
TGF β and the Smad Signal Transduction Pathway

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Abstract

Transforming Growth Factor-beta (TGF β) superfamily members are important regulators of many diverse developmental and homeostatic processes and disruption of their activity has been implicated in a variety of human diseases ranging from cancer to chondrodysplasias and pulmonary hypertension. TGF β family members signal through transmembrane ser/thr kinase receptors that directly regulate the intracellular Smad pathway. Smads are a unique family of signal transduction molecules that can transmit signals directly from the cell surface receptors to the nucleus, where they regulate transcription by interacting with DNA binding partners as well as transcriptional coactivators and corepressors. In addition, more recent evidence indicates that Smads can also function both as substrates and adaptors for ubiquitin protein ligases, which mediated the targeted destruction of intracellular proteins. Smads have thus emerged as multifunctional transmitters of TGF β family signals that play critical roles in the development and homeostasis of metazoans.

Introduction

Transforming Growth Factor β (TGF β) is the canonical member of a large family of polypeptide growth factors. Currently there are well over 50 evolutionarily conserved superfamily members that are found in all metazoan organisms studied. Based on similarity of sequence and function, superfamily members have historically been grouped into families which include: TGF β s; activins and inhibins; Bone Morphogenetic Proteins (BMPs) and Growth and Differentiation Factors (GDFs); and more distantly related molecules such as M \ddot{a} llerian

Inhibitory Substance (MIS) and Glial cell line-Derived Neurotropic Factor (GDNF) (Kingsley, 1994). However as a number of TGF β superfamily members have properties that span these ancestral classifications, it is likely that superfamily ligands actually belong to a continuous spectrum of related factors rather than specific families.

TGF β superfamily signals are utilized at different times and are required in specific tissues throughout development and adulthood. The label Transforming Growth Factor (TGF) was first applied to peptides that, when present in growth medium, conferred a malignant or transformed phenotype on untransformed rat kidney fibroblasts in vitro (Assoian et al., 1984; Roberts and Spom, 1985). Despite these early observations, TGF is somewhat of a misnomer as subsequent work showed it to be a multifunctional factor that regulates an array of biological processes. For example, TGF β can be mitogenic for fibroblasts, whereas it inhibits in vitro proliferation of epithelial and endothelial cells (Moses et al., 1987). During tumorigenesis and wound repair, TGF β chemoattracts and modulates the activity of blood cells (Postlethwaite et al., 1987; Tsunawaki et al., 1988). In addition, TGF β is involved in the initiation of a cascade of events that lead to neovascularization and matrix synthesis. TGF β also exerts control over proliferation and differentiation of a variety of cell types. Targeted disruption of the mouse TGF β 1 gene has profound effects on the development of the immune system and the heart (Letterio et al., 1994; Shull et al., 1992). The broad range of TGF β effects thus make it a central protein during embryogenesis and development (Roberts et al., 1990; Roberts and Spom, 1987).

Other members of the TGF β superfamily are also involved in numerous biological processes. Activins and inhibins are gonad-secreted factors (Lee et al., 1989) that were first described as crucial

regulators of certain endocrine functions including secretion of pituitary hormone (Ling et al., 1986). Bone Morphogenetic Proteins (BMPs), as their name suggests, were originally identified as a group of proteins that cause de novo bone formation in muscle tissue. In addition to these functions, TGF β superfamily ligands control a host of early developmental decisions and mouse knockouts of these factors have provide fundamental insights into ligand functions revealing that TGF β superfamily signalling plays important roles in almost all homeostatic and developmental processes examined (reviewed in Kluppel et al., 1999).

Structural Properties of Mature TGF β Superfamily Ligands

TGF β superfamily members are dimeric molecules that share a conserved structure. Crystallographic analysis reveals that TGF β 2 is comprised of two monomers and each monomer consists of two antiparallel pairs of β -strands that form a flat surface and a separate α -helix (Schlunegger and Grutter, 1992). The α -helix of one subunit interacts with the flat surface of the other subunit to form active dimer. Each monomer contains four intrachain disulfide bonds and one interchain disulfide bond. Based on amino acid sequence conservation, this structure is predicted to be conserved in TGF β 1 through TGF β 5 (Daopin et al., 1992). Two intrachain disulfide bonds form a ring that is threaded by a third intrachain disulfide bond and this arrangement is known as the cystine-knot. Other TGF β superfamily members, including activin, inhibin and BMP7, also possess a conserved arrangement of six cysteines that likely form this cystine-knot conformation (Griffith et al, 1996). Thus, while amino acid sequences between TGF β superfamily ligands vary, dimerization and the cystine-knot are common features of these factors.

Strikingly, the cystine-knot is found in the peptide sequences of a number of growth factors. These factors, that include Platelet-Derived Growth factor (PDGF) and glycoprotein hormone, share no other sequence homology to TGF β and together they define the cystine-knot growth-factor superfamily (Sun and Davies, 1995).

Interestingly, phylogenetic analysis reveals an evolutionary link between these factors and extracellular matrix proteins that also form cystine-knots (Vitt et al., 2001). Cystine-knot containing structures are not found in unicellular yeasts and this observation suggests that the cystine-knot may have evolved with the advent of multicellularity and the need for intracellular communication.

While most TGF β superfamily ligands consist of homodimers, examples of functional heterodimers also exist. For instance TGF β 1.2, a heterodimer of TGF β 1 and TGF β 2, has been identified in vivo and appears to display activity and receptor binding properties intermediate to those of TGF β 1 and TGF β 2 (Cheifetz et al., 1988b). Furthermore BMP4/BMP7 heteromers act as mesoderm inducers in frog embryos and do so at increased potency relative to either homomer (Suzuki et al., 1997). Consistent with BMP4/BMP7 action in vivo, synthetic BMP2/BMP7 heterodimers possess 20-fold the activity of either homodimer in *Xenopus* mesoderm induction assays (Israel et al., 1996). In contrast to enhancing activity, BMP7 can form heterodimers with Nodal and this Nodal/BMP7 heteromeric complex is inhibitory for both Nodal and BMP7 signalling (Yeo and Whitman, 2001). Such a strategy of mixing ligand monomers to achieve novel activity or varying efficacy may help to explain the wide range of TGF β superfamily effects.

Regulation of TGF β Superfamily Ligands

Members of the TGF β superfamily are synthesized as large precursors that are subsequently cleaved to generate mature ligands. TGF β superfamily ligands are initially synthesized as 100 kDa pro-proteins that consist of an amino-terminal pro-region and a carboxy-terminal mature region (Gentry et al., 1988). The pro-region facilitates proper dimerization of these pro-proteins and these dimers are subsequently cleaved by endoproteases at a conserved RXXR amino acid sequence located just upstream of mature TGF β superfamily peptide sequence. Cleavage is thought to be mediated by furins which are pro-protein convertases that process latent precursor proteins into their biologically active forms (Matthews et al., 1994). For TGF β 1, the cleaved

pro-region, known as the Latency-Associated Peptide (LAP), has been shown to remain non-covalently associated with the mature peptide to form a latent TGF β 1 complex also known as the Small Latent Complex (SLC). In this state, the SLC is secreted and undergoes further processing in the extracellular matrix. While SLC-like complexes have not been observed for other superfamily ligands, there are likely analogs as pro-regions can be swapped between ligands and can direct cleavage of heterologous mature regions (Thomsen and Melton, 1993).

