Development 136, 3699-3714 (2009) doi:10.1242/dev.030338

The regulation of TGFβ signal transduction

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Transforming growth factor β (TGFβ) pathways are implicated in metazoan development, adult homeostasis and disease. TGFβ ligands signal via receptor serine/threonine kinases that phosphorylate, and activate, intracellular Smad effectors as well as other signaling proteins. Oligomeric Smad complexes associate with chromatin and regulate transcription, defining the biological response of a cell to TGFβ family members. Signaling is modulated by negative-feedback regulation via inhibitory Smads. We review here the mechanisms of TGFβ signal transduction in metazoans and emphasize events crucial for embryonic development.

Introduction

The human transforming growth factor β (TGFβ) family consists of 33 members, most of which encode dimeric, secreted polypeptides that control developmental processes, ranging from gastrulation and body axis asymmetry to organ-specific morphogenesis and adult tissue homeostasis (reviewed by Derynck and Miyazono, 2008). In addition to TGFβs, this family includes the bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins and nodal. The TGFβ family is conserved throughout metazoan evolution. At the cellular level, TGFβ family members regulate cell growth, differentiation, adhesion, migration and death, in a developmental context-dependent and cell type-specific manner. For example, TGFβ more often inhibits, but sometimes also stimulates, cell proliferation (reviewed by Yang and Moses, 2008). Furthermore, nodal signaling sometimes inhibits, whereas BMP promotes, cell differentiation, as in stem cells (Watabe and Miyazono, 2009). As TGFβ ligands act multifunctionally in numerous tissue types, they also play complex roles in various human diseases, ranging from autoimmune to cardiovascular diseases and cancer (reviewed by Gordon and Blobe, 2008; Massagué, 2008).

Here we review the core components of the TGFβ family and their signaling engines, as part of a Minifocus in this issue on TGFβ signaling (see Box 1), and discuss emerging concepts concerning the regulatory mechanisms of TGFβ pathways at the receptor, cytoplasmic and nuclear level. We also highlight recent discoveries that are of particular developmental relevance.

The TGFβ family

The development of the axes and the asymmetry of the animal body depends on the localized action of extracellular signals, such as the Wnt, nodal and BMP ligands. Gradients of these ligands, their extracellular regulators and the competence of receptors in responding cells, play important roles during tissue morphogenesis (Affolter and Basler, 2007; Smith and Gurdon, 2004). TGFβ family members also contribute to tissue patterning and are important regulators of stem cell self-renewal and differentiation (see Box 2) (De Robertis and Kuroda, 2004; Watabe and Miyazono, 2009).

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The TGFβ morphogens include numerous secreted and conserved polypeptides (Table 1), which emerged at the onset of multicellular (metazoan) life (Huminiecki et al., 2009). Structurally, this family is characterized by a specific three-dimensional fold and by a conserved number and spacing of cysteine residues in the C-terminus of the mature polypeptide (Derynck and Miyazono, 2008). The prototypic TGFβ isoforms (TGFβ1, β2, β3), and the related inhibin β polypeptides that make up the activin and inhibin members, have nine characteristic cysteines, eight of which form four intramolecular disulfide bridges, while one intermolecular bond links the two monomers. The inhibin α polypeptides, BMPs and GDFs have seven cysteines, of which six form intramolecular and one intermolecular bridges. The lefty proteins, GDF3, GDF9 and BMP15A have six cysteines in their mature sequence and lack the intermolecular bridge between the two monomers. The lack of covalent dimers provides regulatory flexibility; for example, lefty forms non-covalent complexes with nodal and binds to the glycosylphosphatidylinositol (GPI)-anchored co-receptor of the epidermal growth factor-Cripto/FRL-1/Cryptic (EGF-CFC) family, leading to the inhibition of nodal signaling (Chen and Shen, 2004).

