Smad-dependent and Smad-independent pathways in TGF-β family signalling

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Transforming growth factor-β (TGF-β) proteins regulate cell function, and have key roles in development and carcinogenesis. The intracellular effectors of TGF-β signalling, the Smad proteins, are activated by receptors and translocate into the nucleus, where they regulate transcription. Although this pathway is inherently simple, combinatorial interactions in the heteromeric receptor and Smad complexes, receptor-interacting and Smad-interacting proteins, and cooperation with sequence-specific transcription factors allow substantial versatility and diversification of TGF-β family responses. Other signalling pathways further regulate Smad activation and function. In addition, TGF-β receptors activate Smad-independent pathways that not only regulate Smad signalling, but also allow Smad-independent TGF-β responses.

The TGF-β superfamily comprises TGF-βs, bone morphogenetic proteins (BMPs), activins and related proteins. These proteins were identified mainly through their roles in development; they regulate the establishment of the body plan and tissue differentiation through their effects on cell proliferation, differentiation and migration. The growth-inhibitory effect of TGF-β signalling in epithelial cells explains its role as a tumour suppressor in carcinomas, although TGF-β expression by tumour cells contributes to cancer progression as well. The current model of induction of signalling responses by TGF-β-related factors (Fig. 1) is a linear signalling pathway from the type II to the type I receptor kinase to Smad activation, resulting in ligand-induced transcription[1–3].

Although there are considerably fewer receptors and Smads than there are ligands, a greater versatility of signalling is possible than might be expected. Combinatorial interactions of type I and type II receptors and Smads in oligomeric complexes allow substantial diversity, and are complemented by the many sequence-specific transcription factors with which Smads cooperate, resulting in context-dependent transcriptional regulation. Other signalling pathways help to define the responses to TGF-β factors, and it is increasingly apparent that TGF-β-related proteins activate not only Smads but also other signalling pathways. These pathways regulate Smad-mediated responses, yet also induce Smad-independent responses. Here we summarize recent progress toward understanding the signalling mechanisms of TGF-β-related factors through Smad-dependent and Smad-independent pathways.

Versatility in receptor interactions and ligand binding

The functional complex of TGF-β family receptors at the cell surface consists of two 'type II' and two 'type I' transmembrane serine/threonine kinase receptors[1–4] (Fig. 2). The latter have a characteristic Gly-Ser (GS) sequence upstream from the kinase domains. In mammals, only five type II receptors and seven type I receptors have been identified[2–4], whereas 29 ligands have been found. In the absence of ligand, type II and type I receptors exist as homodimers at the cell surface. TGF-β1, TGF-β3 and activins bind their type II receptors without needing a type I receptor, whereas BMP-2, BMP-4 and BMP-7 bind primarily to their type I receptors, BMP-RIA or BMP-RIB, although heteromeric BMP receptor complexes provide higher-affinity ligand binding[4]. Some TGF-β ligands—for example, TGF-β2—interact only with type II–type I receptor combinations, suggesting that heteromeric receptor complexes form in the absence of ligand, consistent with the inherent affinity of the receptors for each other[4,5]. Thus, the low-affinity heteromeric receptor complex may provide a surface for ligand binding that conformationally stabilizes the complex. Ligand binding to either homomeric receptor dimer is not sufficient to activate signalling. By contrast, activation of the type I receptor kinase, and consequent signalling, requires phosphorylation of its GS domain by the type II receptor in the heteromeric complex[1,4]. Although ligand binding to type II receptor dimers may induce cytoplasmic-domain autophosphorylation, type II receptor signalling in the absence of type I receptors has not been reported.

Combinatorial interactions in the tetrameric receptor complex allow differential ligand binding or differential signalling in response to the same ligand[4] (Fig. 2). One receptor combination often binds different ligands, and patterns of ligand and receptor expression often dictate which receptors are activated. For example, the type II receptors ActRII and ActRIIB can combine with the type I receptor ActRIB/ALK4 and mediate activin signalling, whereas their interactions with BMP-RIA or BMP-RIB allow BMP binding and signalling instead. The BMP type II receptor BMP-RII can combine with three type I receptors, BMP-RIA, BMP-RIB and ActRI/ALK2, to bind several BMPs and mediate BMP signalling. Thus, a ligand can induce different signalling pathways depending on the composition of the receptor complex. For example, TβRII interacts not only with the 'classical' type I receptor TβRI/ALK5, which activates Smad2 and Smad3, but also with ALK1, which activates Smad1 and Smad5. Differential activation of either receptor in endothelial cells induces different responses to TGF-β, suggesting that a balance in their activation controls the state of the endothelium[6]. In addition, ActRI/ALK2 activation by TGF-β has been implicated in TGF-β-induced epithelial-to-mesenchymal differentiation[7,8].

Further complexity is imposed by heterodimeric TGF-β ligands—for example, inhibins and heteromeric BMPs—which may need to bind to heterodimeric type II or type I receptor combinations. Also, accessory proteins enhance or modify ligand-binding specificity. For example, Nodal acts through ActRIIB and ActRIB/ALK4 (the activin receptor complex), activating Smad2, but efficient ligand binding and signalling require association of fucosylated Cripto, a TGF-α-like transmembrane protein, with ALK4 (ref. 9). Also, betaglycan and endoglin provide high-affinity TGF-β presentation to the signalling TGF-β receptor complex. Thus, cell-surface expression of betaglycan may regulate the TGF-β response[10], and the vascular abnormalities resulting from impaired endoglin func-

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tion are probably explained by defective signalling in endothelial cells by TGF-β ligands that interact with endoglin[11].