The Receptors

TGF β superfamily ligands bind a variety of transmembrane receptors. Receptors for TGF β were first characterized by studies in which radioactively labelled TGF β was chemically cross-linked to cell-surface proteins (Massague and Like, 1985). These analyses revealed that TGF β binds to three types of receptors. These cross-linked receptors segregated according to mobility on SDS-PAGE gels (Cheifetz et al., 1986) and were named type I, type II or type III receptors (Cheifetz et al., 1988b).

Type I and Type II Receptors

Type II receptors comprise a family of related transmembrane serine/threonine receptor kinases. Expression cloning approaches identified the first type II receptors and these preferentially bound activin (ActRII, ActRIIB) (Mathews and Vale, 1991; Mathews et al., 1992) and TGF β 1 (T β RII) (Lin et al., 1992) respectively. Cloning and characterization of additional type II receptors, including those which selectively bind BMPs (BMPRII) (Liu et al., 1995) and MIS (Baarends et al., 1994), reveals that together they comprise a family of highly related serine/threonine kinases. Type II receptors are glycoproteins of approximately 70 kDa. Type II receptors consist of a cysteine-rich extracellular domain, a single-membrane spanning domain and an intracellular serine/threonine kinase domain that is followed by a serine/threonine rich C-terminal extension. In most type II receptors, the kinase domain is capable of autophosphorylation on serine and threonine residues *in vitro* and is constitutively active.

Similarly, type I receptors comprise a family of related transmembrane ser/thr kinases. Use of degenerate primers directed against ActRII identified a number of type I receptors that were called activin receptor-like kinases 1:4 or ALK1-ALK4 (ten Dijke et al., 1993). These, and other type I receptors, were also cloned independently and were named according to specificity of ligand binding. These type I receptors include: the TGF β receptor (ALK5, T β RI) (Franzen et al., 1993; Yamashita et al., 1994b); an activin receptor (ALK4, ActRIB) (Carcamo et al., 1994; ten Dijke et al., 1993); the BMP receptors (ALK3, BMPRIA and ALK6, BMPRIIB and ALK2, ActRI) (Attisano et al., 1993; Ebner et al., 1993; Koenig et al., 1994; Yamashita et al., 1995); as well as other receptors not yet fully characterized (ALK7 and ALK1, TSR1 or Tsk 7L) (Attisano et al., 1993; Ryden et al., 1996; ten Dijke et al., 1994; Tsuchida et al., 1996). Comparison of amino acid sequences reveals that type I receptors are a highly related group of single-membrane spanning kinases. Type I receptors are glycoproteins of approximately 55 kDa and are composed of four regions: an extracellular portion; a cytoplasmic juxtamembrane region; a serine-glycine repeat region (SGSGSG and flanking sequences) known as the 'GS domain'; and a C-terminal serine/threonine kinase domain. Type II and type I receptors are highly related, however amino acid sequences of their extracellular domains vary dramatically. Nonetheless there are characteristic cysteines in the extracellular portions that presumably confer structural relatedness (Ebner et al., 1993) and divergence in extracellular sequences likely confers specificity on ligand-receptor interactions.

Mechanism of Receptor Activation

To initiate signalling, TGF β superfamily ligands bind receptor and these ligand-bound receptors oligomerize. For TGF β and activin, ligand binds to the appropriate type II receptor and this results in a stepwise recruitment of the cognate type I receptor into the complex (Moustakas et al., 1993; Wrana et al., 1992). Some BMP-type ligands bind to type II and type I receptors together in a cooperative, rather than a stepwise, manner (Gilboa et

al., 2000). Cooperative binding of BMPs is suggested by the observation that BMP ligands have low affinity for either receptor type alone, however when ALK2, 3 or 6 and BMPRII receptors are co-expressed, BMP ligands bind with higher affinity (Liu et al., 1995). Upon ligand binding, a heteromeric complex of ligand, a dimer of type II and a dimer of type I is formed (Yamashita et al., 1994b), although the precise mechanism of ligand-dependent receptor recruitment and oligomerization remains unknown.

Once constitutively active type II receptors are brought in proximity to type I receptors, a transphosphorylation event occurs. For TGF β receptors, T β RII transphosphorylates T β RI (Wrana et al., 1994) on conserved residues in the GS domain (Wieser et al., 1995). Multiple serines in this region must be phosphorylated to allow for signal propagation. Transphosphorylation is likely a direct event as the kinase cascade can be recapitulated with baculovirally-expressed receptor complexes (Ventura et al., 1994). Moreover, if only the cytoplasmic domains of type II and type I are fused as a chimeric type I/II receptor, the result is constitutive signalling (Feng and Derynck, 1996). While most work on the mechanism of receptor action has focussed on TGF β 3 receptors, in cases that have been examined, other TGF β superfamily receptor systems follow a similar pattern. Thus upon ligand binding, constitutively active type II receptors transphosphorylate type I receptors.

When type I receptors are phosphorylated, they become activated and thereby specify downstream signalling events. Significantly, mutation of a threonine to aspartate (or glutamate) in the GS domain of T β RI creates a constitutively active receptor (Wieser et al., 1995). This constitutively active receptor can recapitulate known signalling responses of type II/type I heteromeric complexes (Massague and Weis-Garcia, 1996). Analogous activating mutations in BMP type I receptors are also constitutively active and can mediate BMP-type signalling. This class of hypermorphic receptors convincingly demonstrate that type I receptors are sufficient to specify responses downstream of ligand binding.

A controversial report proposes that different regions on type I receptors specify distinct types of signals (Saitoh et al., 1996). T β RI lacking a region of the cytoplasmic juxtamembrane domain can support immediate transcriptional responses, but cannot support growth inhibitory responses to TGF β . T β RI in which either serine 172 or serine 176 is replaced with alanine mimics loss of this juxtamembrane region. In another study, mutations of Ser165 cause an increase in growth inhibition and extracellular matrix formation, but in contrast, a decrease in apoptosis (Souchehnytskyi et al., 1996). In both studies, transcriptional activation signals from mutant receptors were not affected. A third study, however, disputes these results (Dore et al., 1998) and currently the ability of the cytoplasmic juxtamembrane domain to specify growth inhibitory versus immediate transcriptional signals remains an open question.

Type III Receptors

In contrast to type I and type II receptors, type III receptors may play more of an ancillary role as they modulate activity primarily by regulating ligand access to type I and type II receptors. Type III receptors contain two distinct members, a proteoglycan and a glycoprotein known as betaglycan and endoglin, respectively (Cheifetz et al., 1988a). Type III receptors have been identified only for TGF β and it is not known whether correlates of type III receptors exist for other TGF β superfamily ligands.

Betaglycan

Betaglycan exists in two forms, a membrane-bound and a soluble version each of which has opposing effects on TGF β signalling (Andres et al., 1989). The membrane-bound form has a short intracellular tail and can be cleaved by plasmin on its extracellular surface to produce the soluble form (Lopez-Casillas et al., 1991). The usual role of membrane-bound betaglycan is to present ligand to type II receptor (Lopez-Casillas et al., 1993; Wang et al., 1991). In contrast, the soluble form of betaglycan can act as an inhibitor of TGF β action (Lamarre et al., 1994; Lopez-Casillas et al., 1994).

In vivo, the membrane-bound betaglycan seems to be required for TGF β -mediated inhibition of lung vessel branching as antisense betaglycan oligonucleotides cause insensitivity to TGF β in *ex vivo* lung cultures (Zhao et al., 1998). In addition to this TGF β -promoting role, membrane-bound betaglycan can inhibit activin signalling by forming a complex with inhibin and ActRIIB (Lewis et al., 2000). Thus multiple forms of betaglycan can control TGF β /activin signalling by modifying ligand access to the type II receptor.

