gene regulation by TGF-β. Smads themselves can also become ubiquitinated and are degraded by proteasomes. Finally, the inhibitory Smads (I-Smads) block phosphorylation of R-Smads by the receptors and promote ubiquitination and degradation of receptor complexes, thus inhibiting signalling.

Key words: Phosphorylation, Signal transduction, Smad, Transforming growth factor-β, Ubiquitination

Introduction

Members of the transforming growth factor-β (TGF-β) family control growth, differentiation and apoptosis of cells, and have important functions during embryonic development (Derynck et al., 2001; Massagué et al., 2000; Whitman, 1998). The human genome encodes 28 genes that encode members of this family (Venter et al., 2001), including TGF-β isoforms, activins and bone morphogenetic proteins (BMPs). These proteins signal by stimulating formation of specific heteromeric complexes of type I and type II serine/threonine kinase receptors. The type II receptors are encoded by five known mammalian genes, bind to ligands, and phosphorylate and activate the type I receptors, of which there are seven mammalian members (Fig. 1). The available data support the notion that the type I receptors are responsible for the specificity of downstream signalling. The ligands, receptors and their intracellular effectors, the Smads, are conserved in eukaryotes from *Caenorhabditis elegans* and *Drosophila* to mammals (Patterson and Padgett, 2000; Whitman, 1998).

Here, we review the mechanisms by which Smad signalling is regulated, that is, how Smad molecules are activated, translocated to the nucleus, interact with other nuclear partners and how they are degraded.

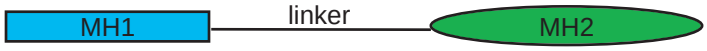
Smads: a conserved family of signal transducers

Smads, the only substrates for type I receptor kinases known to have a signalling function, were first identified as the products of the *Drosophila Mad* and *C. elegans Sma* genes, which lie downstream of the BMP-analogous ligand-receptor systems in these organisms (Patterson and Padgett, 2000; Whitman, 1998). The human genome encodes eight Smad family members (Mad-homologues (*MADH*)), and related proteins are known in the rat, mouse, *Xenopus*, zebrafish, the helminth *Schistosoma mansoni*, *Drosophila* and *C. elegans*. *MADH2*, *MADH4* and *MADH7* map to chromosome 18q21-22, a tumour suppressor locus; *MADH3* and *MADH6* map to chromosome 15q21-22, and *MADH5*, *MADH1* and *MADH8* to chromosomes 15q31, 4 and 13, respectively (Gene encyclopaedia, GeneCards). Smads are ubiquitously expressed throughout development and in all adult tissues (Flanders et al., 2001; Luukko et al., 2001), and many of them (Smad2, Smad4, Smad5, Smad6 and Smad8) are produced from alternatively spliced mRNAs (Gene encyclopaedia, GeneCards). Functionally, Smads fall into three subfamilies (Fig. 1, Fig. 2): receptor-activated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5, Smad8), which become phosphorylated by the type I receptors; common mediator Smads (Co-Smads: Smad4), which oligomerise with activated R-Smads; and inhibitory Smads (I-Smads: Smad6 and Smad7), which are induced by TGF-β family members. The latter exert a negative feedback effect by competing with R-Smads for receptor interaction and by marking the receptors for degradation.

Smads have two conserved domains, the N-terminal Mad homology 1 (MH1) and C-terminal Mad homology 2 (MH2) domains (Fig. 2). The MH1 domain is highly conserved among R-Smads and Co-Smads; however, the N-terminal parts of I-Smads have only weak sequence similarity to MH1 domains. Sequence and structural analyses indicate that the MH1 domain is homologous to the diverse His-Me (histidine-metal-ion) finger family of endonucleases, and it may have evolved from an ancient enzymatic domain that had lost its catalytic activity but retained its DNA-binding properties (Grishin, 2001). The MH1 domain regulates nuclear import and transcription by binding to DNA and interacting with nuclear proteins (Table 1).

The MH2 domain is highly conserved among all Smads. Its structure contains several α-helices and loops, which surround a β-sandwich (Shi, 2001), and it resembles the forkhead-associated (FHA) domain, a phosphopeptide-binding domain common in transcription and signalling factors (Li et al., 2000). The MH2 domain regulates Smad oligomerisation, recognition by type I receptors and interacts with cytoplasmic adaptors and several transcription factors (Table 1).

Table 1. Smad-interacting proteins



Function	Nuclear import cytoplasmic anchoring DNA-binding transcription	Ubiquitination	Oligomerisation cytoplasmic anchoring transcription
Regulatory phosphorylation	CamKII (–) (S2) PKC (–) (S2, S3)	CamKII (–) (S2) Erk (–) (S1-3)	Type I receptors (+) (S1-3, S5, S8)
Receptors			ALK1-7
Oligomerisation			R-Smads, Co-Smad
Cytoplasmic adaptors-effectors	Calmodulin (S1-4) Filamin (S1-6) Importin-β1 (S3)	Filamin (S1-6) ARIP (S3) β-catenin (S4) Microtubules (S2-4) TAK1 (S6)	Axin, Axil (S2, S3) Dab2 (S2, S3) SARA, Hrs/Hgs (S2, S3) STRAP (S2, S3, S6, S7)
Ubiquitination adaptors-substrates	HEF1 (N-ter) (S3)	Smurf1 (S1, S5, S7) Smurf2 (S2, S3, S7)	HEF1 (C-ter) (S3) SCF subunits (S3) APC subunits (S3)
Transcriptional co-activators	pX HBV (S4)	Swift (S1, S2)	MSG1 (S4) p300/CBP (S1-4) P/CAF (S1-4)
Transcriptional repressors	HDAC (?) (S3) Hoxc-8 (S1)	Hoxc-8 (S1) SNIP1 (S1, S2, S4)	SIP1 (S1-3, S5) Ski (S2-4) SnoN (S2-4) TGIF (S2) Tob (S1, S4, S5, S8)
Transcription factors	ATF2 (S3, 4) Jun, JunB, JunD (S3, S4) Lef1/Tcf (S2, S3) Sp1, Sp3 (S2-4) TFE3 (μE3) (S3, S4) VDR (S3) YY1 (S1, S3, S4)	Gli3 ΔC-ter (S1-4) HNF4 (S3) p52 (NFκB) (S3)	AR (S3) BF-1 (S1-4) E1A (S1-3) ERα (S2-4) Evi-1 (S3) FAST (FoXH1) (S2, S3) Fos (S3) GR (S3) Lef1/Tcf (S2, S3) Menin (S2, S3) Milk (S2) Mixer (S2) OAZ (S1, S4) Runx/CBFα/AML (S1-4)

