Protein family review

# The Smads

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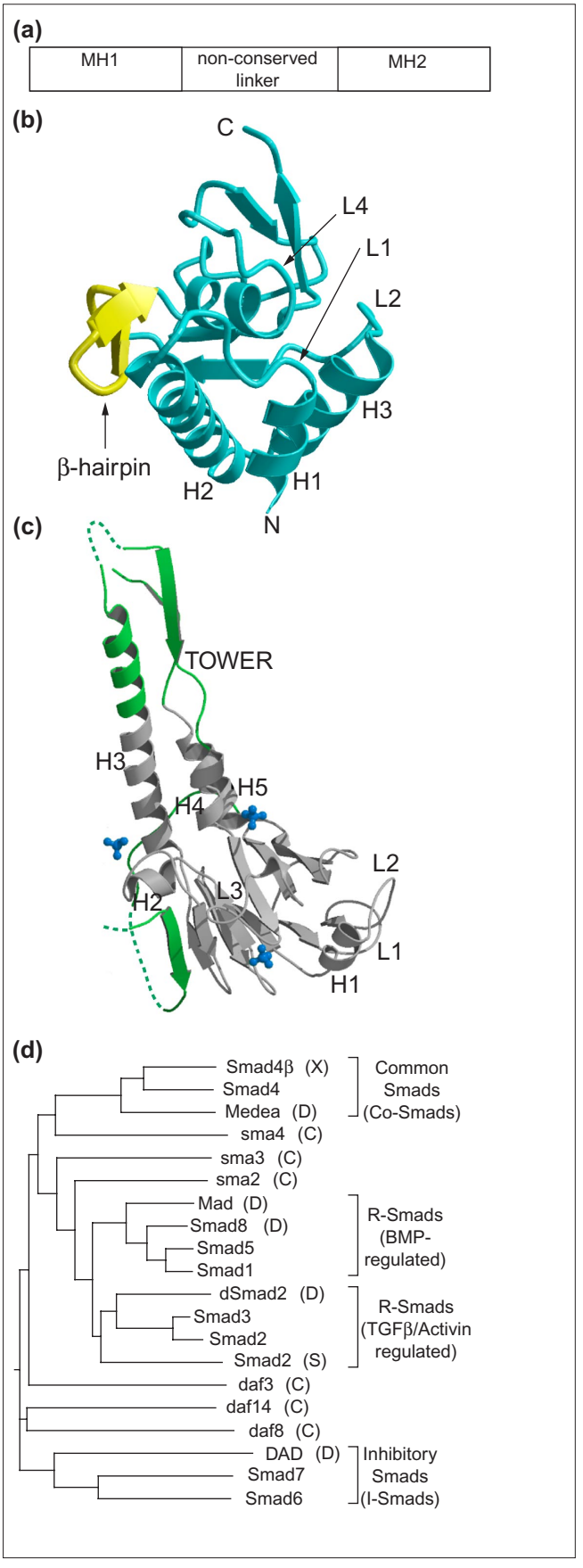
## Summary

The large transforming growth factor-β (TGFβ) superfamily of secreted proteins regulate the growth, development and differentiation of cells in diverse organisms, including nematode worms, flies, mice and humans. Signals are initiated upon binding of TGFβ superfamily members to cell-surface serine/threonine kinase receptors and are then propagated by the intracellular mediators known as Smads. Activation of Smads results in their translocation from the cytoplasm into the nucleus, where they activate or repress transcription together with transcription factors so as to regulate target gene expression. Most Smads consist of two conserved domains, Mad homology (MH) domains 1 and 2, which are separated by a non-conserved linker region. These domains lack enzymatic activity and, instead, Smads mediate their effects through protein-protein and protein-DNA interactions. Targeted disruption of Smad genes in mice has revealed their importance in embryonic development, and a tumor-suppressor role for Smads in human cancers has been described. Smads therefore play an essential role in mediating TGFβ-superfamily signals in development and disease.