Endoglin

Endoglin, which is also implicated in TGF β signalling, is highly related to betaglycan in its transmembrane and intracellular sequences (Cheifetz et al., 1992). Endoglin forms a complex with signalling receptors (Barbara et al., 1999; Yamashita et al., 1994a) and seems to modulate cellular responses to TGF β in a complex manner. Overexpression of endoglin in transfected cells can mitigate TGF β responses (Guerrero-Esteo et al., 1999; Lastres et al., 1996), however defining the precise role of endoglin from overexpression experiments can be problematic. On the other hand, endoglin lack-of-function phenotypes are definitive. Mutations in endoglin result in a human pathology known as hereditary haemorrhagic telangiectasia (HHT) type I (McAllister et al., 1994) a disease characterized by vascular defects. Moreover defects in endoglin lead to defective angiogenesis (Li et al., 1999) and HHT (Bourdeau et al., 1999) in mice. Interestingly, knockout of ALK1 recapitulates HHT in a mouse model (Urness et al., 2000) and, moreover, TGF β s are required for both vasculogenesis and angiogenesis. Though a precise mechanism of endoglin action remains unclear, this relationship between endoglin mutant phenotypes and TGF β action suggests that endoglin may play a role in facilitating, and not inhibiting, TGF β signalling.

Signalling Receptor-Associated Molecules

As part of the complex regulation of TGF β superfamily signalling, TGF β superfamily receptors are negatively regulated by a number of receptor-associated molecules. These include FK506-

binding protein 12 (FKBP 12), BMP and activin membrane bound inhibitor (BAMBI) and BMP receptor associated molecule 1 (BRAM1). FKBP12 associates with T β RI and prevents its phosphorylation by T β RRI (Chen et al., 1997c). Consistent with this observation, a crystal structure of T β RI in complex with FKBP 12 reveals that T β RI is kept in an inactive conformation and that sites for T β RRI transphosphorylation are capped in the presence of FKBP12 (Huse et al., 1999). In spite of these data, FKBP 12 knockout mice do not exhibit increased TGF β activity (Bassing et al., 1998) and this undisturbed phenotype might reflect redundancy in FKBP12 function at the biological level. Next, BAMBI resembles a type I receptor that is truncated on its intracellular surface; its short intracellular domain has weak homology to regions of type I receptors thought to be important for homodimerization (Huse et al., 1999). While the so-called pseudoreceptor BAMBI retains the ability to heterodimerize, it does not transmit signal. It likely works in a dominant negative manner and prevents formation of activated receptor complexes and acts as a general inhibitor of TGF β superfamily signalling (Onichtchouk et al., 1999). Finally, a yeast two-hybrid screen identified the cytoplasmic BRAM1 protein which associates with BMPRI receptor (Kurozumi et al., 1998). The *C. elegans* BRAM1 homolog, *bra-1*, is thought to inhibit BMP-type signalling in amphid neurons (Morita et al., 2001). Thus, FKBP12, BAMBI and BRAM1 are unrelated receptor-binding factors that negatively regulate receptor action by unique mechanisms. Tissue- or time-specific expression of these factors is likely a method of inhibiting TGF β superfamily signalling.

Another set of molecules that can associate with receptor complexes include the TGF β -receptor interacting protein-1 (TRIP-1), protein phosphatase 2A $\beta\alpha$ -subunit (PP2A $\beta\alpha$), and serine-threonine kinase receptor associated protein (STRAP), all of which contain a WD40 repeat. WD40 repeats provide a pliable interaction surface utilized in protein-protein interactions (Neer et al., 1994) and thus diverse WD40-repeat containing proteins can either positively or negatively modify TGF β superfamily effects. TRIP-1 contains five

WD-40 repeats and interacts with T β RII that has heteromerised with type I receptor (Chen et al., 1995). In vitro, TRIP-1 is phosphorylated by TGF β receptor complexes and since TRIP-1 and T β RII are co-expressed throughout development, perhaps similar phosphorylation events also occur in vivo. In signalling assays, overexpression of TRIP-1 acts to inhibit TGF β transcriptional responses by receptor-dependent and receptor-independent mechanisms (Choy and Derynck, 1998). Another WD40-repeat containing protein, PP2A α , associates with the cytoplasmic domain of activated T β RI (Griswold-Prenner et al., 1998). PP2A α is a cytoplasmic protein that regulates the catalytic activity of protein phosphatase 2A and this regulation is implicated in cell cycle control (Mayer-Jaekel et al., 1993). Given that TGF β can regulate cell division, the interaction between PP2A α and TGF β receptor complexes may be of some significance. Importantly, PP2A α has a growth inhibitory effect on cells in culture and this inhibition appears to enhance and is dependent on TGF β receptors (Griswold-Prenner et al., 1998) possibly through regulating S6-kinase activity (Petritsch et al., 2000). Finally, STRAP is a WD40 repeat containing protein that is also phosphorylated by T β RI and acts to inhibit TGF β receptor activity (Datta et al., 1998). In addition to binding receptors, STRAP associates with a known negative regulator of receptors, called Smad7 and may act to bridge Smad7 with receptor complexes (Datta and Moses, 2000). Thus, a number of proteins containing WD40-repeats play distinct and important roles in modifying receptor action. The structural basis underlying TGF β receptor interactions with WD40-repeat proteins is unknown, although this could be an interesting area for developments.

The cytoplasmic domain of T β RI has also been shown to interact with the α -subunit of farnesyl-protein-transferase-alpha (FT- α) (Kawabata et al., 1995). FT- α associates preferentially with activated T β RI and may be a direct target of phosphorylation. FT- α adds isoprenyl or geranylgeranyl moieties to various targets including G-proteins and cytoskeletal components. Currently a functional consequence for TGF β signalling due to FT- α association has not been established as

FT- α is dispensable for studied aspects of TGF β signalling (Ventura et al., 1996). Nonetheless the presence of this (and likely other) receptor-interacting proteins in specific cellular environments might allow for the fine-tuning of TGF β superfamily ligand effects. In addition these interacting proteins might allow for signal integration from a variety of inputs. This growing group of receptor-interacting proteins present experimental opportunities to further study the extensive range of TGF β superfamily receptor action and regulation.

Receptor Trafficking

Mounting evidence suggests that TGF β superfamily receptors are involved in complex receptor trafficking events. In signalling cascades, receptors are typically internalized after ligand binding and upon internalization, receptors can be recycled to the plasma membrane or they can be downregulated through endocytosis (Mellman, 1996). For TGF β receptors, ligand-receptor complexes rapidly internalize in fibroblast cells (Massague and Kelly, 1986). It is unclear what role ligand plays in receptor internalization, since type II receptors have been suggested to be constitutively internalized and possibly recycled in the absence of ligand (Ehrlich et al., 2001; Dore et al., 2001). Furthermore, the half-life of T β RII in mink lung epithelial cells (Mv1Lu) and 293T cells is quite short and is only modestly affected by TGF β signalling (Wells et al., 1997; Kavsak et al., 2000), contrasting T β RI, which is considerably more stable. However, activation of type I receptors, specifically by type II transphosphorylation, leads to rapid down-regulation of occupied receptors that can be mediated by complexes of inhibitory Smads bound to Smurf ubiquitin ligases (Anders et al., 1997; Anders et al., 1998; Ebisawa et al., 2001; Kavsak et al., 2000). Hence internalization may indeed be followed by down-regulation of the occupied receptor and in cells that have a limiting pool of cell surface receptors, this could service to limit TGF signalling. In further support of a role for receptor trafficking and downregulation, proteins of the sorting nexin (SNX) family can also interact with TGF β receptors (Parks et al., 2001). SNX proteins play a role in receptor trafficking for

a variety of tyrosine kinases. Overexpression of SNX proteins inhibit TGF β signalling and this inhibition is consistent with the possibility that SNX proteins may shuttle TGF β receptors to degradative compartments. One important area of future research will be to define how the trafficking routes of the individual TGF β receptors versus the occupied receptor complex function to control cellular responses to TGF β .