A simplified diagram of the three Smad domains is followed by a table of the Smad post-translational modifications and protein-protein interactions known to occur in each domain. The symbols (+ and –) indicate regulatory phosphorylation of Smads that results in functional activation or inhibition, respectively. Entries in more than one domain indicate interactions with or modifications by the same factor at multiple domains. The specific Smad members that are known to exhibit the listed modifications or interactions are shown in parenthesis and are abbreviated as S1-S8 for Smad1 to Smad8, respectively. Proteins, for which the specific Smad domain that they interact with is not yet determined are listed in the centre in stippled boxes. A question mark (?) indicates that HDAC activity but not physical protein interaction has been found to associate with the MH1 domain of Smad3. The names of factors not discussed in the text are: TAK1 (TGF-β activated kinase 1), pX HBV (pX oncoprotein of hepatitis B virus), Swift (*Xenopus* BRCA1 C-terminal domain nuclear protein), MSG1 (melanocyte specific gene 1, transcriptional co-activator), Hoxc-8 (homeobox c-8 transcriptional repressor), SNIP1 (Smad nuclear interacting protein 1, Smad- and p300-associating transcriptional co-repressor), SIP1 (Smad interacting protein 1, zinc-finger/homeodomain repressor), Tob (transducer of ErbB-2, APRO/Btg family of anti-proliferative factors), ATF2 (activating transcription factor 2), Lef1/TCF (lymphoid enhancer-binding factor 1/T cell- specific transcription factor 1), Sp1, Sp3 (Specificity protein 1, zinc finger transcription factor), TFE3 (transcription factor recognising the immunoglobulin enhancer motif μE3), VDR (vitamin D receptor, nuclear hormone receptor), YY1 (yin yang 1, zinc finger transcription factor), AR (androgen receptor, nuclear hormone receptor), BF-1 (brain factor 1 oncoprotein), E1A (early region of adenovirus binding transcription factor 1A), ERα (estrogen receptor α), Evi-1 (Evi-1 oncoprotein), FAST (Forkhead activin signal transducer), GR (glucocorticoid receptor, nuclear hormone receptor), Menin (multiple endocrine neoplasia-type 1 tumour suppressor protein), Milk (Mix 1-related homeobox transcription factor), Mixer (homeobox transcription factor), OAZ (olfactory factor O/E-1-associated zinc finger protein), Runx (runt domain transcription factor), Gli3 ΔC-ter (glioblastoma Kruppel zinc finger transcription factor-3 with deletion of the C-terminal domain), HNF4 (hepatocyte nuclear factor 4, nuclear hormone receptor), NFκB (B cell-specific nuclear factor binding to the intronic κ light chain enhancer). For references see Feng et al. 2000; Furuhashi et al., 2001; Hayes et al., 2001; Itoh et al., 2000a; Itoh et al., 2000b; Kaji et al., 2001; Kardassis et al., 2000; Kim et al., 2000; Lee et al., 2001; Liberati et al., 2001; Matsuda et al., 2001; Padgett and Patterson, 2001; Pardali et al., 2000; Rodriguez et al., 2001; Sasaki et al., 2001; Shimizu et al., 2001; Yahata et al., 2000; Yoshida et al., 2000.

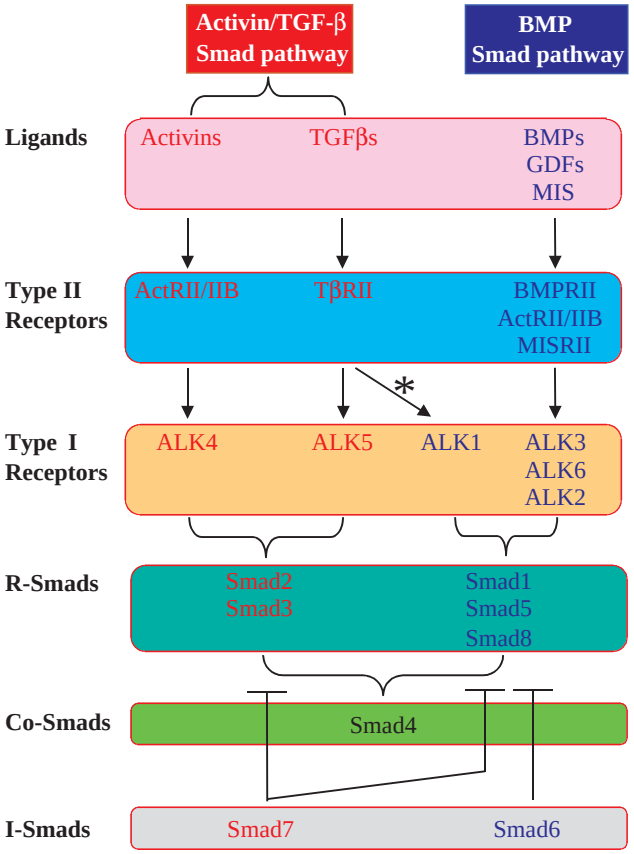


Fig. 1. Signalling specificity in the TGF-β superfamily. Classification of the mammalian Smad signalling cascade into activin–TGF-β (maroon) and BMP (blue) pathways. Representative examples of mammalian ligands (pink shading), type II receptors (red shading), type I receptors (orange shading), R-Smads (green shading), Co-Smads (bright green shading) and I-Smads (grey shading) are depicted in pathways linked by arrows or signs of inhibition. Bifurcation of the TGF-β pathway at the level of type I receptors towards both TGF-β and BMP Smads is marked by an asterisk. Nomenclature of proteins not detailed in the text are growth and differentiation factors (GDFs), Mullerian inhibiting substance (MIS), activin type II and type IIB receptor (ActRII/IIB), TGF-β type II receptor (TβRII), BMP type II receptor (BMPRII), MIS type II receptor (MISRII), activin receptor-like kinases 1 to 6 (ALK1-ALK6). For references see ten Dijke et al. (ten Dijke et al., 2000).