Smads are structurally related signalling effectors

There are eight vertebrate Smads: Smad1 to Smad8. Smad2 and Smad3 are activated through carboxy-terminal phosphorylation by the TGF-β and activin receptors TβRI and ActRIB, whereas Smad1, Smad5 and Smad8 are activated by ALK-1, ALK-2, BMP-RIA/ALK-3 and BMP-RIB/ALK-6 in response to BMP[1–4] or other ligands. These receptor-activated Smads (R-Smads) are released from the receptor complex to form a heterotrimeric complex of two R-Smads and a common Smad4, and translocate into the nucleus. *Xenopus* has two Smad4s, Smad4α and Smad4β, encoded by two genes[12]. Although ubiquitously involved in Smad-mediated transcription, Smad4 is not essential for TGF-β responses: some TGF-β responses occur in the absence of Smad4 and some Smad4-deficient cell lines have a limited responsiveness to TGF-β[13]. The structurally divergent Smad6 and Smad7 act as 'inhibitory' Smads[1–3]. R-Smads and Smad4 contain a conserved MH1 and C-terminal MH2 domain, flanking a divergent middle 'linker' segment[1–3] (Fig. 3). Inhibitory Smads lack a recognizable MH1 domain, but have an MH2 domain. The MH2 domain has limited structural similarity to the phospho-peptide-binding domain FHA[14]. Both the MH1 and the MH2 domains can interact with select sequence-specific transcription factors, whereas the C terminus of the R-Smads interacts with and recruits the related coactivators CREB-binding protein (CBP) or p300 (refs 1–3). With the exception of Smad2, the MH1 domains of Smads can bind DNA, whereas the MH2 domains mediate Smad oligomerization and Smad–receptor interaction (see Fig. 3).

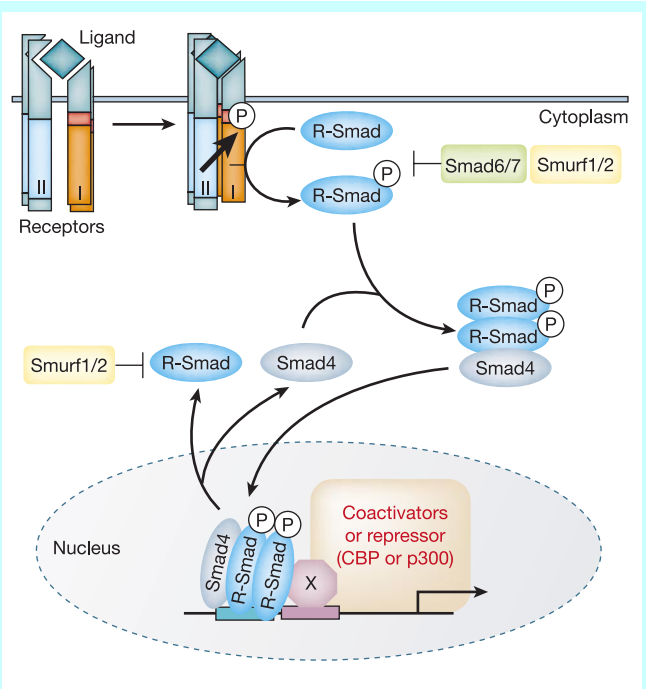


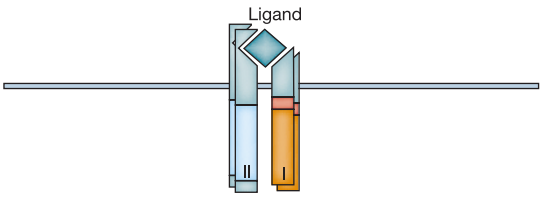
Figure 1 General mechanism of TGF-β receptor and Smad activation. At the cell surface, the ligand binds a complex of transmembrane receptor serine/threonine kinases (types I and II) and induces transphosphorylation of the GS segments (red) in the type I receptor by the type II receptor kinases. The consequently activated type I receptors phosphorylate selected Smads at C-terminal serines, and these receptor-activated Smads (R-Smads) then form a complex with a common Smad4. Activated Smad complexes translocate into the nucleus, where they regulate transcription of target genes, through physical interaction and functional cooperation with DNA-binding transcription factors (X) and CBP or p300 coactivators. Activation of R-Smads by type I receptor kinases is inhibited by Smad6 or Smad7. R-Smads and Smad4 shuttle between nucleus and cytoplasm. The E3 ubiquitin ligases Smurf1 and Smurf2 mediate ubiquitination and consequent degradation of R-Smads, yet can also interact with Smad6/7 and thereby ubiquitinate the type I receptors (not shown).

Regulation of Smad levels

Although differentially controlled during development, R-Smads and Smad4 are expressed in most, if not all, cell types. In contrast to R-Smad expression, expression of the inhibitory Smad6 or Smad7 is highly regulated by extracellular signals. Induction of Smad6 and Smad7 expression by BMP and TGF-β represents an auto-inhibitory feedback mechanism for ligand-induced signalling[1–3]. Accordingly, the downregulation of Smad6 and Smad7 expression during adipocyte differentiation may result from concomitant loss of autocrine TGF-β and BMP signalling[15]. Activation of the epidermal growth factor (EGF) receptor and possibly other tyrosine kinase receptors, interferon-γ signalling through STAT (signal transducer and activator of transcription) proteins, and activation of NF-κB by tumour-necrosis factor-α, also induce Smad7 expression, leading to inhibition of TGF-β signalling[1–3] (Fig. 4).

Ubiquitin–proteasome-mediated degradation controls the levels of Smads post-translationally. The HECT (homologous to the E6-AP carboxy terminus) family E3 ubiquitin ligases, Smurf1 (Smad-ubiquitination-regulatory factor 1) and Smurf2, antagonize TGF-β family signalling by interacting with R-Smads and targeting them for degradation[16]. Smurf-mediated degradation thus controls R-Smad levels and the sensitivity of cells to incoming signals. Smurf1 interacts with Smad1 and Smad5, thereby affecting BMP responses[17], whereas Smurf2 interacts more broadly with different R-Smads, allowing interference with BMP and TGF-β/activin signalling[16,18,19]. Nevertheless, *Xenopus* embryo assays indicate that Smurf1 and Smurf2 primarily target the BMP pathway[17,18].