## Gene organization and evolutionary history

The Smads are a group of related intracellular proteins critical for transmitting to the nucleus signals from the transforming growth factor-β (TGFβ) superfamily at the cell surface (reviewed in [1-10]). Although related to each other, Smads are structurally distinct from other intracellular effector proteins. The prototypic members of the Smad family, Mad and Sma, were first described in *Drosophila* and *Caenorhabditis elegans*, respectively [6,8]. Related proteins in *Xenopus*, humans, mice and rats were subsequently identified, and all family members are now known as Smads, a contraction of the invertebrate gene names. More recently, related proteins have also been described in zebrafish and the helminth parasite *Schistosoma mansoni*. Functional studies have demonstrated that Smads, which range from about 400 to 500 amino acids in length, can be grouped into three subfamilies, the receptor-regulated Smads (R-Smads), the common Smads (co-Smads) and the inhibitory Smads (I-Smads), each of which plays a distinct role in the pathway (Figure 1). Representative members for all three subfamilies have been identified in most animal species, and the highest degree of sequence conservation is observed among the members of each subfamily. For instance, *Drosophila* Mad and human Smad1, members of the R-Smad subgroup, are 82% identical in amino-acid sequence. Across subfamilies, the highest degree of conservation is observed in the carboxy-terminal Mad homology 2 (MH2) domain, with amino acid sequence identities ranging from 32% to 97% in the human Smads.

There are eight Smad family members in mammals, and a search of human genome database suggests that this represents the full complement. The eight human *Smad* genes have been mapped to four chromosomes (Table 1). Three of the *Smad* genes - *Smad2*, *Smad4* and *Smad7* - are closely clustered at 18q21.1, a region that is frequently deleted in human cancers. Three are found on chromosome 15, with *Smad3* and *Smad6* mapping to 15q21-22 and *Smad5* to 15q31. The remaining *Smad* genes, *Smad1* and *Smad8,* are located on chromosomes 4 and 13, respectively. The




intron-exon boundaries for most of the *Smad* genes have been determined in either the mouse or human genome, and in all cases the genes consist of 6-12 exons. Alternatively spliced mRNA species for Smads 2, 4, 5, 6 and 8 have also been described [11].

## Characteristic structural features

Most Smads have two conserved domains at their amino (MH1) and carboxyl (MH2) termini that are separated by a proline-rich linker region of varying length (Figure 1a-c). In the inhibitory Smads, Smad6 and Smad7 and DAD, the MH1 domains are replaced by divergent amino-termini that share regions of similarity within the inhibitory Smad subgroup. Although the structure of a full-length Smad has not yet been determined, crystallographic analysis of individual Smad domains has provided insights into the important structural features of the MH1 and MH2 domains (Figure 1b,c).

### The MH1 domain

Certain R- and co-Smads have DNA-binding activity, binding a core DNA consensus sequence of GNCN. Although this interaction is of relatively low specificity, DNA binding has been shown to be vital for the transcriptional activation of specific target genes. The crystal structure of the MH1 domain of Smad3 bound to an 8 base-pair Smad-binding element (GTCTGTCT) demonstrates that the MH1 domain forms a compact globular fold that uses a highly conserved 11-residue β hairpin to contact DNA in the major groove (Figure 1b [9,12,13]). In Smad2, a 30 amino-acid insertion encoded by exon 3 is thought to displace the β-hairpin loop, providing a structural explanation for Smad2's lack of DNA-binding activity.