Regulation of Smad function by phosphorylation

Phosphorylation of the C-terminal serine residues in R-Smads by type I receptor kinases is a crucial step in TGF-β family signalling (Abdollah et al., 1997; Macías-Silva et al., 1996; Souchelnytskyi et al., 1997). The two most C-terminal serine residues become phosphorylated and, together with a third, non-phosphorylated serine residue, form an evolutionarily conserved SSXS motif in all R-Smads (Abdollah et al., 1997; Souchelnytskyi et al., 1997) (Fig. 2). Substrate specificity is determined by the L45 loop in the type I receptors and, primarily, by the L3 loop in the R-Smad MH2 domain (Fig. 2); thus, TGF-β and activin receptors phosphorylate Smad2 and Smad3, and BMP receptors phosphorylate Smad1, Smad5 and Smad8 (Chen et al., 1998) (Fig. 1). The consequence of R-Smad phosphorylation is the formation of oligomeric complexes with the Co-Smad, Smad4 (see below).

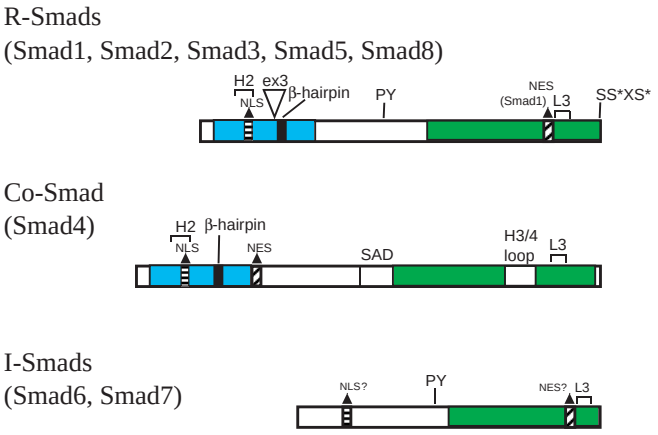


Fig. 2. The Smad family. Diagrammatic representation of the three subfamilies of Smads. The protein diagrams are arbitrarily aligned relative to their C-termini. The MH1 domain is coloured in blue and the MH2 domain in green. Selected domains and sequence motifs are indicated as follows: α-helix H2, L3 and H3/4 loops, β-hairpin, the unique exon 3 of Smad2 (ex3), NLS and NES motifs or putative (?) such motifs, the proline-tyrosine (PY) motif of the linker that is recognised by the Hect domain of Smurfs, the unique SAD domain of Smad4 and the SSXS motif of R-Smads with asterisks indicating the phosphorylated serine residues.

Although 2D phosphopeptide maps of ectopically overexpressed R-Smads are rather simple (Abdollah et al., 1997; de Caestecker et al., 1998; Macías-Silva et al., 1996), analysis of endogenous mammalian Smads reveals >10 different phosphopeptides (Souchelnytskyi et al., 1997; Yakymovych et al., 2001). Other kinases might therefore phosphorylate the Smads. Indeed, the latter contain phosphorylation sites for Erk-family MAP kinases (Kretzschmar et al., 1997), the Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) (Wicks et al., 2000) and protein kinase C (PKC) (Yakymovych et al., 2001) (Table 1).

Erk phosphorylates serine residues in the linker regions of Smad1 (Kretzschmar et al., 1997), Smad2 and Smad3 (Kretzschmar et al., 1999), and substitution of these serines by negatively charged residues inhibits nuclear translocation of Smads and thus signalling. Similarly, CamKII can phosphorylate Smad2 in vitro at linker-region residues Ser240 and Ser260 (as well as at Ser110 of the MH1 domain), which again inhibits nuclear translocation and signalling. Significantly, phosphorylation of Ser240 was observed in vivo upon treatment of cells with epidermal growth factor (EGF) or platelet-derived growth factor (PDGF). PKC phosphorylates Smad2 in vivo and in vitro at Ser47 and Ser110, and Smad3 at the analogous Ser37 and Ser70 (Yakymovych et al., 2001). Phosphorylation of Smad3 by PKC blocks DNA-binding and consequently transcriptional regulation. At the cellular level, this inhibits TGF-β-induced apoptosis and increases susceptibility of cells to loss of contact inhibition (Yakymovych et al., 2001).

In several other cases, the underlying mechanism of Smad phosphorylation remains to be determined. de Caestecker et al., for example, demonstrated that Erk phosphorylates Smad2 in response to EGF or hepatocyte growth factor (HGF) at the C-terminal SSXS motif and thereby activates the Smad pathway (de Caestecker et al., 1998). The molecular mechanisms of

synergistic activation of Smad2/3-mediated transcriptional responses by two other kinases, MEKK-1 and Jun N-terminal kinase (JNK), which phosphorylate unknown residues outside the SSXS motif, also need further investigation (Brown et al., 1999; Engel et al., 1999).

Phosphorylation of the Co-Smad, Smad4, has not been reported in mammals. However, in *Xenopus*, one of two Smad4 isoforms, Smad4β, is phosphorylated, whereas the other, Smad4α, is not (Howell et al., 1999; Masuyama et al., 1999). The Smad4β phosphorylation sites and their importance for signalling remain unknown.

The I-Smads, Smad6 and Smad7, are phosphorylated by as-yet uncharacterised kinases (Imamura et al., 1997; Pulaski et al., 2001). Smad6 phosphorylation sites and their importance for signalling remain unexplored, although phosphorylation may not be mediated by the TGF-β and BMP receptor kinases (Imamura et al., 1997). Smad7 is phosphorylated at Ser249, and this depends on the proliferation status of cells but not on TGF-β receptor signalling (Pulaski et al., 2001). Although phosphorylation of Ser249 regulates the transcriptional activity of Smad7 (Pulaski et al., 2001), its role in regulation of transcription during TGF-β superfamily or independent signalling remains to be uncovered.

Thus, phosphorylation not only activates Smad proteins but also modulates their activity. This provides a mechanism for integration of the Smad pathway with other signalling pathways that modulate TGF-β superfamily signal transduction.

Smad oligomerisation and activation

Following phosphorylation of R-Smads by type I receptors, Smad oligomerisation is thought to occur. Biochemical and structural evidence suggests that the phosphorylated C-terminal tail of R-Smads interacts specifically with the L3 loop of another Smad, which is sufficient to cause their oligomerisation (Correia et al., 2001). Unphosphorylated Smad proteins exist primarily as monomers, and upon phosphorylation, R-Smads form homo-oligomers, which quickly convert to hetero-oligomers containing the Co-Smad, Smad4 (Correia et al., 2001; Kawabata et al., 1998) (Fig. 3). Oligomerisation is assisted by extensive contacts between the loop-helix region of one subunit and the three-helix bundle of another - areas of Smads that contain many evolutionarily conserved residues (Shi, 2001).