Xenopus laevis expresses TGFβs, nodal, activins, BMPs and GDFs (De Robertis and Kuroda, 2004), and additional unique family members, such as the mesoderm-inducer Derrière, and the six nodal-related proteins XNR1-6 (Eimon and Harland, 2002; Onuma et al., 2002; Ramis et al., 2007). *Drosophila melanogaster* has only seven TGFβ family members (Table 1). The BMP-like ligands Decapentaplegic (Dpp) and Screw (Scw) regulate dorsoventral pattering and the differentiation of imaginal discs, such as the wing disc (Affolter and Basler, 2007; Serpe et al., 2005). The BMP-like Glass bottom boat (Gbb) regulates brain and wing disc differentiation (Bangi and Wharton, 2006; Goold and Davis, 2007). The activin-like dActivin (Actβ – FlyBase) and Dawdle (Daw) ligands have tissue-specific roles (for example, in the larval brain), whereas much remains to be understood about the functions of Maverick, the GDF8 (myostatin)-like ligand, and of Myoglianin, which are expressed in endodermal and mesodermal cells (Lee-Hoeflich et al., 2005; Nguyen et al., 2000; Zhu et al., 2008). In *Caenorhabditis elegans*, the BMP-like DBL-1, and the TGFβ-like DAF-7, regulate body length and the dauer pathway, a special environmental adaptation of earthworms, respectively (Table 1) (reviewed by Savage-Dunn, 2005). The other three ligands, TIG-2, TIG-3 and UNC-129, are as yet unexplored.

Box 1. Minifocus on TGFβ signaling

This article is part of a Minifocus on TGFβ signaling. For further reading, please see the accompanying articles in this collection: 'The extracellular regulation of bone morphogenetic protein signaling' by David Umulis, Michael O'Connor and Seth Blair (Umulis et al., 2009); 'Informatics approaches to understanding TGFβ pathway regulation' by Pascal Kahlem and Stuart Newfeld (Kahlem and Newfeld, 2009); and 'TGFβ family signaling: novel insights in development and disease', a review of a recent FASEB Summer Conference on TGFβ signaling by Kristi Wharton and Rik Derynck (Wharton and Derynck, 2009).

Box 2. Role of TGFβ/BMP signaling in embryonic stem cells

Stem cells exhibit self-renewing capacity and pluripotency in generating the multitude of embryonic and adult cell types of the metazoan body (reviewed by Rossi et al., 2008). Growth factors, such as TGFβ and FGF, regulate stem cell self-renewal and differentiation. FGF2, the most widely used growth factor that supports mouse and human embryonic stem cell (ESC) self-renewal in culture, induces TGFβ/activin ligands and receptors while suppressing BMP-like activities (Greber et al., 2007; Ogawa et al., 2007). Furthermore, pharmacological inhibitors of the TGFβ/nodal type I receptor family suppress human and mouse ESC self-renewal (Ogawa et al., 2007). In general, TGFβ inhibits differentiation of pluripotent progenitor cells, whereas BMP induces their differentiation (Watabe and Miyazono, 2009) (Fig. 7A,B).

To promote self-renewal of ESCs, TGFβ/nodal signaling activates SMAD2 and SMAD3, which directly induce *Nanog*, one of the crucial stem cell transcription factors (Xu, R. H. et al., 2008). TGFβ and FGF signaling synergize by enhancing binding of Smad complexes to the *Nanog* promoter. Interestingly, NANOG provides a molecular link for the antagonism between TGFβ (the self-renewing factor) and BMP (the differentiation factor) in ESCs. NANOG binds to SMAD1, inhibiting its transcriptional activity and limiting the BMP signaling potential that promotes early mesodermal differentiation or tissue-specific differentiation later in development (Suzuki et al., 2006). This example is likely to be expanded to additional regulators of ESC self-renewal and differentiation as a result of genome-wide screens for the transcription and signaling factors of these pathways (Chen et al., 2008).

Although specification of body asymmetry is a fundamental function of TGFβ-like proteins during early embryogenesis, the identification of genes that encode a complete TGFβ pathway in the primitive metazoan *Trichoplax adhaerens*, a two-cell-layered animal that lacks obvious body asymmetry, suggests that these morphogens might have played a fundamental role in the specification of the multicellularity that precedes body asymmetry during animal evolution (Huminiecki et al., 2009).

TGFβ secretion and extracellular regulation

All TGFβ ligands are synthesized as precursor proteins with a longer N-terminal pro-peptide followed by a shorter C-terminal mature polypeptide (reviewed by ten Dijke and Arthur, 2007). Intermolecular disulfide linkages pair dimers of these precursors via conserved cysteine residues in the pro-peptide and mature peptide sequence. While precursor proteins are in the secretory pathway, furin-like proteases cleave the pro-peptide from the mature peptide. The TGFβ pro-peptide, called the latency-associated peptide (LAP), continues to scaffold the smaller mature peptide within its core, serving as a chaperone during exocytosis of the complex. It also mediates the deposition of TGFβ in the extracellular matrix (ECM) through its covalent association with large secreted proteins called latent TGFβ-binding proteins (LTBPs), and with ECM proteins, such as fibronectin and fibrillin 1 (reviewed by Rifkin, 2005). Activation of the mature C-terminal dimeric ligands from their matrix-deposited, multi-protein 'cages' relies on several proteases, including elastase (which cleaves fibrillin 1), BMP1/Tolloid family proteases (which cleave LTBPs), and matrix metalloproteases, such as MMP2 (which cleave TGFβ LAPs) (reviewed by ten Dijke and Arthur, 2007).