In addition to down-regulation, ligand-bound receptor internalization may be a mode for transporting ligand from one cell to another. Interestingly, trafficking of DPP receptors appears to be necessary for long-range movement of this ligand (Entchev et al., 2000). Moreover this mode of DPP transport might require dynamin, a protein necessary for clathrin-coated pit internalization (Mellmant 1996). This observation may signify a novel paradigm for the role of trafficking ligand-bound receptors. While these results provide tantalizing hints as to the role of receptor trafficking in TGF β superfamily signalling, a critical advance will be to determine precisely in which subcellular compartment(s) signalling occurs.

Signal Transducers: Smads

Identification of Smads, Downstream Signal Transducers in the TGF β Pathway

After type I receptor activation, TGF β superfamily signalling is mediated by intracellular substrates known as Smads. Genetic approaches in *Drosophila* and *C. elegans* first identified downstream pathway components and one of the best genetically characterized TGF β superfamily pathways is that of the *dpp* gene (Padgett et al., 1987). Maternal effect enhancer screens of *dpp* hypomorphs first revealed components in the black-box downstream of receptors (Raftery et al., 1995). These screens identified novel components in the DPP pathway and the first such molecule cloned was *Mothers-against-Aecapentaplegic (Mad)* (Sekelsky et al., 1995). Mad encodes an intracellular protein that acts genetically downstream of *dpp* and is essential for *dpp* activity (Newfeld et al., 1996). Importantly, a hypomorphic allele of *Mad* is epistatic to an activated *dpp* receptor allele and thus *Mad* activity is downstream of receptor action (Hoodless et al., 1996). At the

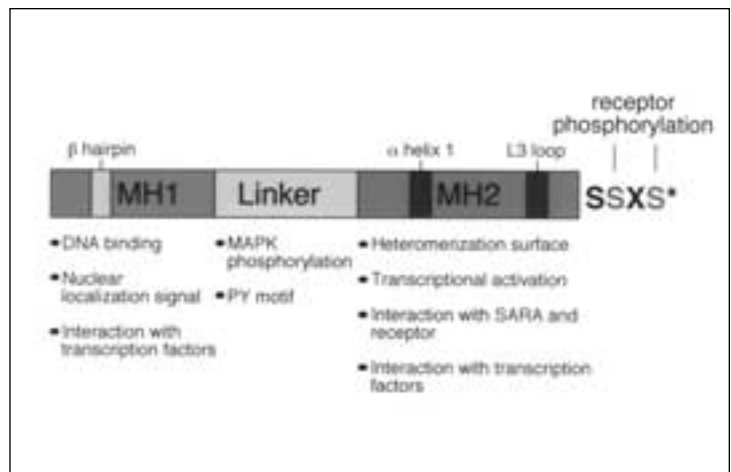


Figure 1. R-Smad Schematic

A schematic representation of R-Smads depicting the location of key structures and functions associated with various regions of the R-Smads is shown. R-Smads are comprised of three regions, two highly conserved Mad homology regions (MH1 and MH2) at the N and C-termini respectively and a non-conserved, intervening linker region. In various R-Smads, the MH1 domain contacts DNA and contains a nuclear localization signal. The MH2 domain is a multifunctional region that is responsible for intra- and intermolecular interactions as well as transcriptional transactivation. Smads are phosphorylated in response to TGF β superfamily signalling on critical serines at the C-terminus. The asterisk indicates the terminus of the protein.

time of its cloning, predicted MAD lacked known motifs however, homologs existed in *C. elegans* and in mammalian sequence databases. Three *C. elegans* loci (*sma-2*, *sma-3* and *sma-4*) display loss-of-function phenotypes that also places them downstream of the worm TGF β -like receptor *dauer-forming-4 (daf-4)* (Savage et al., 1996). Together these molecules foreshadowed a growing family of intracellular signal transducers downstream of receptors.

A large number of MAD-related proteins were subsequently identified from a variety of vertebrates. These proteins are now called Smads in recognition of the founding *sma* and *Mad* genes (Derynck et al., 1996). Characterization of the many Smad proteins reveals 8 members to date that sort into three functional categories of Smads: the receptor-regulated Smads (R-Smads), the Common Smads (C-Smads), and the inhibitory-Smads (I-Smads). R-Smads and C-Smads are intracellular proteins that are each comprised of three parts: the Mad Homology 1 and 2 (MH1 and MH2) regions that are highly conserved sequences

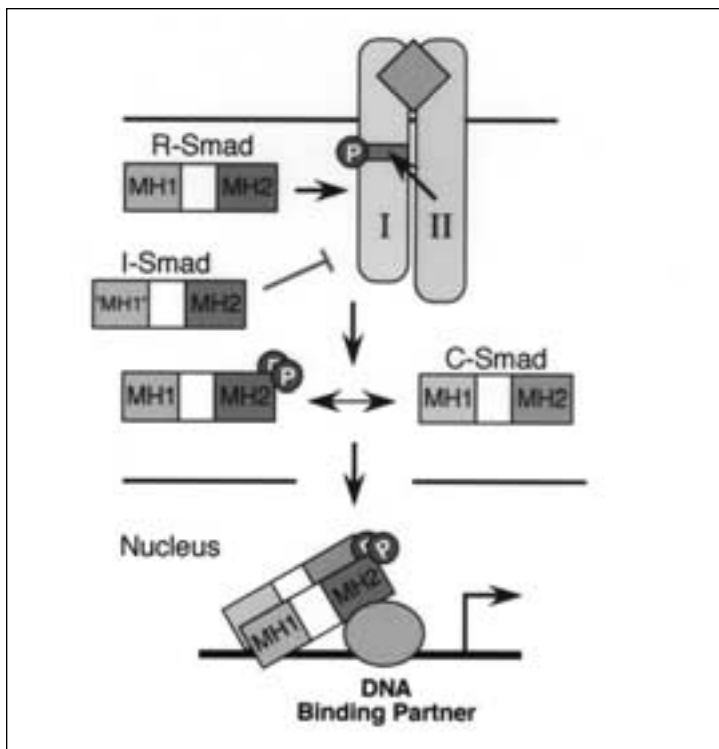


Figure 2. The Canonical Smad Pathway

TGF β /activin and BMPs bind to heteromeric complexes of transmembrane type II and type I receptors. The type I receptor directly phosphorylates R-Smads, which then dissociate from the receptor and bind the common Smad, Smad4. The complex then accumulates in the nucleus where R-Smads associate with different DNA-binding proteins (DBP-BPs) and MH1 domains bind directly to DNA. Thus regulation of distinct target genes is achieved to generate diverse biological responses.

at the N- and C-termini respectively, and an intervening, non-conserved linker section (Fig. 1). I-Smads are similar to R-Smads except that they have a poorly conserved MH1 domain. These three groups of Smads represent a major intracellular pathway for TGF β superfamily signalling that transmits signals directly from the receptor into the nucleus to regulate transcriptional responses (summarized in Fig. 2). Moreover, genetic analysis in the mouse has revealed that all of the Smads analyzed thus far play critical functions at various aspects of development (Table 1).