Inactive, cytoplasmic Smads are intrinsically auto-inhibited through an intramolecular interaction between the MH1 and MH2 domains (Hata et al., 1997). Smad4 also contains a unique loop in its MH2 domain that prevents spontaneous oligomerisation in the absence of signalling (Tada et al., 1999). Receptor-mediated phosphorylation seems to induce conformational changes that relieve the auto-inhibition and possibly expose buried epitopes on the surface of the activated Smads involved in interactions with other components important for nuclear import, transcriptional regulation or degradation.

Early experiments indicated that oligomeric Smads are trimers (Kawabata et al., 1998; Shi, 2001). Equilibrium centrifugation and crystallographic studies have confirmed this in the case of Smad3 (Chacko et al., 2001; Correia et al., 2001). However, Wu et al. have recently proposed a dimeric configuration for the Smad2-Smad4 complex (Wu et al., 2001).

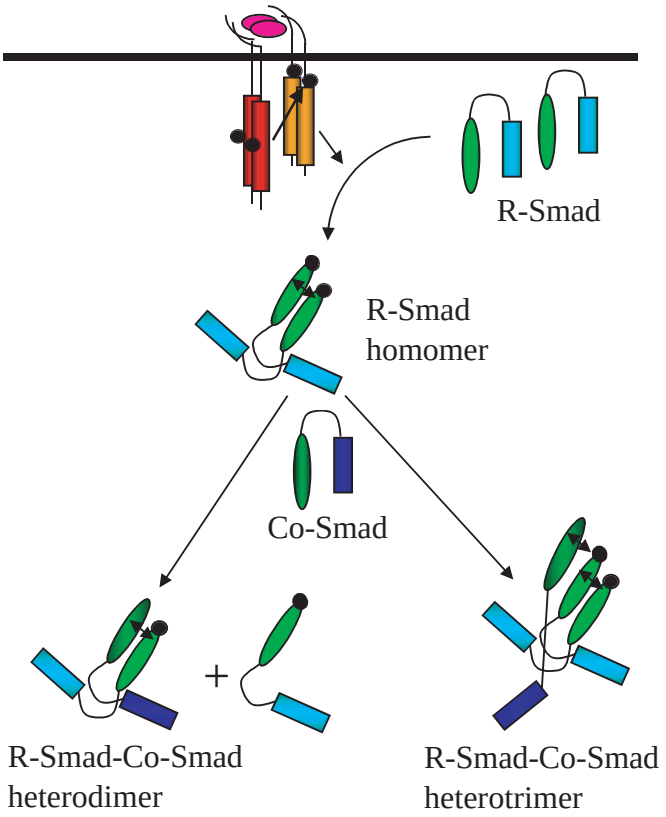


Fig. 3. Smad oligomerisation. Pictorial representation of the plasma membrane receptor kinases that phosphorylate the C-termini of R-Smads (light colour), leading to homo-oligomerisation (a dimer shown for simplicity). Hetero-oligomerisation of R-Smads with the Co-Smad (dark colour) is shown leading to dimers and trimers (see text). The MH1 and MH2 domains are drawn and coloured according to the depiction of Table 1. Small black circles represent the di-phosphate modification of the SXS motif and small, double-headed arrows point to the protein interface between phosphorylated C-termini and the MH2 domain. For references see Chacko et al. (Chacko et al., 2001) and Shi (Shi, 2001).

Thus, different R-Smad-Co-Smad oligomers with distinct stoichiometries are possible (Fig. 3). This notion is supported by a recent analysis of native cellular Smads using gel chromatography (Jayaraman and Massagué, 2000). No information regarding the oligomeric status of BMP-specific R-Smads is currently available. Structural studies of different R-Smad-Co-Smad complexes are needed to resolve the important problem of their stoichiometry.

Organising Smad signalling centres

Recent findings have demonstrated that accessory/scaffolding proteins interact with the type I and II receptors and/or the Smads (Fig. 4). One example is SARA (Smad anchor for receptor activation), a cytoplasmic protein that specifically interacts with non-activated Smad2 and the receptor complex, thus forming a bridge between the receptor and Smad2 and assisting in the specific phosphorylation of Smad2 by the type I receptor (Tsukazaki et al., 1998). The stable interaction of SARA with non-phosphorylated Smad2 also inhibits nuclear import of Smad2 (Xu et al., 2000) (see below). As SARA

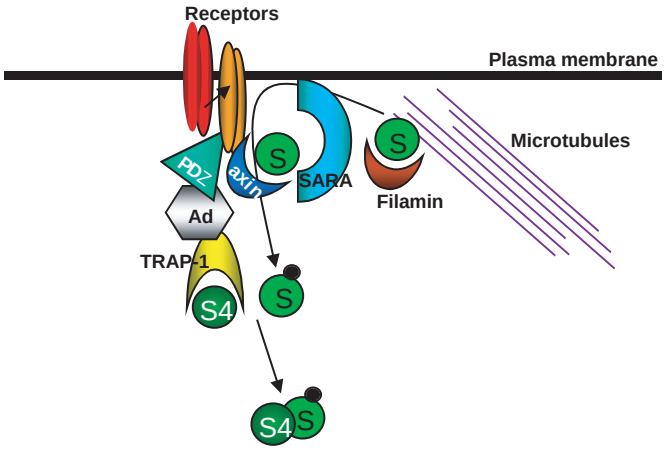


Fig. 4. Smad signalling centres. Pictorial representation of early signalling events of the Smad pathway. A possible but not yet fully documented signalling scenario is shown, initiating at the plasma membrane. R-Smads (S) anchored to microtubules or filamin become mobilised towards SARA and the receptors where multiprotein centres are organised with the aid of scaffolding proteins containing PDZ domains such as ARIPs (PDZ), additional but yet unknown adaptors (Ad) and R-Smad and Smad4 (S4) anchors-activators such as axin and TRAP-1, respectively. This results in R-Smad phosphorylation and R-Smad–Co-Smad oligomerisation. It is worth noting that a similar signalling scenario might become organised at early endosomes, immediately after receptor-mediated endocytosis. For references see Dong et al. and others (Dong et al., 2000; Furuhashi et al., 2001; Sasaki et al., 2001; Tsuchida et al., 2001; Tsukazaki et al., 1998; Wurthner et al., 2001).

contains a FYVE domain, a motif known to bind phosphatidylinositol 3-phosphate, it might anchor Smad2 to the inner leaflet of the plasma membrane or endosomal vesicles. SARA thus provides a first example of how TGF-β signalling centres may be organised at the plasma membrane (Fig. 4), although no SARA-like adaptor proteins have yet been reported in BMP signalling pathways.