Proteasomal degradation also regulates the R-Smad levels after translocation into the nucleus. Thus, C-terminally phosphorylated Smad2 can undergo ubiquitination, and inhibition of proteasomal degradation enhances its nuclear accumulation[20]. Nuclear, C-terminally phosphorylated Smad3 can interact with a Ring-finger protein, Roc1, allowing association with an SCF ubiquitin ligase complex consisting of Roc1, Skp1, Cullin1 and βTRCP1/Fbw1a (β-transducin-repeat-containing protein 1) and consequent



Type II	Type I	R-Smad
BMPRII	ALK-2 (ActRI) ALK-3 (BMP-RIA) ALK-6 (BMP-RIB)	Smad1, Smad5, Smad8
ActRII, ActRIIB	ALK-4 (ActRIB)	Smad2
ActRIIB	ALK-7	Smad2
TβRII	ALK-5 (TβRI) ALK-1 ALK-2	Smad2, Smad3 Smad1, Smad5
AMHR	ALK-3 ALK-2 ALK-6	Smad1, Smad5

Figure 2 Combinatorial interactions of type II and type I receptors define the signalling responses. Only the best-documented receptor combinations and their R-Smads are listed.

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nuclear export and ubiquitin–proteasome-mediated degradation in the cytoplasm[21]. However, only a small fraction of Smad2 and Smad3, in the absence or presence of TGF-β, is ubiquitinated, and, upon TGF-β signalling, phosphorylated Smad2 or Smad3 can form a stable complex with Smurf2 (ref. 19). Thus, the bulk of nuclear Smad2 or Smad3 is not targeted for degradation, but dephosphorylated and relocated to the cytoplasm[22,23].

In contrast to R-Smads, Smad4 is not subjected to ubiquitin-mediated degradation. Instead, sumoylation of Smad4 enhances its stability[24]. However, some tumour-associated mutations allow ubiquitination and/or decrease the stability of Smad4 (ref. 25). Jab1, the CSN5 subunit of the COP9 signalosome that was initially identified as a coactivator for c-Jun, has been implicated in targeting Smad4 for ubiquitination and degradation[26].

Regulation of Smad activation

Ligand-induced interaction of R-Smads with activated type I receptors results in direct phosphorylation of the two distal serines of the C-terminal SSXS motif by the type I receptor kinases. This interaction is specified by sequences in both the receptor and the Smad. The nine-amino-acid L45 loop in the type I receptor kinase domain is the main determinant of receptor signalling and Smad-binding specificity, and interacts directly with the L3 loop in the MH2 domain of the R-Smad. Sequences downstream from the L3 loop also contribute to receptor-binding specificity. The type I receptor's GS sequence, once phosphorylated on serines by the type II receptor, provides an interface with the sequence downstream from the L3 loop, and stabilizes Smad docking and contributes to the Smad-interaction specificity[1,27].

TGF-β ligands induce receptor internalization in endosomes, which may be required for efficient TGF-β signalling through Smads[28,29]. The composition of the heteromeric receptor complex and the interaction of co-receptors not only dictate ligand-binding specificity, but may also confer differential intracellular routeing, thereby regulating receptor signalling. Accordingly, receptor-associated proteins may have a role in vesicular trafficking, as well as facilitating TGF-β-induced receptor internalization and Smad recruitment to the receptors[28,29] (Fig. 4). For example, SARA and Dab2, which are enriched in endosomes and clathrin-coated vesicles, bind both receptors and R-Smads, and promote Smad phosphorylation and TGF-β signalling[30,31]. TGF-β receptors can also associate with caveolin, a protein present in plasma-membrane invaginations called caveolae[32], and the caveolin-positive lipid-raft compartment is required for receptor turnover and regulates receptor availability and R-Smad activation[30]. Thus, multiple protein interactions are likely to control subcellular receptor localization and cell-surface receptor availability. These parameters may in turn control the duration of Smad phosphorylation and activation, and thus give rise to qualitatively different responses resulting from different signalling thresholds.

Cytoskeletal proteins also play a part in the localization and signalling of Smads. Unphosphorylated Smad2 and Smad3 bind microtubule filaments, and TGF-β treatment induces their dissociation. Disruption of microtubules with nocodazole increases this dissociation, and enhances Smad2 phosphorylation and activity[33]. Smads also interact with filamin, a scaffold for intracellular signalling proteins that crosslinks actin. Cells defective in filamin expression have impaired TGF-β signalling and Smad2 phosphorylation, which can be rescued by ectopic filamin expression[34]. Finally, TGF-β induces phosphorylation of ELF, a β-spectrin, and its association with Smad3 and Smad4. Lack of ELF results in mislocalization of Smad3/4 and loss of TGF-β-dependent transcription[35].

Smad6 and Smad7 also regulate activation of R-Smads[1–3]. Smad6 and Smad7 inhibit TGF-β family signalling through binding of their MH2 domains to the type I receptor, thus preventing recruitment and phosphorylation of effector Smads[1–3] (Fig. 4). Smad6 also interferes with the heteromerization of BMP-activated Smads with Smad4, preventing the formation of an effector Smad complex. In addition, recruitment of a complex of Smad7 with Smurf1 or Smurf2 to the type I TGF-β receptor results in receptor ubiquitination by the Smurf proteins and targets the receptor for degradation[36,37], possibly at caveolin-containing compartments[30], leading to inhibition of R-Smad activation.