Smad-dependent activation of target promoters depends upon translocation of R-Smad-co-Smad complexes from the

**Figure 1**
Primary structure and relationships of Smads. **(a)** The conserved Mad homology 1 (MH1) and Mad homology 2 (MH2) domains are separated by a proline-rich non-conserved linker region. **(b)** Structure of the Smad3 MH1 domain (reproduced with permission from [13]). H2, helix H2; β, β hairpin, which contacts DNA. **(c)** Structure of the Smad4 MH2 and Smad activation domains (reproduced with permission from [26]). H1-H5, helices; L1-L3, loops. New features not present in the first Smad4 MH2 crystal structure [17] are colored green. The location of bound sulfate ions from the crystallization medium are in blue. **(d)** Relationship dendrogram for the Smad family, including members from *Drosophila* (D), *C. elegans* (C), *S. mansoni* (S) and human (the remainder). The subgrouping of Smads into the common Smads (co-Smads), receptor-regulated (R-Smads) and inhibitory (I-Smads) is indicated. *C. elegans* Smads, which have not been subject to extensive biochemical characterization, have been excluded from this subgrouping. This dendrogram was generated using the MacVector program.

**Table 1**

**Chromosomal localization of human Smads**

| Name | Previous names | Chromosomal localization | Gene name | OMIM ID | Unigene accession number |
|---|---|---|---|---|---|
| TGFβ/activin-regulated R-Smads | | | | | |
| Smad2 | MADR2, JV18-1 | 18q21.1 | MADH2 | 601366 | NM005901 |
| Smad3 | JV15-2 | 15q21-q22 | MADH3 | 603109 | NM005902 |
| BMP-regulated R-Smads | | | | | |
| Smad 1 | MADR1, JV4-1, Dwarfin A, bsp-1 | 4q28 | MADH1 | 601595 | NM005900 |
| Smad 5 | DwarfinC, JV5-1 | 15q31 | MADH5 | 603110 | NM005903 |
| Smad 8 | Smad9, MADH6 | 13q12-q14 | MADH9 | 603295 | NM005905 |
| Common Smad | | | | | |
| Smad 4 | DPC4 | 18q21.1 | MADH4 | 600993 | NM005359 |
| Inhibitory Smads | | | | | |
| Smad 6 | JV15-1 | 15q21-q22 | MADH6 | 602931 | AF035528 |
| Smad 7 | | 18q21.1 | MADH7 | 602932 | NM005904 |

Further information for each Smad is available by searching under the Gene Name in the Gene Card database [11] and using the OMIM ID number at the Online Mendelian Inheritance in Man database [32].

cytoplasm to the nucleus. R-Smads enter the nucleus after receptor-mediated phosphorylation, whereas Smad4 requires association with an R-Smad partner for nuclear accumulation. A basic helix (H2) in the MH1 domain, consisting of a typical nuclear localization signal (KKLKK), has been shown to be essential for Smad3 nuclear import (Figure 1b [14,15]). Translocation of Smad3 into the nucleus requires interaction with importin β, and it is interesting to note that the presence of the insertion encoded by exon 3 in the MH1 domain of Smad2 prevents its interaction with importin β [15]. This result and other data [16] suggest that other determinants, such as the MH2 domain, may also be involved in regulating nuclear accumulation of R-Smads. The interaction of Smads with several transcription factors, including Jun, TFE3, Sp1 and Runx, also occurs through the MH1 domain; detailed analysis of the determinants for these types of interactions have not yet been conducted, however.

## The MH2 domain

The MH2 domain does not bind DNA and instead is a multifunctional region that mediates differential association with a wide variety of proteins. Many of these interactions serve to provide specificity and selectivity to Smad function. The first crystal structure of a Smad MH2 domain to be solved was that of the co-Smad Smad4 [9,12,17]. This study revealed that the MH2 domain is composed of five α helices (H1 to H5) and three loops (L1, L2 and L3) that enclose a β sandwich (Figure 1c [17]). Smads exist as monomers and trimers, and although there is some controversy as to the precise composition, stoichiometry and formation of the oligomers, it is clear that the MH2 domain is critical for mediating interactions in the oligomers. Analysis of the trimeric Smad4 crystal demonstrated that the loop-helix region (L1, L2, L3 and H1) of one subunit makes extensive contacts with the three-helix bundle (H3, H4 and H5) of another subunit and that many conserved residues in Smads are located within the trimer interface [17].