A second FYVE-domain-containing protein, Hrs, also facilitates Smad2 signalling and cooperates with SARA-mediated signalling (Miura et al., 2000). The adaptor proteins disabled 2 (Dab2), 14-3-3ε and the negative regulator of Wnt signalling Axin provide additional examples of proteins that link the receptor complex with Smad2 and Smad3 and assist in signal propagation (Furuhashi et al., 2001; Hocevar et al., 2001; McGonigle et al., 2001). The mechanism that organises such Smad signalling centres and its links to receptor endocytosis (Doré et al., 2001), degradation (Kavsak et al., 2000) and signalling crosstalk is an important topic that deserves further analysis.

Several other proteins with possible roles in Smad anchoring have recently been described. Microtubules can anchor inactive Smads in the cytoplasm (Dong et al., 2000). Activation by a ligand results in dissociation of the Smads from the microtubule network. In fact, pharmacological disruption of microtubules leads to aberrant and constitutive activation of the Smad pathway. It is possible that microtubules serve as tracks for intracellular Smad movement. Filamin, an actin crosslinking factor and scaffolding protein, also associates with Smads and positively regulates transduction of Smad signals (Sasaki et al., 2001). Another example of a receptor- and Smad-associating scaffolding protein is caveolin 1, which interacts with the type I receptor and mediates localisation of the receptor complexes to caveolae, thus inhibiting Smad2-mediated signalling (Razani et al., 2001). Proteins of the sorting nexin (SNX) family of vesicle- and receptor-trafficking adaptors also interact with TGF-β receptor complexes (Parks et al., 2001). Similarly, ARIPs (activin receptor interacting proteins) associate with Smad2 and enhance Smad2-mediated signalling in response to activin (Tsuchida et al., 2001). In addition, GIPC (GAIP-interacting protein, C-terminus) is a scaffolding protein for Gα subunits that associates with clathrin vesicles and interacts with the proteoglycan-like, type III TGF-β receptor, enhancing Smad-mediated signalling (Blobe et al., 2001). Both ARIPs and GIPC are PDZ-domain-containing proteins that serve as multiprotein-complex organising centres (Harris and Lim, 2001). Finally, TRAP1 (TGF-β receptor type I associated protein 1) associates with Smad4 and is proposed to serve as a Smad4 anchor that lies proximal to the receptor complex and might assist formation of R-Smad-Co-Smad oligomers (Wurthner et al., 2001).

The available data support the notion that interactions between TGF-β superfamily receptors and Smads with adaptor/scaffolding proteins are an important regulatory mechanism. Proper receptor localisation in plasma membrane or endocytic vesicle microdomains, their proximity to cytoplasmic anchors that hold the Smads and the ability of such complexes to be mobilised between various cytoplasmic compartments are exciting new aspects of the regulation of Smad signalling (Fig. 4). Such mechanisms could provide cell-context specificity, allowing differential regulation of the basic Smad pathway.

Nucleocytoplasmic shuttling

All R-Smads, mammalian Smad4 and *Xenopus* Smad4α reside in the cytoplasm. In contrast, *Xenopus* Smad4β and I-Smads localise to the cell nucleus (Howell et al., 1999; Itoh et al., 2001; Itoh et al., 1998; Masuyama et al., 1999). Coprecipitation experiments indicate that phosphorylated R-Smads quickly form complexes with the Co-Smad, possibly prior to nuclear translocation (Lagna et al., 1996). This notion is enhanced by studies of Smad4 mutants that cannot translocate to the nucleus yet oligomerise efficiently with R-Smads (Morén et al., 2000). Early studies established that nuclear translocation of R-Smads is independent of Smad4, whereas translocation of Smad4 after TGF-β signalling seems to require the presence of an activated R-Smad (Hoodless et al., 1999; Liu et al., 1997).

The nuclear import mechanisms of Smad1, Smad2 and Smad3 have been analysed in detail (Kurisaki et al., 2001; Xiao et al., 2000; Xiao et al., 2001; Xu et al., 2000). The MH1 domains of all eight Smads each contain a lysine-rich motif that in the case of Smad1 and Smad3 has been shown to act as a nuclear localisation signal (NLS) (Xiao et al., 2000; Xiao et al., 2001) (Fig. 2). In Smad3, C-terminal phosphorylation results in conformational changes that expose the NLS so that importin β1 can bind and mediate Ran-dependent nuclear import (Kurisaki et al., 2001; Xiao et al., 2000) (Fig. 5). In contrast, Smad2, which has the same lysine-rich sequence in its MH1 domain, is released from the anchoring SARA after C-terminal phosphorylation and then translocates into the nucleus by a

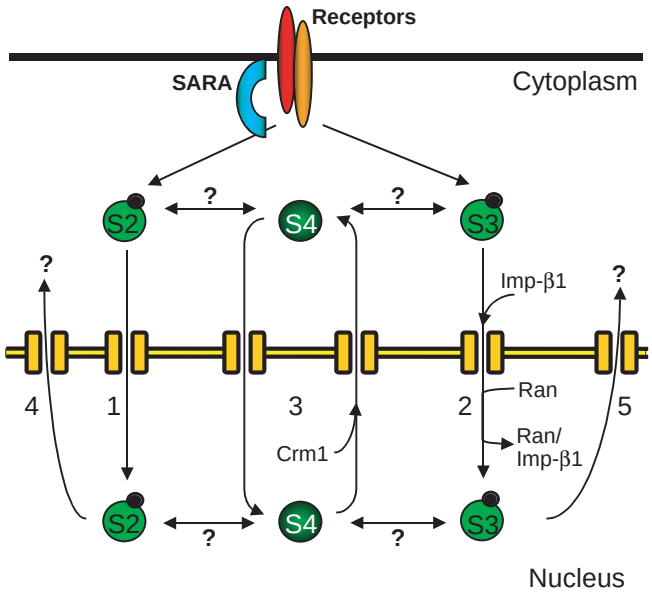


Fig. 5. Smad nucleocytoplasmic shuttling. The five pathways shown are: Smad2 nuclear import after release from SARA (pathway 1); Smad3 nuclear import mediated by importin-β1 and Ran (pathway 2); Smad4 shuttling mediated by the exportin Crm1 (pathway 3); putative Smad2 (4) and Smad3 (5) export pathways marked with question marks. Horizontal double arrowheaded lines indicate possibilities of Smad oligomerisation in the cytoplasm or nucleus. Smad2 (S2), Smad3 (S3) and Smad4 (S4) are shown as monomers and the actual stoichiometry of the Smad complexes is not depicted here. Small black circles represent the di-phosphate modification of the SXS motif. For references, see Kurisaki et al. (Kurisaki et al., 2001) and Massagué (Massagué, 2000).