Heteromeric interactions of activated Smads

After C-terminal phosphorylation, the R-Smad dissociates from the type I receptor, presumably because of conformational changes that also allow transition from the primarily monomeric, unphosphorylated R-Smad to an oligomeric complex[38,39]. Activated R-Smads form oligomers in which the phosphorylated C terminus contacts the phosphoserine-binding pockets in the L3 loop region

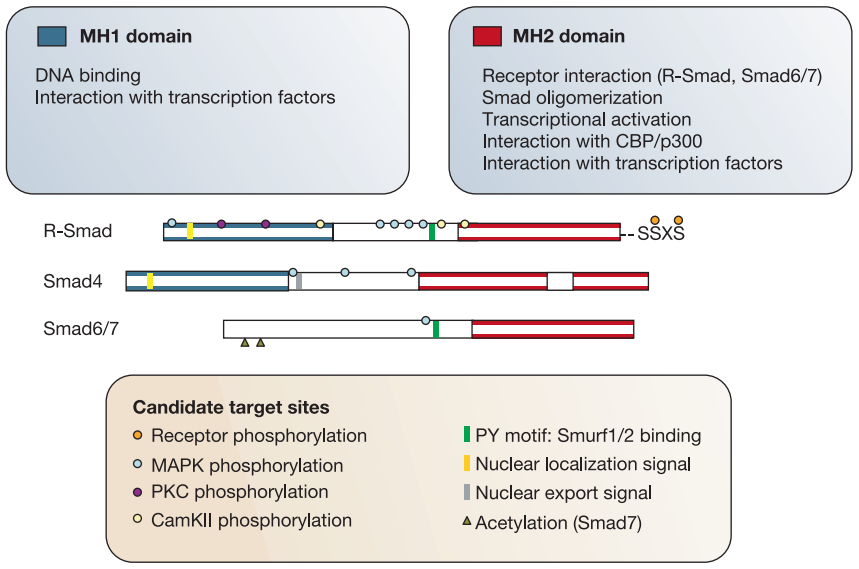

Figure 3 Structural organization and role of the domains of Smads, and candidate target sites for kinase pathways. Such pathways include Erk MAPK and JNK, as well as CamKII and PKC. The significance of candidate MAPK phosphorylation sites in Smad4 and Smad6/Smad7 is not known.

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of a neighbouring R-Smad or Smad4. Endogenous phosphorylated R-Smads may acquire different stoichiometries—heterotrimers with two R-Smads and one Smad4, or heterodimers[38,39]. Smad heterodimers and heterotrimers may both exist in transcription complexes, depending on the interacting transcription factors; for example, complexes of Smad2–Smad4 with Fast-1 or Fast-3 contain two Smad2s and one Smad4, whereas Smad3–Smad4 complexes at the c-Jun promoter may be heterodimers[40]. Most TGF-β-induced transcription responses are mediated by Smad3 and Smad4, whereas activin responses are mediated by Smad2 and Smad4, suggesting a complex of two Smad3s or two Smad2s, respectively, with Smad4. Some TGF-β responses—for example, induction of $p15^{Ink4B}$ expression—require concomitant activation of Smad2 and Smad3 with Smad4 (ref. 41). The heterotrimeric model allows incorporation of two different R-Smads into the complex and may impose differential signalling thresholds upon gene expression.

Nuclear translocation of Smads

TGF-β receptors remain active for at least 3–4 h after ligand binding, and continuous receptor activation maintains the Smad complexes in the nucleus, where they regulate gene expression[22]. Nuclear import of a Smad complex follows 'classical' nuclear translocation paradigms, established through studies of other proteins. Without ligand stimulation, R-Smads localize in the cytoplasm, whereas Smad4 is distributed in the nucleus and cytoplasm[22,42,43]. Nuclear import of R-Smads does not require Smad4, although Smad4 cotranslocates with the R-Smads. Nuclear import of Smad1 and Smad3 is conferred by a lysine-rich nuclear localization sequence (NLS) in the MH1 domain that is conserved in all R-Smads[43]. C-terminal phosphorylation of the MH2 domain and consequent conformational changes may expose the NLS and allow importin-β binding[44,45]. In contrast to Smad3, nuclear import of Smad2 may be independent of importin-β, owing to an insertion in its MH1 domain, and may require the MH2 domain instead[22,23,43,45]. In the nucleus, R-Smads are constantly dephosphorylated, resulting in dissociation of Smad complexes and export of inactive Smads to the cytoplasm[22,23]. Nucleocytoplasmic shuttling of Smad2 requires its MH2 domain to interact with nucleoporins CAN/Nup214 and Nup153 (ref. 23). In contrast to ligand-dependent import of R-Smads, Smad4 continuously shuttles between the nucleus and cytoplasm owing to the combined activities of a constitutively active NLS in the MH1 domain and a nuclear export signal (NES) in the linker region, whose activity depends on the nuclear transport receptor CRM1 (refs 22, 42). This NES may be masked in the complex with R-Smads, allowing Smad complexes to accumulate in the nucleus. Remarkably, *Xenopus* Smad4β, which lacks the NES, localizes almost exclusively in the nucleus, whereas Smad4α, which has an NES, is predominantly cytoplasmic in unstimulated cells[12,42].

Smad7 and Smad6 are localized in the nucleus in some cells in the absence of TGF-β[46,47]. Their activities in transcription assays[48,49] and the interaction of Smad7 with CBP/p300 (ref. 50) suggest that they are transcription (co)factors, which might explain their cooperation with TGF-β signalling in inhibiting adipocyte differentiation and stimulating cell proliferation[15]. TGF-β induces Smad7 export, whereas BMP induces export of Smad6 (refs 46, 47). TGF-β-induced export of Smad7 may require an interaction with Smurf1 or Smurf2 (ref. 47), whereas BMP-induced cytoplasmic localization of Smad6 may require association with a cytoplasmic retention protein[46].