Mechanism of Signalling via Smads

Type I Receptors Phosphorylate R-Smads. Type I receptors directly phosphorylate R-Smads in response to TGF β superfamily signalling. Smad 1

is phosphorylated in response to BMP addition and this phosphorylation is achieved by type I BMP receptor kinases (Hoodless et al., 1996; Kretschmar et al., 1997b). Moreover endogenous *Drosophila* MAD is phosphorylated *in vivo* within 15 m of ligand addition (Newfeld et al., 1997). Similarly, Smad2 is phosphorylated by TGF β and activin receptors (Eppert et al., 1996; Nakao et al., 1997b; Yingling et al., 1996) on two terminal serines in an SSXS motif and these phosphorylations are required for activity (Abdollah et al., 1997; Macias-Silva et al., 1996; Souchelnytskyi et al., 1997) Mutant Smad2 bearing alanines substituted for these terminal serines cannot be phosphorylated and instead associates constitutively with activated T β RI. Together these observations support the model that activated type I receptors can directly phosphorylate Smads.

While type I receptors are thought to phosphorylate R-Smads directly, other essential factors may be present in the signalling complex. Disabled-2 (Dab2) is an adaptor molecule that is required for signalling in the context of some tyrosine signalling pathways. Interestingly, Dab2 associates with TGF β receptors, Smad2 and Smad3 and Dab2 may be required for the phosphorylation of these R-Smads (Hocevar et al., 2001). Accordingly Dab2 may be an essential component of the TGF β signalling pathway that helps to transmit TGF β signals from receptors to Smads as part of a multiprotein complex.

Subgroups of R-Smads Specify BMP or TGF β Signals.

Upon phosphorylation by type I receptors, R-Smads specify downstream biological responses and subgroups of R-Smads transduce subsets of TGF β superfamily signals. For instance in *Xenopus*, overexpression of Smad1 yields ventral mesoderm—a typical BMP response, whereas Smad2 overexpression results in dorsal mesoderm—a TGF β /activin response (Graff et al., 1996; Thomsent 1996). These and related studies have led to the conclusion that Smads 1, 5 and 8 comprise the BMP-responsive group of Smads while Smads 2 and 3 constitute the TGF β /activin responsive group. In general Smads in these two groups mediate ligand-dependent effects and thus

two subgroups of R-Smads are primary effectors of either BMP or TGF β /activin signalling. Consequently, this dichotomy of R-Smads suggests a similar convenient categorization of ligands as BMP-type or TGF β -type.

Specificity of R-Smad receptor interactions is ensured by determinants on R-Smads and receptors. On R-Smads, a solvent-exposed region of the MH2 known as loop β (L3) (Chen et al., 1998; Lo et al., 1998) and α -helix-1 (aH1) (Chen and Massague, 1999) determine the specificity of R-Smad/receptor interactions (Figure 1). Exchanging amino acids in these regions between R-Smads in the subgroups causes a switch in signalling specificity. R-Smads also contain a highly positively charged surface groove adjacent to L3 and this basic surface can bind phosphoserines and is postulated to bind the phosphorylated GS domain (Wu et al., 2001; Huse et al., 2001; Wu et al., 2000). Additionally, a region on T β RI known as loop-4-5 (L45) lies adjacent to the GS domain and specifies interactions with Smad2 (Feng and Derynck, 1997; Huse et al., 1999). One model proposes that L45/L3 interactions provide specificity while GS domain/basic groove interactions increase the overall strength of binding (Shi, 2001).

R-Smad Access to Receptor

Since type I receptors directly phosphorylate R-Smads, it is a reasonable expectation that appropriate machinery is required to help cytoplasmic R-Smads localize to their membrane-bound target receptors (Fig. 3). Two proteins, Smad anchor for receptor activation (SARA) (Tsukazaki et al., 1998) and Hgs (Miura et al., 2000), each contain a FYVE-domain for membrane localization and also bind Smad2 and Smad3. The FYVE-domain is a double zinc-finger that binds phosphatidylinositol-3-phosphate (PI-3P) in the plasma membrane (Wurmser et al., 1999). By binding the membrane and the target R-Smad, SARA and Hgs may act to recruit Smad2 to T β RI. SARA binds TGF β /activin responsive Smads but not BMP responsive Smads. These associations depend on a critical arginine residue that is present in TGF β /activin responsive R-Smads, but is not present in Smad1 (Wu et al., 2000). Interestingly,

Table 1. Smads and the Knockout Phenotypes in Mice

Type Member	Loss-of-function phenotype(s) (L) or Other (e.g. hypermorph) characterization (O)	Reference(s)
R-Smads - TGFβ/activin		
Smad2	(L) embryonic lethal, defects in proximal/distal axis, extraembryonic ectoderm and epiblast	(Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998)
Smad3	(L) metastatic colon cancer, diminished T-cell response to TGF β , accelerated wound healing	(Ashcroft et al., 1999; Datto et al., 1999; Yang et al., 1999b; Zhu et al., 1998)
R-Smads - BMP		
Smad1	(O) mediates BMP signalling. (L) Defects in extraembryonic tissues and germ cell formation	(Hoodless et al., 1996; Kretzschmar et al., 1997b; Tremblay et al., 2001)
Smad5	(L) die between E9.5 and E11.5, multiple defects in amnion, gut, heart, face, neural tube, heart looping, embryonic turning, angiogenesis defects, mesenchymal apoptosis	(Chang et al., 1999; Chang et al., 2000; Yang et al., 1999a)
Smad8	(O) mediates BMP signalling	(Chen et al., 1997b)
C-Smads		
Smad4	(L) embryonic lethal, heterozygotes have intestinal tumours, variable requirement in murine cells	(Sirard et al., 1998; Sirard et al., 2000; Takaku et al., 1999; Takaku et al., 1998)
I-Smads		
Smad6	(L) viable, multiple cardiovascular abnormalities, aortic ossification, elevated blood pressure	(Galvin et al., 2000)
Smad7	(O) antagonist of TGF β superfamily signalling	(Hayashi et al., 1997; Nakao et al., 1997a)

SARA and Hgs are enriched in the early endosome and recent studies have suggested that endocytosis through the clathrin pathway is important for TGF β signal transduction (Itoh et al., 2002; Panopoulou et al., 2002; Penheiter et al. 2002). Also, a BMP-type SARA has not yet been identified. Smads can also associate with microtubules and thereby possibly preventing spurious activation of the pathway; this regulatory step precedes the interaction of R-Smads and SARA (Dong et al., 2000). While key players that are likely required for membrane localization have been identified, further work is needed elucidate precise mechanisms of Smad delivery to, and association with membrane-bound receptors.