cytosolic-factor-independent import activity that requires a region of the MH2 domain (Xu et al., 2000) (Fig. 5). The difference between the two R-Smads of the TGF-β and activin pathways is due to the presence of the unique exon 3 in the MH1 domain of Smad2 (Kurisaki et al., 2001) (Fig. 2). Thus, the lysine-rich sequence of the Smad MH1 domain may not be fully functional in all Smads, perhaps because of the unique structural determinants in each Smad. Whether the two different mechanisms of Smad2 and Smad3 nuclear import also reflect differences in their oligomerisation status remains unclear.

The identification of alternatively spliced forms of Smad4 in *Xenopus* led to the discovery that Smad4 constitutively enters the nucleus and that its cytoplasmic localisation in unstimulated cells is due to active nuclear export (Pierreux et al., 2000; Watanabe et al., 2000) (Fig. 5). This export is mediated by a unique leucine-rich nuclear export signal (NES) localised in the linker region of Smad4 and is catalysed by the exportin Crm1 (Fig. 2; Fig. 5). Smad4 therefore continuously shuttles in and out of the nucleus. R-Smad–Co-Smad oligomerisation might therefore occur in the nucleus or at least en route to the nucleus.

Along with other Smads, Smad1 has recently been shown to have both ligand-dependent import and constitutive export activities (Xiao et al., 2001). The latter depends on an NES in the MH2 domain, which is N-terminal to the L3 loop (Fig. 2). This is conserved among all Smads but proposed to be active only in certain Smads. Smad2 and Smad3 also exit the nucleus, but this occurs after prolonged treatment with TGF-β (Pierreux et al., 2000). The putative NESs in Smad2 and Smad3 have not been identified. It is also unclear whether their export mechanisms depend on specific exportins. Finally, I-Smads are constitutively imported to the nucleus and are exported to the cytoplasm in response to TGF-β or BMP signalling (Itoh et al., 1998; Itoh et al., 2001). The functional NLSs and NESs and the mechanisms of regulation of I-Smad nucleocytoplasmic shuttling have not been characterised yet.

The physiological significance of selective regulation of the subcellular distributions of different Smads is hard to understand at this point, when only limited comparative analyses of these mechanisms are available.

Nuclear signalling

All Smads have transcriptional activity (Itoh et al., 2000b; Massagué and Wotton, 2000). Heteromeric R-Smad–Co-Smad complexes are the transcriptionally relevant entities in vivo (Fig. 6). I-Smads have also been shown to have transcriptional activities, the significance of which remains to be elucidated (Bai et al., 2000; Hill-Kapturczak et al., 2000; Pulaski et al., 2001).

Smad3 and Smad4 bind directly but with low affinity to Smad-binding elements (SBEs), which have the minimal sequence motif 5′CAGAC3′, through a conserved β-hairpin loop in the MH1 domain (Fig. 2). Additional MH1 sequences, such as α-helix 2, contribute to SBE DNA-binding by Smad3 (Kusanagi et al., 2001) (Fig. 2). In contrast, Smad2 cannot bind to the SBE because of its unique exon-3-encoded sequence (Fig. 2) (Yagi et al., 1999).

Smad3 and Smad4 also associate with GC-rich motifs in promoters of certain genes, which demonstrates a relaxed DNA-binding specificity of the Smad MH1 domain (Labbé et al., 1998). The BMP-responsive Smads also have highly conserved β-hairpin loops and thus are predicted to bind to SBEs, as has been recently demonstrated in the case of Smad5 (Li et al., 2001). Alternatively, BMP-dependent R-Smads can directly, but very weakly, bind to GC-rich motifs in several *Drosophila* promoters and one mammalian (*MADH6*) promoter (Ishida et al., 2000; Kim et al., 1997).

All the above examples involve Smad-mediated activation of gene expression. Recently, however, the first examples of Smad-dependent gene repression were uncovered. The DNA elements involved do not resemble SBEs or GC-rich motifs (Alliston et al., 2001; Chen et al., 2001). Whether Smads associate directly with such elements remains to be examined.

The fact that GAL4-Smad chimeras exhibit transcriptional activity in mammalian and yeast cells indicated that Smads might associate with the basal transcriptional machinery (Liu et al., 1996). The transactivation function of Smads maps to the MH2 domain and is mediated by direct association of the MH2 domain with co-activators of the p300 and P/CAF (p300- and CBP-associating factor) families (Itoh et al., 2000a; Itoh et al., 2000b) (Fig. 6). Smad4 appears to play a crucial role in regulating the efficiency of transactivation of the Smad complexes in the nucleus. This is thought to involve the unique Smad-activation domain (SAD) of Smad4, which allows stronger association with the p300/CBP co-activators and confers a unique conformation on the Smad4 MH2 domain (Chacko et al., 2001; de Caestecker et al., 2000b) (Fig. 2).