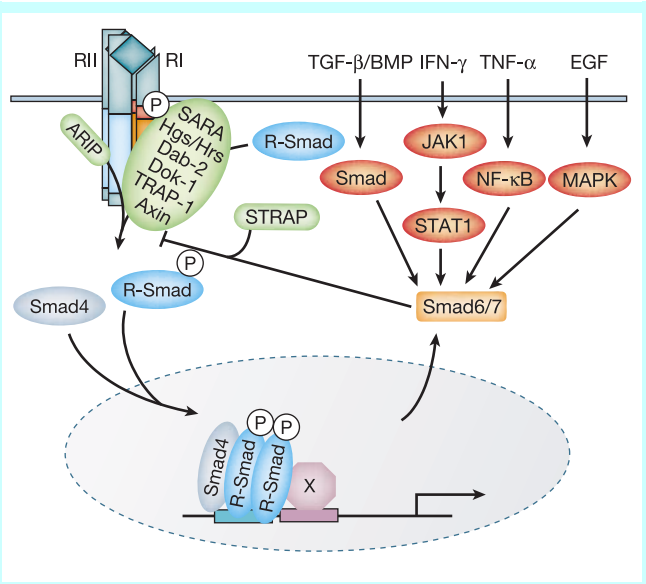

Figure 4 R-Smad activation is regulated by receptor-interacting proteins and Smad6/Smad7. SARA, Hgs/Hrs, Dab2, Dok-1, TRAP-1 (TGF-β-receptor-associated protein), Axin and ARIP (activin-receptor-interacting protein) (green) interact with type I or type II receptors and R-Smads (see refs 1–3 and references therein). SARA or Hrs and Dab2 stabilize the Smad2/Smad3 interactions with TGF-β type I receptors and function in internalization with the endocytic machinery in endosomes. Other proteins, such as the RasGAP-binding protein Dok-1 (ref. 96), the PDZ-domain protein ARIP1 and axin also probably control subcellular localization of receptors and link Smad2/Smad3 to the receptors. TRAP-1, a homologue of the yeast sorting protein Vam6p, interacts with TGF-β or activin type I receptors first, and then with Smad4 upon receptor activation, possibly facilitating Smad4 interaction with activated Smad2 or Smad3. Smad6 and/or Smad7 expression can be induced by several signalling pathways, including TGF-β/BMP signalling through Smads, and attenuates R-Smad activation. STRAP interacts with type I and type II receptors and with Smad7, thus stabilizing the interaction of Smad7 with the receptor complex.

Regulation of Smad activity by kinase pathways

C-terminal phosphorylation by the type I receptor is the key event in Smad activation[1–3]; however, other kinase pathways further regulate Smad signalling (Fig. 3), as suggested by the complex phosphorylation patterns of endogenous Smads. Smad2 phosphorylation and transcription in response to EGF and hepatocyte growth factor, which act through receptor tyrosine kinase receptors, challenge the belief that only TGF-β ligands activate Smads[51].

The Erk mitogen-activated protein kinase (MAPK) pathway, stimulated by the activation of tyrosine kinase receptors and/or Ras, targets R-Smads. Erk MAPK phosphorylates the MH1 domain of Smad2 and the linker segments of Smad1, Smad2 and Smad3 (refs 52, 53). Tyrosine kinase receptor activation and oncogenic Ras inhibit ligand-induced nuclear translocation of activated Smads[52], which could explain the impaired TGF-β response in some cells with hyperactive Ras signalling. Other studies have not found impaired nuclear translocation of Smads in Ras-transformed cells or in cells with activated MAPK signalling[51,53,54]. Furthermore, the cooperation between Ras/MAPK and TGF-β signalling in tumour cell behaviour does not seem to be compatible with the defective Smad signalling in Ras-transformed cells[55]. Other kinases may phosphorylate Erk MAPK sites, as suggested by the phosphorylation of these sites in Smad2, but not Smad1, during development[56].

Phosphorylation of Smads can also result from the activation of MAPK/Erk kinase kinase 1 (MEKK1), which acts downstream from Ras and upstream from growth-factor-induced Erk MAPK and stress-activated SAPK/JNK (c-Jun N-terminal kinase) pathways. MEKK1 activation enhances Smad2 phosphorylation, heteromerization with Smad4, nuclear translocation and transcriptional activity[57]. JNK phosphorylates Smad3 outside its C-terminal SSXS motif, and enhances TGF-β-induced nuclear translocation and transcription[54]. The induction of Erk MAPK and JNK signalling by TGF-β itself (see below) may regulate Smad activation and signalling.

Activation of Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) also results in Smad2, Smad3 and Smad4 phosphorylation, inhibits TGF-β-induced nuclear import and transcriptional

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activity of Smad2, and affects Smad heteromerization. CamKII phosphorylates Smad2 in the linker segment and the MH1 domain[58]. The phosphorylation of the MH1 domains of Smad2 and Smad3 by protein kinase C (PKC), which abrogates DNA binding of Smad3, suggests a regulatory role for PKC in Smad-mediated transcription[59]. In contrast to R-Smads, mammalian Smad4 is not regulated by phosphorylation. In *Xenopus*, Smad4β, but not Smad4α, is phosphorylated, but the role of this phosphorylation is unknown[12]. Smad6 and Smad7 are also phosphorylated independently of TGF-β stimulation. Mutation of a serine that is phosphorylated in Smad7 alters its transcriptional activity when fused to a DNA-binding domain[49].

TGF-β-induced transcriptional activation versus repression

TGF-β proteins activate transcription through physical interaction and functional cooperation of DNA-binding Smads with sequence-specific transcription factors and the coactivators CBP and p300 (Fig. 5). R-Smads (except Smad2) and Smad4 bind to preferred DNA sequences with a 100-fold lower affinity than the interacting high-affinity DNA-binding transcription factors, yet their DNA binding is required for transcriptional activation. Selective DNA binding to a subset of promoters that bind a potential Smad-interacting transcription factor defines the promoters that are activated in response to the ligand. The number of DNA-binding transcription factors with which Smads can functionally interact is impressive[1–3], and these are also often regulated by multiple signalling pathways. Besides the essential CBP or p300 coactivators, other coactivators and co-repressors that interact with Smads define the level of transcriptional activation. Smad4 itself acts as a key coactivator that enhances ligand-induced transcription by stabilizing the interaction of the R-Smads with DNA and CBP/p300. This model of transcriptional activation has been reviewed extensively elsewhere[1–3] and will not be discussed here.