Propagation of TGFβ signals is mediated by the direct association of R-Smads with the TGFβ receptor complex. The R-Smads are then directly phosphorylated by the type I TGFβ receptor kinase on the last two serines of a conserved SSXS motif located at the extreme carboxyl terminus of the MH2 domain. Biochemical analyses have shown that specific Smad-receptor interactions are mediated by the L3 loop in the R-Smads and the L45 loop in the type I receptor. Further insights into this interaction were provided by the structure of the Smad2 MH2 domain crystallized in complex with the Smad-binding domain (SBD) of SARA (Smad anchor for receptor activation), a protein that functions to recruit Smads to the TGFβ receptor [9,12,18]. The overall topology of the R-Smad MH2 domain is similar to that of Smad4, but the R-Smads also have a basic pocket on one surface that lies adjacent to loop 3 [18]. As Smad4 does not interact with receptors, it is thought that this basic pocket may serve as a docking site for the phosphorylated and activated type I receptor. The crystal structure of the Smad2 MH2 domain in complex with the SARA SBD also revealed an unusual arrangement in which the 40-residue SBD is in an extended conformation that forms a proline-rich coil, an α helix and a β strand that contacts the three helix bundle (H3, H4 and H5) and a β strand of the Smad2 MH2 domain. It is thought that the interaction of SARA with the β sheet is required for specificity, whereas contact with the three-helix bundle contributes to the binding affinity. It is currently not clear whether other proteins that interact with the Smad MH2 domain might also adopt a similar interaction interface; functional studies have indicated that α-helix 2 of the MH2 domain is important for the interaction of Smad2 with the transcription factor FAST (FoxH1) [19].

### The Smad linker region

The linker region that connects the MH1 and MH2 domains contains a number of important peptide motifs. These include potential sites for phosphorylation by mitogen-activated protein kinases (MAPKs) - this phosphorylation can block R-Smad function [1-5] - and a nuclear export signal located within exon 3 of the co-Smad Smad4 [15,20,21]. R-Smads and I-Smads also contain a conserved proline-tyrosine (PY) motif that mediates interaction with the WW domains in the Smad-interacting proteins Smurf1 and Smurf2. Smurfs are E3 ubiquitin ligases of the C2-WW-HECT domain class that catalyze ubiquitin-mediated degradation of certain Smads and Smad-associated proteins, including the nuclear oncoprotein SnoN and the TGFβ-receptor complex [22-25]. The linker region of Smad4 also contains a Smad activation domain (or SAD) that is required for transcriptional activation. A crystal structure of a fragment of Smad4 that includes the SAD and MH2 domain revealed that the SAD contacts a Smad4-specific sequence in the MH2 domain [9,12,26]. This stabilizes a glutamine-rich α-helical extension termed the TOWER, which, together with the proline-rich SAD, may form a transcriptional activation surface [26].

## Localization and function

### Developmental expression patterns

In general, all Smads are widely expressed throughout embryonic development and in most adult tissue and cell types. Analysis of mouse embryos has revealed, however, that there is some variation in the pattern, timing and level of expression of the individual Smads [27,28]. For instance, the inhibitory Smads, Smad6 and Smad7, are highly expressed in the developing cardiovascular system, although each also displays distinct expression patterns in non-cardiovascular tissues, including intramembranous bone and testis. The co-Smad Smad4 is ubiquitously expressed throughout embryonic development, with particularly high levels being detected in the epithelial crypts of the gut. Interestingly, the R-Smads display overlapping expression patterns; at least one of the BMP-regulated Smads (Smad1, Smad5 and Smad8) and one TGFβ/activin-regulated Smad (Smad2 or Smad3) is expressed in every tissue [27,28].