As mentioned above, Smad signalling can also lead to repression of gene expression. Smad3 has been reported to

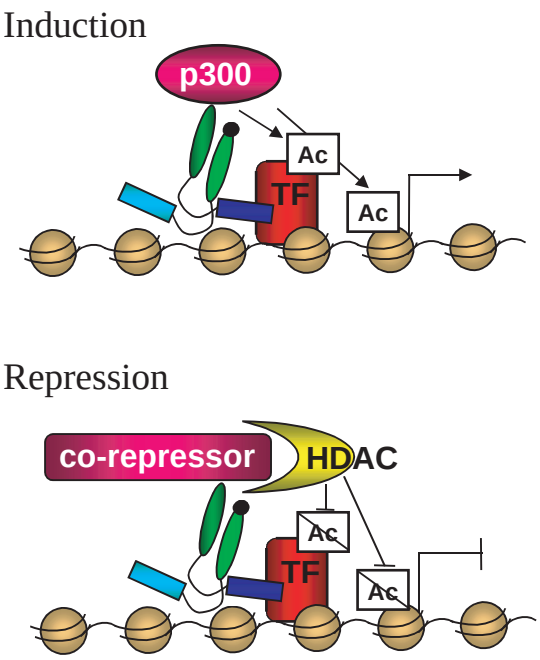


Fig. 6. Transcriptional regulation by Smads. Two examples, one for gene induction and one for gene repression are shown. Chromatin in nucleosomal configuration is depicted by an arrow indicating promoter activation and a vertical line depicting promoter silencing. Smads are shown as heterodimers of phosphorylated (small black circle) R-Smad–Smad4 according to Fig. 2. Smads interact with DNA-binding transcription factors (TF) and recruit co-activators (p300) or co-repressors that sequentially associate with HDACs. The former results in transcription factor and histone acetylation (Ac), whereas the latter leads to deacetylation. These models take into account only the role of protein acetylation in transcriptional regulation. For references, see Massagué and Wotton (Massagué and Wotton, 2000).

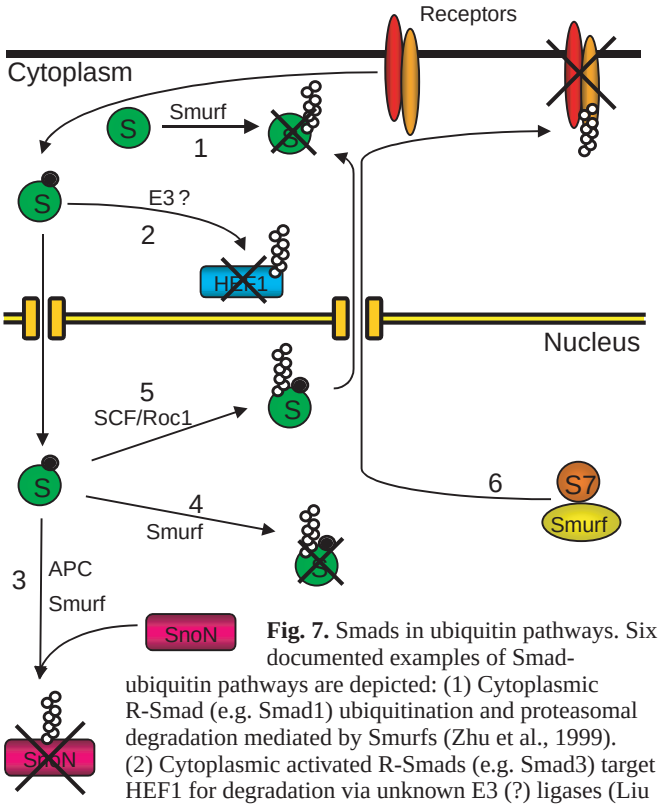


Fig. 7. Smads in ubiquitin pathways. Six documented examples of Smad-ubiquitin pathways are depicted: (1) Cytoplasmic R-Smad (e.g. Smad1) ubiquitination and proteasomal degradation mediated by Smurfs (Zhu et al., 1999). (2) Cytoplasmic activated R-Smads (e.g. Smad3) target HEF1 for degradation via unknown E3 (?) ligases (Liu et al., 2000). (3) Nuclear R-Smads (e.g. Smad3) target the co-repressor SnoN for degradation via Smurfs or the APC that act as E3 ligases (Bonni et al., 2001; Stroschein et al., 2001). (4) Nuclear R-Smads (e.g. Smad2) are degraded after Smurf-mediated ubiquitination (Lin et al., 2000; Lo and Massagué, 1999; Zhang et al., 2001). (5) Nuclear R-Smads (e.g. Smad3) are ubiquitinated by the action of the SCF^{Fbw1a}/Roc1 E3 ligase complex, exported to the cytoplasm and finally degraded there (Fukuchi et al., 2001). (6) The TGF-β signal induces Smad7-Smurf association, export to the cytoplasm and targeting of the receptor kinases that become degraded (Ebisawa et al., 2001; Kavsak et al., 2000). R-Smads (S, with small black circles indicating C-terminal phosphorylation) and Smad7 (S7) are depicted as circles. The poly-ubiquitin chain is shown as a multi-circle attachment and an X indicates proteasomal degradation of the target protein.

associate with histone deacetylase (HDAC) activities through its MH1 domain, but whether Smads interact directly with HDACs remains unclear (Liberati et al., 2001). Alternatively, Smads can interact with co-repressors that recruit HDACs (Fig. 6). These co-repressors include the homeodomain DNA-binding protein TGIF (Wotton et al., 1999) and the proto-oncogene products Ski and SnoN (Liu et al., 2001). Such co-repressors appear to modulate the nuclear activity of Smads, and their levels of expression define the level of Smad transcriptional activity.

SnoN illustrates an interesting example of a nuclear feedback loop (Liu et al., 2001; Stroschein et al., 2001). SnoN basal levels have been proposed to maintain TGF-β-responsive genes in a repressed state. When a cell is stimulated by TGF-β, the incoming nuclear Smads target SnoN for ubiquitination and degradation (see below), thus relieving repression and possibly allowing other Smad complexes to activate transcription of target genes. One such gene is *SnoN* itself, which presumably re-represses target genes as soon as the nuclear Smad signal declines. Whether such a model applies generally to many TGF-β superfamily gene targets or to selected groups of genes remains to be elucidated.

Therefore, the required transcriptional specificity of the Smad pathway is achieved through multiple SBE motifs in promoters of Smad-target genes, which confer higher Smad-binding affinity, and additional transcription factors that cooperate with the Smads (Fig. 6). The in vivo characteristics of such transcriptional complexes and their dynamic interaction with chromatin remain largely unexplored. However, the list of Smad-interacting transcription factors is large (Table 1), providing a mechanistic basis for cell-type- and context-specific gene regulation. Because Smad-interacting transcription factors have been recently reviewed exhaustively (Itoh et al., 2000b; Massagué and Wotton, 2000), we do not discuss all cases of such transcription factors here. The plethora of interacting proteins provides a mechanistic explanation for the documented crosstalk between the Smad pathway and many other signalling networks, which range from the Ras/MAPK pathway to the Wnt/β-catenin and nuclear hormone signalling cascades (Itoh et al., 2000b; Massagué, 2000).