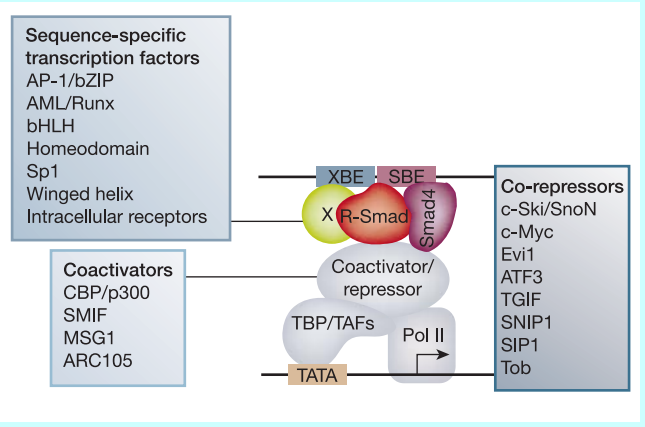

Figure 5 The R-Smad–Smad4 complex cooperates with sequence-specific transcription factors (X) that bind with high affinity to a cognate DNA sequence (XBE), yet also binds with lower affinity to a Smad-binding DNA element (SBE) to activate transcription in response to TGF-β ligand. R-Smads interact directly with the essential CBP or p300 coactivator, and Smad4 serves as coactivator for R-Smads by stabilizing the R-Smad interaction with CBP/p300. Other Smad-interacting coactivators, such as SMIF, MSG1, Swift and ARC105, further define the level of Smad-mediated transcription activation (see refs 1–3, 97, 98 and references therein). Smad-interacting co-repressors downregulate Smad-mediated transactivation. Several of these are proto-oncogenes—for example, c-Ski and the related SnoN, c-Myc[99,100] and Evi1—linking malignant transformation to repression of TGF-β/Smad-induced transcription. Other Smad co-repressors—for example, the homeodomain proteins TGIF (TGF-β-induced factor) and SNIP1 (Smad nuclear interacting protein)—repress not only TGF-β/Smad-mediated transcriptional activation, but also Smad-independent transcription. Interaction of Tob with BMP-activated Smads represses BMP-activated gene expression, whereas its interaction with Smad2 represses interleukin-2 expression in T cells.

Many genes are activated in response to TGF-β ligands, whereas others are transcriptionally repressed. Smad co-repressors that inhibit transcriptional activation by Smads (Fig. 5) have not been implicated in TGF-β-induced repression, raising the question of what defines transcriptional activation versus repression by Smads.

TGF-β inhibits cell-cycle progression by regulating the transcription of cell-cycle regulators. Among them, c-Myc and Id family members are downregulated by TGF-β[60,61]. In cells with TGF-β-induced downregulation of c-Myc expression, Smad3 represses c-Myc transcription in association with the transcription factors E2F4 and E2F5, and the co-repressor p107. This complex is pre-assembled in the cytoplasm and, in response to TGF-β treatment, translocates into the nucleus, where, in association with Smad4, it binds to a Smad–E2F-binding site in the c-Myc promoter and represses c-Myc expression[60]. In Id1 downregulation, TGF-β-activated Smad3 directly induces ATF3 expression, and ATF3 and Smad3 then form a complex that represses the Id1 promoter[61]. Similarly, SIP1, induced by TGF-β, downregulates E-cadherin expression[62].

Apart from the repression of transcription of Id members in several differentiation lineages, TGF-β also inhibits myoblast, osteoblast and adipocyte differentiation through functional repression of key transcription factors that drive these differentiation pathways. Smad3 represses transcription by Runx2/CBFA1 (core-binding factor, runt domain, α-subunit) in osteoblastic differentiation[63], MyoD and other myogenic basic helix–loop–helix (bHLH) transcription factors in myoblasts[64], and CCAAT/enhancer-binding proteins (C/EBPs) in adipocyte differentiation[65]. Smad3 represses MyoD function by physically interacting with the HLH domain of MyoD, obstructing MyoD function dimerization with E12/E47, which is required for efficient DNA binding to E-box sequences[64]. Smad3 does not interfere with DNA binding of Runx2 or C/EBPs, and represses the transcription function of C/EBP[63,65]. Whether Smads repress or activate transcription also depends on the cell type and the promoter sequence. Smad3 cooperates with Runx proteins to activate transcription in epithelial cells, and represses transcription from the same promoter in mesenchymal cells. The Runx-recognition-sequence context defines Smad-mediated activation versus repression[63]. Finally, the MH1 domain of Smad3 can interact with histone deacetylases[66]. Thus, distinct mechanisms, depending on the interacting transcription factor, the promoter and the intracellular context lead to Smad-mediated repression and determine whether Smads activate or repress transcription.

Regulation through receptor proteins

TGF-β receptor complexes are nodal points for multiprotein assemblies that regulate receptor function, routeing, and Smad and non-Smad signalling pathways. These interactions are likely to depend on the activation and subcellular localization of the receptors and vary throughout the 'life cycle' of the receptor complex.

In addition to anchoring proteins that link Smads to their receptors (Fig. 4), several WD-40 repeat proteins can associate with TGF-β receptors (Fig. 6). For example, TGF-β-receptor-interacting protein 1 (TRIP-1) interacts with the ligand-bound TGF-β type II receptor and is phosphorylated by it. Increased TRIP-1 levels repress TGF-β-induced transcription, and some mutants enhance TGF-β responses, presumably through interference with endogenous TRIP-1 (ref. 67). TRIP-1 also participates in the translation-initiation factor complex eIF3, possibly linking TRIP-1 to TGF-β-induced translational regulation. Such a linkage is also suggested for the translation factor eIF2α, which, similarly to TRIP-1, interacts with the type II receptor and downregulates the gene-expression response[68]. The regulatory Bα subunit of protein phosphatase 2A (PP2A), another WD-40 repeat protein, can also directly interact with type I TGF-β receptors, and enhances the anti-mitogenic signalling of TGF-β[69]. Upon receptor activation, PP2A-Bα binds

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to type I receptors, possibly activating PP2A-mediated signalling (see below). A third WD-40 protein, STRAP, can associate with the type I and type II TGF-β receptors, and with Smad2, Smad3 and Smad7. STRAP stabilizes the Smad7 association with the receptor and inhibits TGF-β-dependent transcription, probably by interfering with Smad2 or Smad3 binding to the receptor[70].