### Function

Members of the TGFβ superfamily signal by inducing the stable assembly of heteromeric complexes of transmembrane type I and type II serine/threonine-kinase receptors. Within this complex the type II receptor kinase phosphorylates the type I receptor, which subsequently initiates downstream signaling to the Smad pathway. Smads then propagate the TGFβ signal from the cell surface into the nucleus [1-5].

As mentioned above, three functional classes of Smads have been defined, each of which plays a distinct role in the signaling pathway (Figure 2). The activated type I receptors associate with specific R-Smads and phosphorylate them on the last two serines of a conserved carboxy-terminal SSXS motif. The recognition of different R-Smads by the various type I receptor kinases is highly specific. Thus, the TGFβ and activin type I receptors, TβRI (ALK5) and ActRIB (ALK4), respectively, activate both Smad2 and Smad3, which are closely related, whereas ALK1 and the BMP type I receptors ALK2, ALK3 and ALK6 all target Smads 1, 5 and 8. Phosphorylated R-Smads then dissociate from the receptor and form a heteromeric complex with the co-Smad, Smad4. Although in mammals there is only one co-Smad, in *Xenopus* a second co-Smad, Smad4β, has been identified.

The R-Smad-co-Smad heteromeric complex then translocates to the nucleus to modulate the activity of specific promoters. Although Smads can directly bind DNA with low affinity and low specificity, they rely on interactions with various DNA-binding partners to target specific genes for transcriptional regulation. For instance, the TGFβ/activin-regulated Smads, Smad2 and Smad3, directly associate with DNA-binding partners such as FoxH1 (FAST), AP-1, TFE3, Sp1, Mixer, Runx2, LEF1/TCF and Miz1. Much less is known about the binding partners of BMP-regulated Smads, but identified nuclear partners include OAZ, Runx2 and Hoxc-8/9. Once localized to appropriate target promoters, Smads can then positively or negatively regulate transcriptional activity by recruiting coactivators, such as CBP/p300, or corepressors, including TGIF and Ski/Sno, which bind deacetylases.

Unlike the R-Smads, the I-Smads, Smad6 and Smad7, are potent antagonists of TGFβ signaling pathways. I-Smads, which do not have a carboxy-terminal SSXS motif, function by stably binding to activated receptor complexes, thus blocking access to and phosphorylation of the respective R-Smads by the type I receptor kinase. In addition, Smad7 can concomitantly induce ubiquitin-mediated degradation of active receptor complexes through its ability to recruit Smurfs, members of the C2-WW-HECT domain E3 ligase family [24,25]. Unlike the R- and co-Smads, which translocate from the cytoplasm to nucleus upon activation of the signaling pathways, Smad7 resides in the nucleus, and ligand stimulation results in its export into the cytoplasm where it can bind to receptors to manifest its inhibitory effects. In addition to TGFβ-independent signals, expression of *I-Smad* genes is stimulated by TGFβ and BMPs, thereby providing for negative feedback of the pathway.

### Important mutants

Currently, five of the eight *Smad* genes have been disrupted by homologous recombination in mice (Table 2 and reviewed in [29]). Consistent with their observed expression in early embryos, mice lacking *Smad2*, *Smad4*, or *Smad5* all have an early embryonic lethal phenotype. In the case of *Smad2* and *Smad4*, null mutant mice have defects in mesoderm induction and anterior-posterior axis formation, whereas *Smad5* null mice die later, between embryonic days 9 and 11.5, and display defects in angiogenesis. In contrast,

**Table 2**

**Phenotypes of Smad-deficient mice**

| Protein removed | Viability | Phenotype | References |
|---|---|---|---|
| Smad2 | Embryonic lethal (before E8.5) | Defects in mesoderm induction, anterior-posterior and left-right patterning, extra-embryonic tissues and endoderm formation | [33-37] |
| Smad3 | Viable | Variable phenotypes including defects in T-cell and splenocyte responsiveness; metastatic colon cancer, accelerated wound healing, and degenerative joint disease | [38-42] |
| Smad4 | Embryonic lethal (E6.5-8.5) | Defects in gastrulation and anterior development, epiblast proliferation and egg cylinder formation. Heterozygotes have intestinal tumors | [43-46] |
| Smad5 | Embryonic lethal (E9.5-11.5) | Defects in angiogenesis, vasculogenesis, left-right axis determination, and primordial germ cell development | [47-50] |
| Smad6 | Viable | Multiple cardiovascular abnormalities: defects in cardiac valves and outflow tract septation; aortic ossification, and elevated blood pressure | [51] |