The ability of the TGFβ LAP to maintain the ligand in an inactive state is conserved among some TGFβ family ligands, such as GDF8 and GDF11 (Ge et al., 2005; Wolfman et al., 2003). However, the nodal, BMP4 and BMP7 pro-peptides do not act as extracellular antagonists, but instead regulate mature ligand stability and processing, including ligand degradation in lysosomes, which limits ligand availability (Degnin et al., 2004; Dick et al., 2000; Le Good et al., 2005). Similarly, the nodal pro-peptide associates with its EGF-CFC family co-receptor Cripto in secretory vesicles near the cell surface (Blanchet et al., 2008). Cripto also forms complexes with mature nodal and enhances signaling via the receptor kinase complex (see below) (Bianco et al., 2004). Recent evidence demonstrates that the signaling Cripto-nodal-receptor complex enters a specialized endocytic pathway that is characterized by the protein flotillin, possibly en route to its final degradation. Interestingly, many other TGFβ ligands are inactivated in the extracellular space by antagonists, such as noggin and chordin, which inhibit BMPs, and follistatin, which inhibits activins (Gazzerro and Canalis, 2006; Harrison et al., 2005). These extracellular antagonists help to establish the morphogen gradients that pattern early embryos, as discussed in an accompanying review (Umulis et al., 2009) (see Box 1).

The TGFβ receptor family

All TGFβ ligands transmit biological information to cells by binding to type I and type II receptors that form heterotetrameric complexes in the presence of the dimeric ligand (reviewed by Wrana et al., 2008). Five type II and seven type I receptors exist in humans and other mammals, and are characterized by a cytoplasmic kinase domain that has strong serine/threonine kinase activity and weaker tyrosine kinase activity, which classifies them as being dual-specificity kinases (Table 1) (reviewed by ten Dijke and Heldin, 2006). The type I receptors are also known as activin receptor-like kinases (ALKs), a nomenclature that is employed to tackle the problem of one ligand signaling via many receptors, or many ligands signaling via the same receptor. TGFβ ligands also interact with co-receptors that either facilitate or limit receptor kinase signaling. In addition to the EGF-CFC/Cripto co-receptors discussed above, type III receptors, such as endoglin and the proteoglycan betaglycan (TGFβR3; TβRIII), regulate TGFβ signaling in mammals, as does the repulsive guidance molecule (RGM, also known as Dragon) family of co-receptors (reviewed by Wrana et al., 2008) (Table 1).

Ligand binding links the constitutively active type II receptor kinases to the dormant type I receptor kinases, allowing the type II receptor to phosphorylate the juxtamembrane part of the cytoplasmic domain of the type I receptor (Fig. 1), turning on receptor kinase activity (reviewed by Wrana et al., 2008). Recent structural analysis of TGFβ and BMP ligands bound to their respective type I and type II receptor ectodomains shows that TGFβ ligands contact both receptors tightly, whereas the evolutionarily more ancient BMPs associate more loosely with their receptors (Groppe et al., 2008). Binding of TGFβ to TβRII (TGFβR2) creates the interface required for TβRI (ALK5; TGFβR1) type I receptor recruitment to the complex.

D. melanogaster has five TGFβ family receptors, including the type II receptors Punt (Put) and Wishful thinking (Wit), which bind the BMP-like ligands Dpp, Gbb and Scw during fly development and which form complexes with the type I receptors Thickveins (Tkv) and Saxophone (Sax) (Table 1) (Affolter and Basler, 2007; Goold and Davis, 2007; Serpe et al., 2005). Put and Wit also pair with the type I receptor Baboon (Babo) to mediate activin-like signals from dActivin and Daw (Zhu et al., 2008). The accompanying review by Umulis et al. (Umulis et al., 2009) discusses how, in the developing wing disc, a gradient of BMP-like signaling activity is achieved by the dual contribution of Dpp and Gbb, which differentially bind to distinct receptor complexes.