TGF-β receptors also interact with the immunophilin FKBP12 at a conserved Leu-Pro motif adjacent to the GS domain of unliganded type I receptors. FKBP12 decreases TGF-β signalling by inhibiting type I receptor phosphorylation by the type II receptor[71], which correlates with decreased receptor internalization and Smad phosphorylation[72]. Type I receptors defective in FKBP12 binding have elevated basal signalling, but normal signalling in response to TGF-β, and inactivation of FKBP12 expression produces a phenotype consistent with TGF-β receptor hyperactivity[73]. Thus, FKBP12 may act as a safeguard against leaky TGF-β receptor signalling. Although FKBP12 can have a role in calcineurin and NF-AT signalling, there is no evidence that TGF-β induces FKBP12-dependent signalling. Also the α-subunit of farnesyl transferase can interact with the type I receptor, but no response has been correlated with this interaction[74].

Smad-independent signalling through MAPK pathways

Besides Smad-mediated transcription, TGF-β activates other signalling cascades, including MAPK pathways (Fig. 6). Some of these pathways regulate Smad activation, as described above, but others might induce responses unrelated to transcription.

TGF-β can activate the Erk, JNK and p38 MAPK kinase pathways. Activation with slow kinetics in some cases may result from Smad-dependent transcription responses, but the rapid activation (5–15 min) in other cases suggests independence from transcription[1]. Studies using Smad4-deficient cells, or dominant-negative Smads, support the possibility of MAPK pathway activation that is independent from Smads[54]. In addition, mutated TGF-β type I receptors, defective in Smad activation, activate p38 MAPK signalling in response to TGF-β[75].

The mechanisms of Erk, JNK or p38 MAPK activation by TGF-β and its biological consequences are poorly characterized. Rapid activation of Ras by TGF-β in epithelial cells may implicate Ras in TGF-β-induced Erk MAPK signalling[76]. JNK and p38 MAPK signalling are activated by various MAPK kinase kinases (MAPKKKs) in response to many stimuli. Both TGF-β and BMP-4 can activate TGF-β-activated kinase 1 (TAK1), a MAPKKK family member[77]. Perhaps XIAP (X-linked inhibitor of apoptosis) links TGF-β/BMP receptor activation to TAK1 signalling[77]; however, a direct interaction between XIAP and type I receptors has not been demonstrated, and XIAP-deficient mice respond to TGF-β[78]. MEKK1 may also function upstream of TGF-β-mediated activation of MAPKKs; thus, MEKK1 and TAK1 could activate JNK through MAPK kinase 4 (MKK4), and p38 MAPK through MKK3 or MKK6, in response to TGF-β. Because TAK1 can phosphorylate and activate IκB kinase, thus stimulating NF-κB signalling, TGF-β/BMP signalling may induce NF-κB signalling. Further characterization of this web of interactions will provide insight into the activation of MAPK pathways by TGF-β ligands.

TGF-β-induced activation of the Erk and JNK pathways can result in Smad phosphorylation and regulate Smad activation[51–54]. Also, TGF-β-induced activation of Ras/Erk MAPK signalling can induce TGF-β1 expression, thereby amplifying the TGF-β response and inducing secondary TGF-β responses[76]. Activation of MAPK pathways by TGF-β may also affect transcription responses through direct effects on Smad-interacting transcription factors—for example, the JNK substrate c-Jun or the p38 MAPK substrate ATF-2 (activating transcription factor 2)—allowing convergence of TGF-β-induced Smad and MAPK pathways[1–3]. The dual ability of TGF-β to activate Smads and MAPK signalling has a role in TGF-β-induced epithelial-to-mesenchymal transdifferentiation, which depends in part on the Erk and/or p38 MAPK pathways[75,79,80]. Although this convergence often results in cooperativity, these pathways may also counteract each other. For example, Smad6 can bind to TAK1 and downregulate its activity[81], whereas Smad7 can enhance and sustain JNK activation[82]. Also, c-Jun inhibits Smad2 signalling through association with Smad co-repressors, and this interaction is regulated by JNK signalling[83]. Thus, the balance between direct activation of Smads and MAPK pathways often defines cellular responses to TGF-β.