Heteromerization with C-Smad and Nuclear Entry

Once phosphorylated, R-Smads heteromerise with the C-Smad, Smad4. Smad4 independently identified as DPC4 (Hahn et al., 1996), lacks the SXS sequence and is phylogenetically more dis-

Table 1

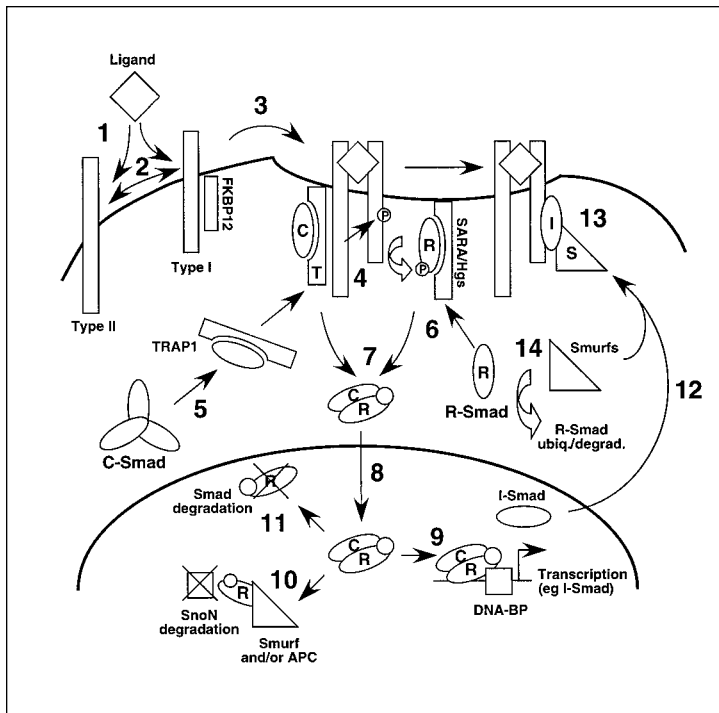


Figure 3. Model of Intracellular TGF β Signalling Events

This schematic depicts the complex molecular activities that underlie TGF β signalling. Arrows depict movement or enzyme activity and events are not necessarily sequential. 1) Dimeric mature ligand binds receptors in sequential (TGF β) or cooperative (BMP) mode. 2) Type II and type I receptors form heteromeric complexes. 3) Heteromeric complex appears to colocalize in an endosomal compartment shared by SARA. 4) Type II receptor transphosphorylates type I thereby activating the type I. 5) Trimeric C-Smad changes multimerisation state and is brought to receptor complex by TRAP1. 6) Trimeric R-Smad associates with SARA, is brought to receptor complex and is phosphorylated on terminal serines by type I receptor. 7) Phosphorylated R-Smad associates with C-Smad and this complex translocates to the nucleus. 8) Smads accumulate in the nucleus. 9) Smads bind DNA-binding proteins, to regulate transcriptional responses. 10) Smads can also recruit the ubiquitin ligases Smurf and APC to target the corepressor SnoN for degradation. 11) R-Smad will be degraded by Smurf-independent mechanisms. 12) In addition to being transcriptionally induced, Smad7 is exported from the nucleus and binds the receptor to inhibit signalling. 13) The I-Smads bound to receptor also act as adapters for Smurf ubiquitin ligases which can degrade receptor complexes. 14) Smurfs can also directly target R-Smads for degradation.

tantly related to R-Smads than R-Smads are to each other. Smad4 is not a direct substrate of receptors, instead Smad4 interacts with both TGF β - and BMP-activated R-Smads (Lagna et al., 1996; Zhang et al., 1997). The *Drosophila* homolog of Smad4, MEDEA, functions in a similar manner as it associates with activated MAD

(Das et al., 1998; Hudson et al., 1998; Wisotzkey et al., 1998).

As described above for R-Smad/SARA interactions, C-Smads also appear to be shuttled to the membrane, in this case by TBRI associated protein-1 (TRAP1). TRAP1 is a protein that preferentially interacts with activated T β RI receptor in the yeast two-hybrid assay (Charng et al., 1998). In cells, TRAP1 associates strongly with activin and TGF β receptors in their basal state and TRAP1 is released upon receptor activation. Importantly, TRAP1 also interacts with Smad4 (Wurthner et al., 2001; Fig. 3). Perhaps increasing the local concentration of Smad4 in membrane-proximal domains permits efficient heteromeric complex formation.

Upon heteromeric complex formation, Smad complexes enter the nucleus (Baker and Harland, 1996; Hoodless et al., 1996). While R-Smads can accumulate independently of C-Smads, the latter are brought along into the nucleus upon their association with R-Smads (Wisotzkey et al., 1998). In the case of Smad3, nuclear import occurs in an importin β -dependent manner (Kurisaki et al., 2001; Xiao et al., 2000b). Consistent with this phenomenon, a distinct nuclear localization signal (KKLKK) resides in the N-terminus of Smad3 (Xiao et al., 2000a). Smad2 however, bears an insert which prevents its interaction with importin β and thus other distinct sequences in the MH2 domain are thought to allow for nuclear localization. In addition to regulated nuclear import, Smad4 contains a nuclear export sequence (NES) in its linker region (Watanabe et al., 2000). Heteromeric complex formation inactivates this NES and this inactivation further contributes to nuclear accumulation of the complex. Thus a number of mutually compatible sequence-dependent mechanisms regulate proper Smad subcellular localization.

While R-Smad and C-Smad heteromeric complexes are necessary for most TGF β superfamily signalling, heteromerisation of R- and C-Smad may not be required for all signalling responses. In *Drosophila* some R-Smad responses are supported in C-Smad deficient cells (Wisotzkey et al., 1998) and in murine cells some non-essential

roles for Smad4 have been observed (Sirard et al., 2000). Such a variable requirement for Smad4 may allow for some of the pleiotropic effects of TGF β superfamily action.

Although it is known that Smads exist as homomers and heteromers, the precise stoichiometries of R-Smads, C-Smads and R/C-Smad complexes remain unclear. X-ray crystallographic analysis of Smad4 MH2 (Shi et al., 1997) and a slightly larger transcriptionally active fragment of Smad4 (Qin et al., 1999) reveals that this protein exists in a trimeric arrangement. Interestingly, three amino acids that are thought to participate in hydrogen bonding in Smad4 trimers are conserved across Smads and this conservation hints at the possibility that all Smads may exist as trimers. However, gel filtration, sedimentation and structural studies suggest the Smads are monomers in their basal state and oligomerize into trimers upon signalling (Kawabata et al., 1998). This observation of Smads as monomers in their basal state is consistent with monomeric Smad observed in the Smad2 MH2/SARA SBD crystal structure (Wu et al., 2000). Furthermore, recent structural studies indicate that phosphorylation may drive R-Smad trimer assembly by stabilizing phosphorylation-independent MH2 domain interactions (Wu et al., 2001; Qin et al., 2001). However there is considerable controversy regarding the stoichiometry of R-Smad/C-Smad heteromeric complexes. Some argue that a trimer is formed (Kawabata et al., 1998) whereas others conclude that heterodimers form (Jayaraman and Massagué 2000; Wu et al. 2001). Furthermore, a recent study on Smad1 and Smad3 bearing pseudoactivating mutations reveals trimers of these R-Smads and heteromers containing two Smad3 and one Smad4 (Chacko et al., 2001; Qin et al., 2001). The apparent observation of Smads in both heterodimeric and heterotrimeric states suggests that the multimeric state of Smads may vary in a context-dependent manner. Further such observed heterogeneity in the stoichiometry of complexes might reflect differing *in vivo* spa-

tiotemporal states and varying functions and signalling efficacies of these complexes. This should be an exciting and challenging area of investigation in the future as very little is understood of how the stoichiometry of signalling complexes in general affect biological responses.

DNA binding and Smad-mediated Activation and Repression of Transcription.