Table 1. TGFβ pathways in humans, flies and worms

	H. sapiens					
Pathway	BMP	GDF	Activin	TGFβ	AMH	Inhibitors
Ligand	BMP2, 4 BMP5, 6, 7 BMP8A, 8B BMP9, 10	GDF5, 6, 7 GDF9b GDF10, 11 GDF15 (MIC1) --- GDF1, 3 GDF8 (MYO) GDF9	Inhibin βA Inhibin βB Nodal	TGFβ1 TGFβ2 TGFβ3	AMH (MIS)	BMP3 Inhibin α Inhibin βC Inhibin βE LEFTYA LEFTYB
RII	BMPRII ActRIIA, ActRIIB	BMPRII ActRIIA, ActRIIB	ActRIIA ActRIIB	TβRII	AMHRII	N/A
RI	BMPRIA (ALK3) BMPRIB (ALK6) ALK2 ALK1	BMPRIA (ALK3) BMPRIB (ALK6) ALK2 --- ActRIB (ALK4) ALK7 TβRI (ALK5)	ActRIB (ALK4) ALK7	TβRI (ALK5) --- ALK1 ALK2 BMPRIA (ALK3)	BMPRIA (ALK3) BMPRIB (ALK6) ALK2	N/A
RIII	RGMa, b, c (+)	Cripto 3 (+)	Cripto 3 (–) Cripto 1 (+)	TβRIII (+) Endoglin (+) Cripto 3 (–)	?	TβRIII (–) Cripto 3 (–)
R-Smad	SMAD1, 5, 8	SMAD1, 5, 8 --- SMAD2, 3	SMAD2, 3	SMAD2, 3 --- SMAD1, 5, 8	SMAD1, 5, 8	N/A
Co-Smad	SMAD4	SMAD4	SMAD4	SMAD4	SMAD4	N/A
I-Smad	SMAD6, 7	SMAD6, 7	SMAD7	SMAD7	SMAD6, 7	N/A

	D. melanogaster			*C. elegans*	
Pathway	BMP	Activin	Other	Sma/Mab	Dauer
Ligand	Dpp Gbb Scw	dActivin Daw	Mav Myo	DBL-1	DAF-7
RII	Put Wit	Put Wit	?	DAF-4	DAF-4
RI	Tkv Sax	Babo	?	SMA-6	DAF-1
RIII	?	?	?	?	?
R-Smad	Mad	dSmad2	?	SMA-2 SMA-3	DAF-8 DAF-14
Co-Smad	Medea	Medea	?	SMA-4	DAF-3 (?)
I-Smad	Dad	?	?	TAG-68 (?)	TAG-68 (?)

Receptors are listed as type II (RII), type I (RI) and type III (RIII) co-receptors. Dashed lines separate groups of ligands or receptors based on the division into BMP and TGFβ/activin-like pathways. Ligands, type I receptors and R-Smads are color-coded: blue, BMP-like pathways; red, TGFβ/activin-like pathways. Question marks indicate unassigned signaling relationships. For two human ligands, GDF8 and GDF15, we provide their alternative names [myostatin (MYO) and macrophage inhibitory cytokine 1 (MIC1)], as the latter are more commonly used in the literature. LEFTYA and B are also known as LEFTY2 and 1, respectively. The co-receptor TβRIII is also known as betaglycan. Cripto 1 and Cripto 3 are also known as TDGF1 and TDGF3, respectively. In the RIII group (+) or (–) indicates positive or negative effects, respectively, on signaling by each co-receptor. N/A, not applicable.

C. elegans has three TGFβ family receptors (Table 1) (Patterson and Padgett, 2000). In the dauer pathway, DAF-7 signals via the type II receptor DAF-4 and the type I receptor DAF-1. In the Sma/Mab pathway, which regulates body length, tail development and innate immunity, DBL-1 signals via DAF-4 and the SMA-6 type I receptor. Which of these receptors mediate signals by UNC-129, TIG-2 and TIG-3 remains unknown.

Finally, *T. adhaerens* has one type II and three type I receptors (Huminiecki et al., 2009), which is compatible with a model in which the type II receptor represents the ligand-recognizing core, whereas the type I receptor is the downstream signaling effector that defines biological responses and that has diverged more rapidly to serve the new developmental processes of more complex organisms.