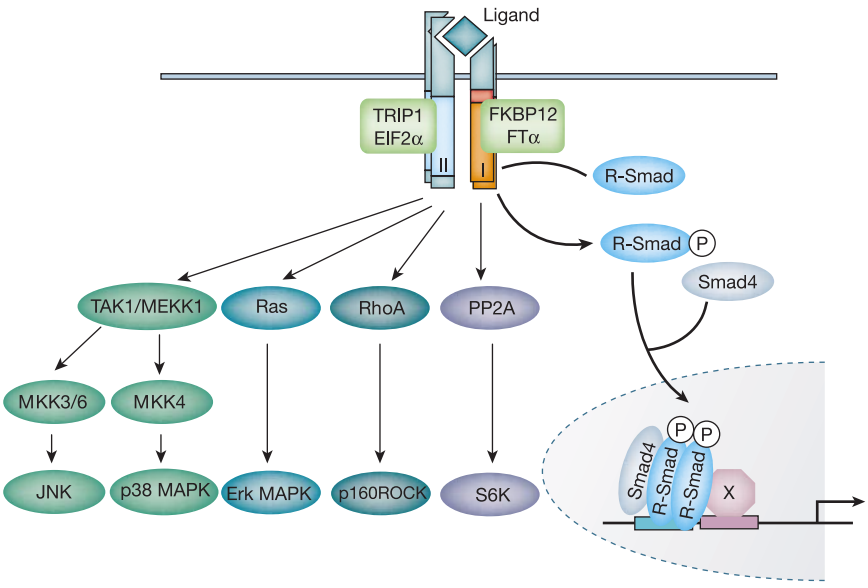

Figure 6 TGF-β receptor signalling through Smad-independent pathways. Apart from proteins that interact with receptors and Smads (see Fig. 4), other proteins (green) can associate with the type II or type I receptors and regulate TGF-β ligand signalling without an apparent direct effect on Smad activation. In addition, the activated receptor complex activates non-Smad signalling pathways, such as MAPK, PP2A/p70^{S6K}, RhoA and TAK1/MEKK1. Only the best-characterized pathways are shown.

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Other TGF-β-induced signalling pathways

Depending on the cell line, TGF-β can rapidly activate Rho-like GTPases (Fig. 6), including RhoA, Rac and Cdc42, although delayed activation of RhoA and Cdc42, because of new protein synthesis, has also been observed[79,84,85]. TGF-β also enhances the expression of RhoB[86], possibly by inhibiting its proteasomal degradation, and induces the expression of NET1, a RhoA-specific guanine exchange factor that mediates RhoA activation[87]. Finally, Ras activation in response to TGF-β may also lead to activation of a Rho-like GTPase.

Rac and Cdc42 regulate JNK and p38 MAPK pathway activation, presumably by directly interacting with MAPKKKs upstream of JNK and p38 MAPK, whereas Rho, Rac and Cdc42 affect the cytoskeletal organization[88]. Activation of small GTPases by TGF-β may play a part in gene-expression responses. RhoB counteracts[86], whereas Rac1 contributes to[79], TGF-β-induced gene expression, and Rho-dependent activation of JNK signalling contributes to Smad activation[54]. These GTPases also mediate TGF-β-induced changes in cytoskeletal organization and epithelial-to-mesenchymal transdifferentiation. Activation of Rac1, RhoA and p38 MAPK, an effector of Cdc42, is required for rapid membrane ruffling and lamellipodia formation in response to TGF-β[85]. Activation of RhoA and its effector kinase $p160^{ROCK}$, as well as Cdc24, p38 MAPK and Smad signalling have been implicated in TGF-β-induced stress-fibre formation and epithelial-to-mesenchymal transdifferentiation[75,79,84,85,87].

TGF-β can also activate phosphatidylinositol-3-kinase (PI3K), as indicated by phosphorylation of its effector Akt[89,90]. This activation can be direct, with possible involvement of RhoA[89], but can also result from TGF-β-induced TGF-α expression and consequent EGF receptor activation[90]. Chemical inhibition of PI3K activity reduces TGF-β-induced Smad2 phosphorylation and transcription, and epithelial-to-mesenchymal transdifferentiation[89]; however, inhibition of PI3K at lower inhibitor concentrations did not affect TGF-β-induced epithelial-to-mesenchymal differentiation[7,91]. The role of PI3K in differentiation remains to be clarified.

TGF-β may also signal through PP2A, which consists of a catalytic C subunit and a regulatory A subunit, with which a regulatory B subunit can interact. Upon TGF-β binding, the Bα subunit of PP2A associates with the activated type I receptor[69]. Because Bα inhibits the activity of the A/C dimer, this recruitment is expected to enhance PP2A activity. Upon TGF-β stimulation, the A and C subunits, as well as Bα, interact with $p70^{S6K}$, a kinase with a key role in translational control and cell-cycle progression[92]. The resulting dephosphorylation and decreased activity of $p70^{S6K}$ is thought to contribute to TGF-β-induced growth arrest independently of Smads[92], suggesting a role for PP2A in the response to TGF-β. Protein phosphatase 1 (PP1) has also been implicated in TGF-β signalling. Its catalytic subunit (PP1c) interacts with *Drosophila* SARA, and negatively regulates Dpp signalling by affecting type I receptor phosphorylation[93].

Finally, TGF-β signalling also regulates protein stability. Most notably, TGF-β increases the degradation of the Smad co-repressor SnoN[24,94,95] and the TβRI receptor[36,37]. TGF-β-induced degradation of SnoN involves Smad2 or Smad3 as docking proteins to target it for proteasomal degradation. Thus, Smad2/3 interact with SnoN and Smurf2 or the anaphase-promoting complex, which then serve as E3 ubiquitin ligases for SnoN[19,94,95]. Similarly, in response to TGF-β, Smad7 interacts with Smurf1 or Smurf2, and interaction of this complex with TβRI leads to receptor ubiquitination and degradation[36,37]. It remains to be determined whether other Smad-interacting proteins are subject to TGF-β-induced degradation, and whether ubiquitin-mediated degradation contributes to the cellular response to TGF-β family signalling.

Future perspectives

Extensive progress has provided insight into the complex regulation and roles of Smads in transcription responses, and the effects of signalling crosstalk on Smad function. The demonstration of Smad-independent TGF-β signalling pathways and the regulation of TGF-β signalling by receptor-associated proteins illustrate that our understanding now transcends the linear model of Smad signalling as an effector of TGF-β signalling. Incorporation of genomic and proteomic approaches, combined with RNA interference technology and mouse genetic manipulation, will advance our understanding of the plasticity of the cellular response to TGF-β, and the roles of TGF-β signalling components in coordinating signalling events and maintaining cell and tissue homeostasis.

Note added in proof: A recent study[101] demonstrated direct interaction of the cytoplasmic domain of BMP-RII with the cytoskeletal regulator LIM kinase 1 as well as BMP-induced regulation of the kinase activity of LIM kinase 1. These data provide evidence for direct signalling by a type II receptor, through LIM kinase 1, to the cytoskeleton. □

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