Smad3-deficient mice are viable. Several groups have independently targeted the *Smad3* gene, and each reports distinct phenotypes, including defects in T-cell or splenocyte responsiveness, presence of colorectal cancers and the development of a degenerative joint disease resembling osteoarthritis. Mice lacking the inhibitory Smad Smad6 have cardiovascular abnormalities, including hyperplasia of the cardiac valves and outflow tract septation defects, suggesting that Smad7 cannot substitute for Smad6 even though both are highly expressed in the cardiovascular system. The phenotypes of mice lacking Smads 1, 7 and 8 remain to be determined. It is interesting to note that mice heterozygous for *Smad2*, *Smad3* or *Smad4* also display varying defects, indicating that *Smad* gene dosage is important [29].

TGFβ is a potent inhibitor of cellular proliferation of many normal cell types, suggesting that loss of TGFβ responsiveness may be an important step during tumor progression. Consistent with this, mutations in *Smads* have been implicated in a number of human cancers (reviewed in [2,7,30]. *Smad4* was originally identified as a potential tumor-suppressor gene in pancreatic carcinoma, and mutations in colorectal, lung and pancreatic tumors have also been reported. *Smad4* is also mutated in families with familial juvenile polyposis, an inherited syndrome associated with an increased risk of gastrointestinal cancer. Consistent with these observations, *Smad4* heterozygote mice develop intestinal polyposis and invasive carcinomas. *Smad2* has also been shown to harbor mutations in colorectal and lung tumors. Thus, Smads that mediate TGFβ signals appear to represent a class of tumor-suppressor genes important in human cancer. To date, there is no evidence that inactivating mutations in the other Smads are associated with cancers or other human diseases.

## Frontiers

Since the first genetic description of Smads in 1995 and the initial biochemical characterizations of the proteins in 1996, our understanding of how Smads function to mediate TGFβ signaling has grown considerably. The availability of complete human and *Drosophila* genome sequences has confirmed that the full complement of Smads is now known. In addition, structural, biochemical and cell-biological approaches have culminated in the development of a model that provides a molecular description of how Smads transmit TGFβ superfamily signals (Figure 2). With this basic framework in hand, current research efforts are directed towards reaching a more detailed mechanistic understanding of the signaling process. An area of particular interest is how localization of Smads and their association with other proteins is controlled. The phosphorylation of Smads by the TGFβ receptor complex is essential for initiating the signaling cascade, and recruitment of R-Smads to the receptor is thought to be facilitated by SARA, but the subcellular compartment in which these events occur, and even whether there is a SARA-like protein for the BMP-regulated Smads, is not known. Insights into what determines the subcellular localization and nuclear accumulation of Smads will also be invaluable for enhancing our understanding of how their nuclear activities are manifested. Recent evidence has shown that Smads associate with E3 ubiquitin-ligases, are themselves ubiquitinated and degraded, and can serve as adapters to mediate ubiquitin-mediated degradation of other proteins [22-25,31]. Thus, it will be important to understand how a cell maintains the delicate balance between Smad and ubiquitin-ligase protein levels to ensure appropriate responsiveness to TGFβ-superfamily signals. Although Smads are known to function in the nucleus as transcriptional regulators, little is understood of what determines whether Smads positively or negatively regulate transcription. Furthermore, very few DNA-binding partners for the BMP-regulated Smads are known.