The Smad family

The activated type I receptor phosphorylates cytoplasmic proteins of the Smad family in their C-terminal regions (Figs 1 and 2). Smads consist of three domains: (1) an N-terminal Mad-homology 1 (MH1) domain that can interact with other proteins and carries nuclear localization signals (NLSs) and a DNA-binding domain; (2) a middle linker domain that interacts with prolyl-isomerases and ubiquitin ligases and that is enriched in prolines and phosphorylatable serines or threonines; and (3) a C-terminal MH2 domain that binds to type I receptors and can interact with other proteins, and that mediates Smad homo- and hetero-oligomerization and mediates the transactivation potential of nuclear Smad complexes (Fig. 2) (reviewed by ten Dijke and Heldin, 2006). The C-terminal phosphorylation of receptor-activated (R) Smads allows

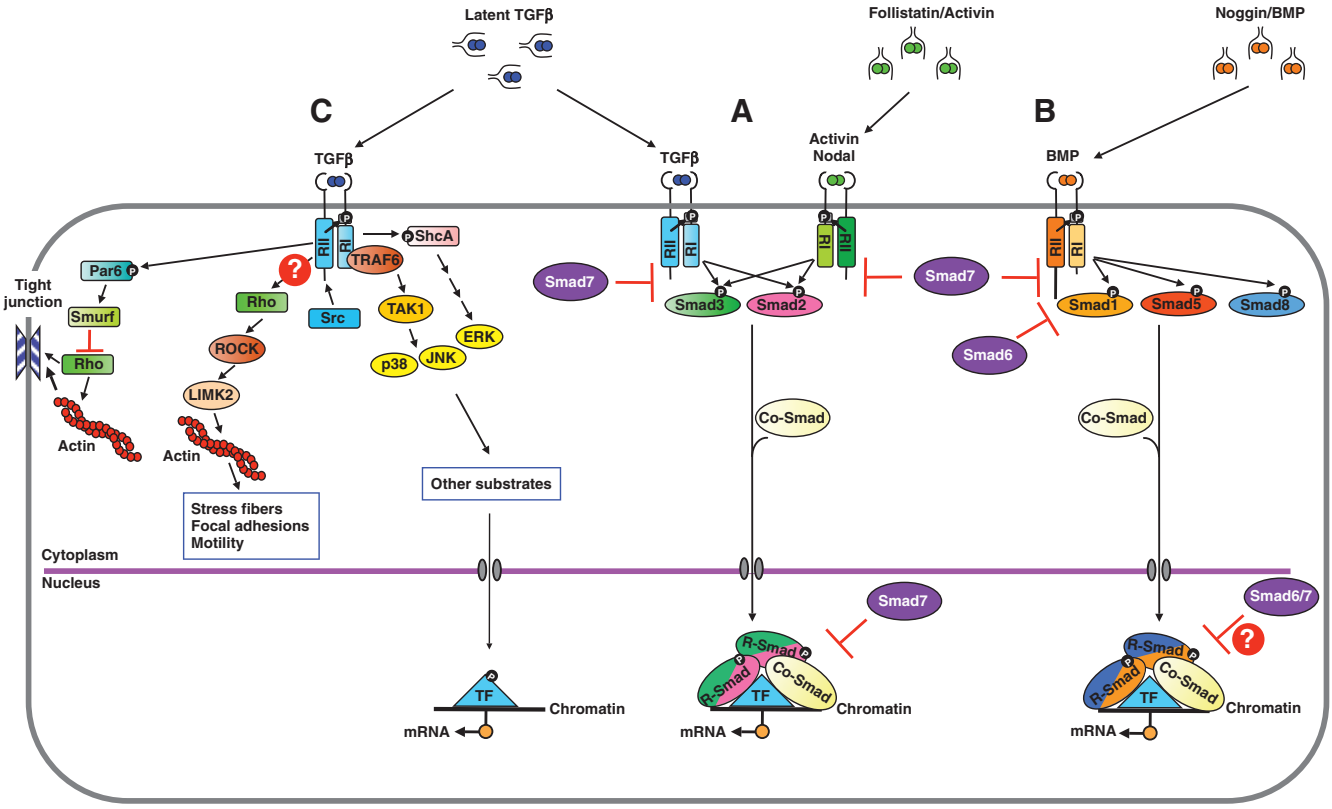

Fig. 1. TGFβ and BMP signaling. (**A**,**B**) The (A) TGFβ and activin/nodal and (B) BMP pathways, with their corresponding Smad proteins and mechanisms of inhibition by I-Smads (Smad6/7). The latent TGFβ complex and the extracellular antagonists, follistatin (bound to activin) and noggin (bound to BMP) are shown. (**C**) Non-Smad signaling pathways downstream of the TGFβ receptors [RI, TβRI (ALK5) and RII, TβRII (TGFβR2)]. The nuclear Smad complexes that lead to gene regulation are shown for each pathway. In these complexes, the Smad trimer most likely contains two R-Smad (identical or different) and one Co-Smad subunit. In addition to the major signaling pathways shown, TGFβ also activates BMP R-Smads in certain contexts (see text). BMP (bone morphogenetic protein), Erk (extracellular signal-regulated kinase), LIMK2 (LIM domain kinase 2), JNK (Jun N-terminal kinase), p38 (p38 MAPK), PAR6 (partitioning-defective 6 homolog), Rho (Ras homolog), ROCK (Rho-associated, coiled-coil-containing protein kinase), SHCA (SH2 domain-containing sequence A), Smurf (Smad ubiquitylation regulatory factor), Src (Rous sarcoma virus oncoprotein), TGFβ (transforming growth factor β), TAK1 (TGFβ-activated kinase 1), TF (transcription factor), TRAF6 (tumor necrosis factor α receptor-associated factor 6).