Smad degradation and roles in protein ubiquitination pathways

Protein ubiquitination and subsequent proteasomal degradation

is a common regulatory mechanism (Ciechanover et al., 2000). Recently, a novel class of E3-type, Hect-domain ubiquitin ligase, designated Smurf, has been shown to interact with the Smads and been implicated in their ubiquitination (Fig. 7). Smurf1 regulates the abundance of Smad1 in the cytoplasm of unstimulated cells (Zhu et al., 1999). C-terminally phosphorylated Smad2 is another target. Once engaged in transcriptional complexes, Smad2 is eventually ubiquitinated and degraded by proteasomes (Lo and Massagué, 1999). In this case, Smurf2 is one of the E3 ligases involved (Lin et al., 2000; Zhang et al., 2001).

C-terminally phosphorylated nuclear Smad3 is also ubiquitinated after completion of its transcriptional role, and ubiquitination is mediated by the SCF/Roc1 E3 ligase complex (Fukuchi et al., 2001) (Fig. 7). In the case of Smad3, proteasomal degradation occurs in the cytoplasm, and the SCF/Roc1 complex assists in nuclear export of Smad3. Interestingly, overexpression of p300 enhances ubiquitination of phosphorylated Smad3, suggesting that ubiquitination follows the transcriptional role of Smad3. Whether specific exportins are involved in the export of ubiquitinated Smads or whether the associated ubiquitin ligases, such as Smurfs or the SCF complex, mediate such export, remains unanswered.

Finally, proteasomal degradation of Smad4 occurs in tumour cells, which either harbour deleterious mutations in *MADH4* or express activated oncoproteins such as Ras; the specific ubiquitination mechanism is as yet elusive (Maurice et al., 2001; Morén et al., 2000; Saha et al., 2001; Xu and Attisano, 2000). Whether mediated by Smurfs or other E3 ligases, the ultimate degradation of nuclear Smads after prolonged ligand stimulation has been firmly established as a mechanism that shuts off the signalling pathway.

Recent findings underscore additional roles of proteasomal degradation in the control of Smad signalling. The Smurfs can also regulate ubiquitination and degradation of other target proteins, including the TGF-β receptor complex and the transcriptional co-repressor SnoN (Bonni et al., 2001; Ebisawa et al., 2001; Kavsak et al., 2000; Stroschein et al., 2001) (Fig. 7). Smurf1 and Smurf2 associate with the nuclear I-Smad Smad7 after stimulation by TGF-β (Ebisawa et al., 2001; Kavsak et al., 2000). The I-Smad–Smurf complex is exported to the cytoplasm and ubiquitinates the receptors on the cell surface or endosomal membranes; these are then targeted for degradation in proteasomes and lysosomes. Whether Smad6 plays a similar adaptor role in ubiquitination remains to be examined. An additional inhibitor that recruits Smad7 to the receptor is STRAP, a WD domain protein that binds to both the MH2 domain of Smad7 and the type I receptor (Datta and Moses, 2000). It would be interesting to test whether STRAP participates in Smurf-mediated ubiquitination of the type I receptor. A search for Smad-interacting proteins uncovered the human enhancer of filamentation 1 (HEF1), whose levels are regulated by proteasomal degradation induced by the Smad pathway (Liu et al., 2000). Thus, the paradigm of Smads acting as mediators of ubiquitination of cellular proteins may be extensive and involve various different mechanisms and molecular partners (Fig. 7).

Alternatively, when entering the nucleus, activated Smad2 and Smad3 can interact with Smurf2 and with the anaphase-promoting complex (APC) and thus stimulate ubiquitination of SnoN, for example, with which the Smads and E3 ligases interact. This leads to the efficient and rapid elimination of the SnoN co-repressor (Bonni et al., 2001; Stroschein et al., 2001) (Fig. 7). The mechanisms by which TGF-β signals might discriminate among Smurfs that target the Smads themselves, as opposed to other protein targets, such as SnoN or the receptors, when uncovered, may point to novel means of regulation of Smad signalling.

Perspectives

Clearly, important aspects of the cell biology of the Smad pathway are yet to be understood. Among those, the elucidation of the TGF-β-receptor-endocytosis and Smad-activation mechanism, as well as the mechanism of oligomerisation and the stoichiometry of various Smad complexes are primary goals. The mechanisms of nucleocytoplasmic shuttling also deserve further clarification. Similarly, the in vivo mechanisms of gene activation and repression in the context of chromatin need to be addressed, and the ubiquitin-mediated shut-off pathways for the different Smads must be analysed systematically. Although it may seem that the Smad pathway mediates all known physiological and pathological effects of the various TGF-β superfamily ligands, extensive literature exists that implicates alternative signalling pathways in such cellular effects (de Caestecker et al., 2000a; Derynck et al., 2001; Massagué, 2000). Elucidation of the signalling effectors that link the receptors to these alternative pathways and their functional crosstalk with the Smads is of primary importance.

The recent advent of functional genomics and the ability to globally monitor gene expression at the RNA and protein levels provides an important approach for the future. A major quest is to identify co-regulated groups of genes that respond to TGF-β superfamily signals and classify them on the basis of their mode and kinetics of regulation, the functions of the encoded proteins and the cell type and developmental context. Indeed an effort exploiting oligonucleotide and cDNA microarray analysis has already revealed a large number of genes that are regulated by TGF-β (Akiyoshi et al., 2001; Chen et al., 2001; Verrecchia et al., 2001; Zavadil et al., 2001). For this task, the use of cells derived from mice in which specific genes are inactivated will be valuable, as exemplified by recent reports that have assigned differential outputs to the Smad2, Smad3 and Smad4 signals (Piek et al., 2001; Sirard et al., 2000). In addition, since the Smad pathway provides a plethora of interacting signalling factors, proteomic screens will provide the complete repertoire of Smad-interacting proteins, and again a major goal should be to associate these factors and signalling networks with the physiology or pathology of specific cell types. To this end, systematic analysis of model organisms, especially invertebrates, will consolidate the biological relevance of complex signalling networks (Padgett and Patterson, 2001). The new technologies also hold promise for a better understanding of the contribution of Smads to various disease conditions and thus may provide novel drug targets.

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