Upon entering the nucleus, Smads can directly bind target DNA sequences via their MH1 domains. An amino-terminal fragment of *Drosophila* MAD binds directly to an enhancer of the vestigial (vg) gene and this binding is required for vg transcription (Kim et al., 1997). Smad3 and Smad4 can directly bind to regions in the plasminogen activator inhibitor 1 (PAI-1) promoter (Dennler et al., 1998) and Smad4 can bind DNA in response to TGF β (Yingling et al., 1997). A co-crystal of the Smad1 MH1 and DNA demonstrates that sequence-specific DNA binding depends on a β -hairpin found in the MH1 and three amino acids in this hairpin make hydrogen bonds with nucleotides in Smad target DNA (Shi et al., 1998).

Smads bind weakly to GC-rich sequences. Smad1 binds DNA with an affinity of approximately 5×10^{-7} M (Shi et al., 1998) and this relatively low affinity likely requires assistance from other DNA-binding factors. Various studies with differing results have claimed to define different consensus Smad binding sites (Dennler et al., 1998; Johnson et al., 1999; Shi et al., 1988; Zawel et al., 1998). However it is unlikely that there is a single consensus Smad binding site and Smads bind DNA with relatively low specificity and numerous G/C rich sequences are also bound by Smad MH1 domains (Labbe et al., 1998). A preponderance of Smad binding sites throughout promoters is not incompatible with a broad biological role for Smads.

DNA elements that can bind Smads occur frequently in the genome, thus partner DNA-binding is essential for defining specificity of gene targets. Indeed, Smads can associate with DNA-binding partners. The first such protein identi-

fied was the *Xenopus* winged-helix/forkhead factor, the forkhead activin signal transducer 1 (FAST1) (Chen et al., 1996), now known as FoxH1. Factors such as FoxH1 are critical for recruitment of Smads to appropriate DNA targets. The *Xenopus* FoxH1 or its mammalian counterpart bind in a complex with Smad2 and Smad4 to the promoters of the activin-response gene *Mix. 2* (Chen et al., 1998) and *goosecoid* (*gsc*) genes respectively (Labbe et al., 1998). In the mouse, FoxH1 plays a key role in mediating specific activities of Smad2 and Smad3 during gastrulation (Hoodless et al., 2001; Yamamoto et al., 2001).

One of the striking aspects to emerge from investigation of Smads has been that these factors associate with a wide range of DNA binding partners as well as transcriptional coactivators and corepressors (reviewed in Attisano and Wrana, 2000). For instance Smads bind to partners that include Jun, Atf2, TFE3, vitamin D receptor, OAZ and many others as well as to transcriptional coactivators such as CBP/p300, MSG and SMIF. Additionally Smads can bind the corepressor TGIF, which recruits the transcription-silencing histone deacetylases (HDACs) and thereby represses transcription of Smad-bound promoters/enhancers as well as HDAC itself. Another pair of related Smad-interacting proteins, the nuclear oncoproteins Ski (Luo et al., 1999) and SnoN, associate with R-Smads and recruit nuclear β -Irepressor (N-CoR) to repress transcription (Stroschein et al., 1999). Thus Smads, in conjunction with sequence-specific DNA binding partners and transcriptional coactivators or corepressors can direct a vast array of transcriptional responses.

Variable transcriptional responses are directed by peptide sequences in MH2 and MH1 domains and these sequences regulate binding of associated factors or binding to DNA. For instance, the C-terminus of Smad1 acts as a transcriptional trans activator (Liu et al., 1996). Similarly, Smad4 requires its MH2 and a proline-rich stretch just upstream, known as the Smad4 activation domain (SAD), to direct transcrip-

tional responses (Liu et al., 1997a; Qin et al., 1999). Mutations in these regions can abrogate transcriptional responses, likely by disrupting association with transcriptional activators. In addition, differences in the MH1 domains of highly related Smads can confer dramatic changes in their ability to activate transcription. Smad2 and Smad3 have nearly identical MH1 domains but have opposing activities on the *gsc* promoter; while Smad2 activates, Smad3 represses (Labbe et al., 1998). Smad3 binds *gsc* DNA while Smad2 does not and this difference may account for the observed opposing activities. Interestingly, using a different CAGA containing promoter another study shows a related but opposite result, Smad3 activates while Smad2 represses transcription (Dennler et al., 1999). In this case, as in the previous study, Smad3 binds DNA but Smad2 does not. The latter study further argues that a short insert in the MH1 of Smad2 is responsible for preventing Smad2 from binding DNA. While it is not entirely clear why Smad2/3 have opposing activities on different promoters, their opposite activities seem to depend on their DNA binding statuses on each promoter.

Antagonistic Smads: I-Smad.

In contrast to R-Smads that propagate TGF β superfamily signals, I-Smads inhibit TGF β superfamily signalling. Smad6 and Smad7 were first identified in endothelial cells exposed to conditions of non-laminar shear stress (Topper et al., 1997). TGF β superfamily ligands induce the expression of Smad6 and 7 messages and Smad6 or 7 proteins prevent TGF β signalling by interacting constitutively with activated T β RI and preventing access of R-Smads (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997a). I-Smads have a poorly conserved MH1 domain but possess an MH2 that lacks a C-terminal SXS and in a manner opposite to R-Smads, Smad7 translocates from nucleus to cytoplasm in response to signalling (Itoh et al., 1998). In addition, I-Smads also recruit Smurf ubiquitin ligases to catalyze degradation of the receptor complex. I-Smads thus provide a tight layer of control on

Smad activation by closing a negative feedback loop (Fig. 3). Such a feedback loop might allow a tight readout of ligand activity and I-Smads might thus delimit Smad activation to provide appropriate spatiotemporal control of ser/thr kinase receptor activity during development.

Ubiquitin-Mediated Degradation of Smads and Receptors

Many cellular processes are controlled by degradation of proteins and a number of TGF β superfamily components are subject to degradation. Specific degradation of proteins is accomplished by diverse mechanisms including lysosomal degradation and the ubiquitin-proteasome system. In the latter mechanism, ubiquitin protein moieties are covalently linked to proteins which are targeted for degradation. Enzymes ensuring that only appropriate proteins are destroyed include ubiquitin-ligase enzyme 1 (E3) and ubiquitin-conjugating enzyme 2 (E2). C2-WW-HECT domain containing E3 ligases recognize the sequence PPXY (PY-motif) in their targets and mediate ubiquitination of those targets by facilitating the conjugation of ubiquitin side-chains (Huibregtse et al., 1995). Ubiquitinated proteins targeted for degradation are ultimately destroyed by a multiprotein complex known as the proteasome.

R-Smads are regulated by ubiquitin-mediated degradation. The Smad ubiquitination regulatory factors 1 and 2: (Smurf1 and Smurf2) are both E3 ligases that facilitate Smad destruction. Consistent with a requirement for E3 ligase targets, R-Smads contain PY-motifs. Interestingly, Smurf1 triggers the degradation only of Smad1 (Zhu et al., 1999) and inhibits BMP signalling in *Xenopus* embryos. Smurf2 has a more controversial role. One study argues that Smurf2 preferentially targets Smad 1 (Zhang et al., 2001), a second study reports that Smurf2 preferentially mediates the destruction of Smad2 (Lin et al., 2000), while a two other reports show that Smurf2 does not mediate R-Smad destruction (Kavsak et al., 2000; Bonni et al., 2001); these conflicting data await clarification.