them to associate with the common-mediator (Co) Smad, SMAD4. The resulting Smad oligomer is thought to consist of a trimer of two R-Smads and a single SMAD4 (such as a SMAD2-SMAD2-SMAD4 complex, a SMAD3-SMAD3-SMAD4 complex, or a SMAD2-SMAD3-SMAD4 complex), which is then shuttled into the nucleus. Nuclear Smad complexes bind to chromatin, and, together with other transcription factors, regulate target gene expression (Fig. 1) (reviewed by Massagué et al., 2005; Schmierer and Hill, 2007). TGFβ- or BMP-specific Smad complexes induce the expression of the inhibitory (I) Smads, SMAD6 and SMAD7 (Figs 1 and 2), which negatively regulate signaling strength and duration, thus forming a negative-feedback loop (reviewed by Itoh and ten Dijke, 2007).

All genomes sequenced to date possess the three fundamental classes of Smad proteins: R-, Co- and I-Smads (Table 1) (Huminiecki et al., 2009). In *D. melanogaster*, BMP-like signaling is mediated by a single R-Smad (Mad) and a single Co-Smad (Medea), despite the existence of two type I receptors (Tkv, Sax) (Affolter and Basler, 2007). In the activin-like pathways, the type I receptor Babo signals via dSmad2 (Smox) (an R-Smad) and Medea. A single I-Smad (Dad) also operates during wing imaginal disc development (Tsuneizumi et al., 1997). Dad binds and inhibits signaling from the Dpp/Scw/Gbb type I receptors Tkv and Sax, but not from the dActivin type I receptor Babo (Kamiya et al., 2008).

In *C. elegans*, the Sma/Mab pathway engages two R-Smads, SMA-2 and SMA-3, that signal with a single Co-Smad, SMA-4 (Patterson and Padgett, 2000). The dauer pathway has two R-Smads, DAF-8 and DAF-14, which have more divergent MH1 domains but conserved MH2 domains that suggest activation by the corresponding type I receptors. The dauer pathway Co-Smad is possibly DAF-3, which presents peculiar developmental characteristics. Unlike the Co-Smads in other organisms and SMA-4 in *C. elegans*, the DAF-3 loss-of-function phenotype is distinct from those of loss-of-function mutations in the ligand, receptors or R-Smads of this pathway (Patterson et al., 1997). Furthermore, the receptors and R-Smad seem to negatively regulate the function of DAF-3. Thus, DAF-3 is classified as a Co-Smad only on the basis of sequence similarity to other Co-Smads (Huminiecki et al., 2009). Finally, TAG-68 is classified as an I-Smad based on phylogenetic arguments, although functional evidence for such a role is currently absent (Savage-Dunn et al., 2003). *T. adhaerens* also has three distinct Smad classes, which suggests that the three different functional features of Smad proteins evolved early during metazoan evolution (Huminiecki et al., 2009).

A fundamental feature of all TGFβ signaling pathways is their division into TGFβ-like and BMP-like cascades, a classification based on the specificity of interaction between the so-called L45 loop of the