Cells receive multiple simultaneous signals, and the interaction of the TGFβ pathway components with effectors of other signaling pathways has been described. Thus, future efforts will also focus on developing a better understanding of how

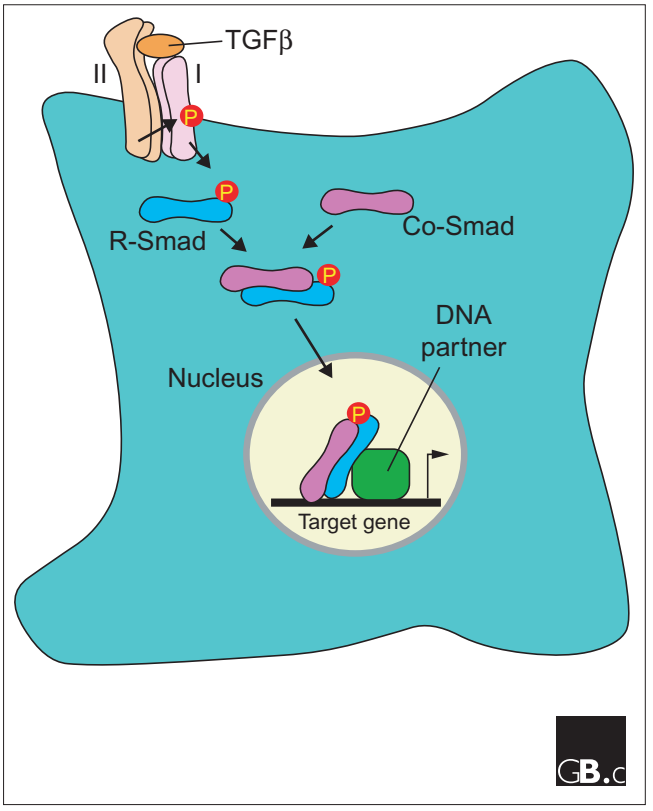



**Figure 2**
The TGFβ signal-transduction pathway. Ligand binding induces activation of TGFβ receptor complexes, which then directly phosphorylate (P) specific R-Smads. These R-Smads associate with the common (co) Smad Smad4 and then translocate to the nucleus, where they interact with a variety of DNA-binding partners to regulate gene expression. See text for further details.

and whether Smads cross-talk with other signaling pathways. Current research efforts, including the search for novel Smad-interacting proteins, will undoubtedly shed light on these questions and may reveal new insights that challenge existing paradigms.

TGFβ superfamily members play critical roles in numerous developmental events from cell-fate determination to organogenesis, and there is great interest in understanding these events. Examination of the effects of gene disruption in mice has revealed important information on the role Smads play in the earliest events. With the exception of Smad3 and Smad6, however, mice deficient in Smads die early in embryonic life; future work directed towards understanding their role in later development will thus require the generation of conditional alleles. In addition, the phenotype of mice lacking Smad1, Smad7 or Smad8 is eagerly awaited. These studies in mice will be bolstered by the analysis of Smad function in several other genetically manipulatable model systems, including *C. elegans*, *Drosophila* and zebrafish.

TGFβ signaling has been implicated in a wide variety of human disorders, including fibrosis, hypertension, osteoporosis, atherosclerosis and cancer, making this pathway an excellent target for therapeutic intervention [2,7,30]. Furthermore, mutations in components of the TGFβ signaling pathway have been associated with a number of hereditary diseases including persistent Müllerian duct syndrome, hereditary hemorrhagic telangiactasia, hereditary chondrodysplasia, familial primary pulmonary hypertension and hereditary non-polyposis colorectal cancer [2,30]. Of the Smads, only Smad4 has been shown to be associated with a hereditary disease, namely juvenile polyposis syndrome. Thus, it will be important to determine whether other hereditary syndromes can be attributed to mutations in Smads. In addition, the pathological implications of Smad hemizygosity or Smad dysfunction in other diseases, including cancer, is a worthy undertaking, as this may provide a target for the development of novel clinical treatments.

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