In addition to being degraded through Smurfs,

Smads can also serve as adapters for Smurf-mediated degradation of Smad-associated proteins (Fig. 3). For instance, Smurfs in concert with Smad7 mediate the destruction of activated receptors. Both Smurf1 and Smurf2 translocate to activated T β RI and bind to the receptor complex using Smad7 as an adapter (Ebisawa et al., 2001; Kavsak et al., 2000). Once bound to the receptor these proteins initiate the destruction of activated receptor complexes. Also, Smad2 can act as an adapter for Smurf2 and recruit SnoN for ubiquitin-dependent degradation (Bonni et al., 2001). R-Smads can also facilitate APC-dependent degradation of SnoN (Stroschein et al., 2001; Wan et al., 2001), suggesting that they may play a more general role in the ubiquitin-proteasome system as adaptors that regulate steady-state levels of proteins in response to TGF β family signals.

Finally, phosphorylated R-Smads can also be degraded by Smurf-independent pathways. For instance, Smad2 lacking its PY-motif is still ubiquitinated (Lo and Massague, 1999) and this degradation requires Smad2 to be localized to the nucleus. Such nuclear localization-dependent ubiquitination may serve to limit spurious DNA binding in the nucleus by destroying Smad2 that is not tightly bound to target DNA. Additionally, this mode of degradation may simply down-regulate Smad2 signals. In a related manner, Smad3 bound to transcription factors is recognized by a protein called ROC 1 and in turn this complex becomes part of a larger E3 ubiquitin ligase complex (Fukuchi et al., 2001). This assembly is exported from the nucleus and is subject to degradation (Wan et al., 2002). Smad4 can similarly be targeted for destruction by the Jab 1 ubiquitin ligase. These modes of destruction of nuclear R-Smads provide yet more ways of restricting TGF β superfamily ligand effects.

Cross-talk with Other Signalling Pathways

Cells *in vivo* are subject to inputs from multiple signalling pathways. As more signalling pathways are elucidated, it becomes increasingly clear that signalling pathways do not work in isolation of each other (Jordan et al., 2000). Signalling cas-

cadetes may intersect or their activities may depend on the output of other simultaneous signals. TGF β superfamily signalling can be influenced by and can modulate other distinct signalling pathways. Understanding how and when Smads cross-talk therefore represents an important arm of TGF β signalling research.

Mitogen Activated Protein Kinases (MAPKs) Involved in TGF β Signalling

MAPK signalling intersects with TGF β superfamily pathways. MAPKs are a family of serine/threonine kinases that comprise signalling cascades and MAPK signalling is required for many facets of cellular regulation (Chang and Karin, 2001). MAPK cascades are initiated downstream of signals that activate certain receptor tyrosine kinases (RTKs) and the multifunctional GTPase effectors related to the oncoprotein Ras (Boguski and McCormick, 1993). TGF β family members can also activate these MAPK cascades and in various systems TGF β has been reported to activate Erk, JNK and p38 (reviewed in Moustakas et al., 2001).

Multiple interactions between MAPK pathways and TGF β superfamily pathways have been described and a number of these result in inhibition of TGF β superfamily signalling. For example, Smad1 is phosphorylated in its linker region by MAPKs and this phosphorylation appears inhibitory (Kretzschmar et al., 1997a). Furthermore Smad2 and Smad3 phosphorylation downstream of activated Ras also leads to inhibition of TGF β signalling (Kretzschmar et al., 1999). Some controversy exists for this role of MAPK. Others have reported minimal effects of MAPK activation on Smad2 function (Lehman et al., 2000) and studies in *Drosophila* support a role for MAPK-mediated inhibition of TGF β signalling that does not require phosphorylation of R-Smad (Kubota et al., 2000). Furthermore, in certain systems MAPK activity may be required to activate TGF β superfamily signalling and under some conditions TGF β and BMP signalling appears to require co-activation by Ras (Yue et al., 1999; Yue and Mulder, 2000). Complementary genetic evidence exists for this positive role of

MAPK in the example of *Drosophila* endoderm development (Szuts and Bienz, 2000; Szuts et al., 1998). Given the diversity of RTK signalling, opposite effects on TGF β superfamily signalling (inhibitory versus activating) are not necessarily mutually incompatible, and the precise *in vivo* mechanisms whereby MARK and TGF β signals intersect both positively and negatively awaits further research.

TGF β 3 Associated Kinase I (TAK1)

TAK1 signalling also interconnects with TGF β superfamily signalling. TAK1 is a MAPK kinase kinase (MAPKKK) and its activity is upregulated by TGF β and BMP. In addition, TAK1 modifies TGF β transcriptional responses (Yamaguchi et al., 1995). In *Xenopus* activated TAK 1 can mimic overexpression of BMP signalling in the early *Xenopus* embryo (Shibuya et al., 1998) and this role is linked to the BMP receptors by the *Xenopus* inhibitor of apoptosis protein (XIAP) (Yamaguchi et al., 1999). While the precise role of TAK1 awaits a knockout, these initial results indicate a potentially important, positive role for TAK1 in TGF β superfamily signalling.

Wingless/WNT Pathways

Along with TGF β superfamily pathways, the *Drosophila wingless (wg)* pathway and its mammalian correlate, the wingless/int or Wnt pathway play crucial roles in embryonic development and tumor progression (Akiyama, 2000). Together these pathways positively or negatively interact to regulate a range of biological effects. For instance, genetic Control of *Drosophila optomotor-blind (omb)* (Grimm and Pflugfelder, 1996), a gene involved in wing formation, and activation of the extradenticle homeodomain protein in the embryonic midgut (Mann and Abu-Shaar, 1996) are dependent on *dpp* and *wg*. In *Xenopus*, TGF β and Wnt pathways are required for formation of anterior endomesoderm (Zorn et al., 1999) and certain target genes are synergistically activated by both pathways which cooperatively pattern mesoderm using TGF β /activin (Crease et al., 1998) or BMPs (Hoppler and Moon, 1998). In addition, simultaneous repression of Wnt and BMP sig-

nalling is required for head induction in amphibians (Glinka et al., 1996). Together these observations are good circumstantial evidence in favour of wg/TGF β cross-talk.

Consistent with these biological data, TGF β and Wnt pathway effectors interact directly. First, a protein-protein interaction complex may exist between β -catenin, lymphoid enhancer binding factor-1/T cell-specific factor (Lef1/Tct) and Smad4 (Nishita et al., 2000). Furthermore, a TGF β -dependent interaction between Smad3 and Lef1 has also been demonstrated and shown to regulate synergistic induction of WNT target genes (Labbe et al., 2000). In another interesting example, the inhibitory Wnt pathway protein, axin, associates with Smad3 and facilitates its phosphorylation by TGF β receptors (Furuhashi et al., 2001). Mutants of axin which fail to bind Smad3, inhibit Smad3 phosphorylation which suggests that axin association with Smad3 is required for signalling. Together these results suggest a certain interdependence between TGF β and WNT pathways. It is unknown how extensive this interplay will turn out to be, but it may be a critical factor in governing the precise execution of complex developmental programs and may be important in the initiation or progression of human cancer

Conclusions.

Elucidation of the molecular components of the TGF β superfamily signal transduction pathways has provided important insights into the fundamental molecular events that underlie developmental processes and human disease. Indeed, many human syndromes and illnesses, both hereditary and spontaneous, have been attributed to mutations in components of TGF β family signalling pathway. For instance mutations in receptors are associated with Hereditary Hemorrhagic Telangiectasia, Primary Pulmonary Hypertension, Persistent Mullerian Duct Syndrome, Juvenile Polyposis Syndrome and colorectal and gastric carcinomas. Mutations in Smads have also been associated with cancers, particularly those of the colon and gastrointestinal tract. Undoubtedly, further elucidation of the molecular mechanisms in

this signalling pathway promises to provide new insights into cellular regulation and physiology in health and disease.

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