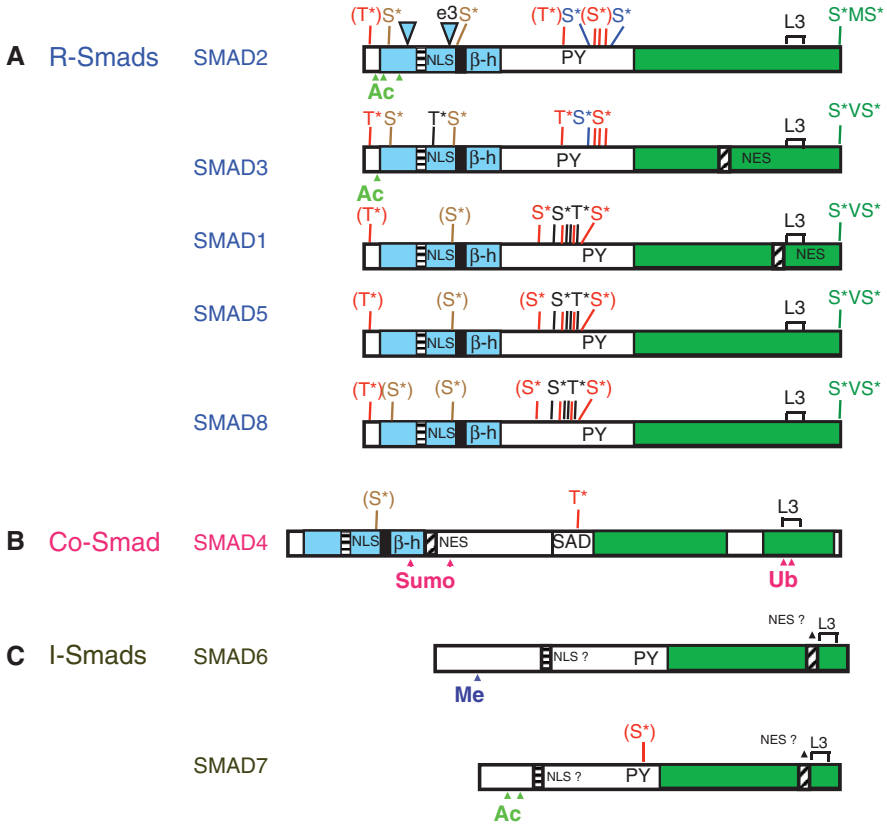

Fig. 2. The Smad family. Simplified structures of the eight human Smad proteins divided into (**A**) Receptor-activated (R) Smads; (**B**) common-mediator (Co) Smad; and (**C**) inhibitory (I) Smads. The conserved N-terminal Mad-homology 1 (MH1) (blue) and C-terminal MH2 (green) domains are shown. Highlighted are the nuclear localization signal (NLS, striped box); the two unique inserts of SMAD2 (triangles), the second of which corresponds to exon 3 (e3); the β-hairpin domain that binds to DNA (β-h, black box); the proline-tyrosine (PY) motif in the linker domain that is recognized by the WW domain of Smurf family proteins; the Smad activation domain (SAD) at the linker-MH2 border; the nuclear export signal (NES, hatched box); and the L3 loop of the MH2 domain. Phosphorylatable serine and threonine residues are shown; S/T* indicates experimentally proven phosphorylation sites; (S/T*) indicates a conserved residue with a predicted phosphorylation motif that awaits experimental validation. The C-terminal serines that are phosphorylated by the type I receptor kinases are shown in green (S*VS*, S*MS*); red S/T residues are phosphorylated by the MAPKs ERK1/2; brown S/T residues are phosphorylated by protein kinase C and by calmodulin-dependent kinase II; blue S/T residues are phosphorylated by cyclin-dependent kinases; and black S/T residues are phosphorylated by glycogen synthase kinase 3β. Sumoylation (Sumo), ubiquitylation (Ub), methylation (Me) and acetylation (Ac) sites are indicated with colored arrowheads.

type I receptors and the L3 loop of the MH2 domains of R-Smads (Fig. 2) (reviewed by ten Dijke and Heldin, 2006). TGFβ/activin pathways signal via SMAD2, SMAD3, and BMP/GDF pathways via SMAD1, SMAD5 and SMAD8. However, in a diversity of cell types in culture, such as endothelial, immortalized epithelial, adenoma and carcinoma cell lines and even NIH-3T3 fibroblasts and chondrocytes (Daly et al., 2008; Finnson et al., 2008; Goumans et al., 2003; Liu et al., 2008), TGFβ signaling can also activate SMAD1 and SMAD5. Originally, TGFβ was shown to bind to two type I receptors, thus activating SMAD2 and SMAD3 via TβRI, and SMAD1, SMAD5 and SMAD8 via the BMP type 1 receptor ALK1 (ACVRL1). ALK1 is expressed mainly in endothelial cells, where SMAD2, SMAD3 signaling inhibits and SMAD1, SMAD5, SMAD8 signaling promotes proliferation and migration (Goumans et al., 2003). However, TGFβ can also activate SMAD1 and SMAD5 via two additional mechanisms. First, in immortalized EpH4 mouse mammary epithelial cells and in MDA-MB-231 human mammary carcinoma cells, TGFβ induces SMAD1 and SMAD5 C-terminal phosphorylation via heteromeric receptor complexes that form between TβRII and TβRI, as well as between TβRII and the BMP type I receptors ALK2 (ACVR1) and BMPRIA (ALK3) (Daly et al., 2008). Second, in 4T1 mouse mammary carcinoma cells, TGFβ leads to SMAD1 and SMAD5 phosphorylation without the requirement of a BMP-like type I receptor because the TβRI receptor kinase can directly phosphorylate SMAD1/5 (Liu et al., 2008). These findings suggest that a re-evaluation of TGFβ family signaling is needed through the elucidation of the type I receptors and Smad pathways that function in specific physiological and developmental contexts.

TGFβ family signaling via non-Smad signaling proteins

Other proteins mediate TGFβ signaling in addition to Smads (see also Moustakas and Heldin, 2005), and here we describe the mechanistically best-established examples (Fig. 1C). TβRII phosphorylates the polarity protein PAR6, which regulates the local degradation of the RHOA small GTPase that controls the assembly of intercellular tight junctions in mammalian cells (Ozdamar et al., 2005). As tight junctions disassemble, epithelial architecture disintegrates, followed by de-differentiation known as the epithelial-to-mesenchymal transition (EMT), an important developmental and disease-associated process that is regulated by TGFβ signaling (reviewed by Moustakas and Heldin, 2007). During EMT, in

addition to tight junctions, adherens junctions and desmosomes of polarized epithelial cells are destroyed and remodeled to give rise to mesenchymal-like cells that are motile and invasive. It should be noted that, in addition to the above direct mechanism of tight junction disassembly, TGFβ elicits EMT via Smad signaling, leading to the transcriptional induction of major inducers of this differentiation process (Thuault et al., 2008).

Whereas the TGFβ-PAR6 pathway locally degrades RHOA in a breast epithelial cell culture model, other studies have demonstrated the positive activation of Rho GTPase signaling by TGFβ and BMP receptors in diverse cell types (reviewed by Kardassis et al., 2009). However, the mechanism of Rho activation by TGFβ receptors remains unclear (Fig. 1C).

In a distinct mechanism, the TGFβ type I receptor phosphorylates both serine and tyrosine residues in the SHCA (SHC1) adaptor, which then recruits the adaptor protein GRB2 and the Ras guanine exchange factor (GEF) son of sevenless (SOS) in mammalian cells (Fig. 1C) (Lee, M. K. et al., 2007). This leads to activation of the Ras-Raf-MEK-Erk mitogen-activated protein kinase (MAPK) signaling cascade, which can regulate cell proliferation or migration. Future work might decipher to what extent a specific biological response to TGFβ receptor signaling depends on its strong serine/threonine, or on its weaker tyrosine kinase, activity.

The tyrosine kinase Src can phosphorylate Tyr284 in the cytoplasmic domain of the TβRII receptor, leading to GRB2 and SHC recruitment and to the activation of the p38 MAPK pathway (Galliher and Schiemann, 2007). Src-dependent TβRII phosphorylation regulates breast cancer cell proliferation and invasiveness, possibly without affecting the Smad signaling output (Galliher-Beckley and Schiemann, 2008).

As a final example, TGFβ-induced receptor heterotetramers recruit the ubiquitin ligase tumor necrosis factor α receptor-associated factor 6 (TRAF6) to the TβRI cytoplasmic domain in mammalian cells (Fig. 1C). TRAF6 ubiquitylates and activates the catalytic activity of the TGFβ-activated kinase 1 (TAK1; MAP3K7), leading to activation of the p38 and c-Jun N-terminal kinase (JNK) cascades, which regulate apoptosis or cell migration (Sorrentino et al., 2008; Yamashita et al., 2008). The TβRI kinase activity is dispensable for this pathway (Sorrentino et al., 2008).

The developmental significance of these non-Smad pathways remains to be elucidated. However, it has recently been shown that both p38 MAPK and Smad signaling play important roles downstream of TGFβ during mouse palate and tooth development (Xu, X. et al., 2008). In *Xenopus*, the adaptor protein TRAF4 has positive signaling roles in mediating both the BMP and nodal signals that regulate neural crest differentiation and migration (Kalkan et al., 2009). TRAF4 is a substrate of the ubiquitin ligase Smad ubiquitylation regulatory factor 1 (SMURF1), which polyubiquitylates and promotes TRAF4 degradation, thus limiting the activity of the BMP and nodal pathways in the *Xenopus* neural plate. Signaling via multiple effectors enables the TGFβ pathways to be controlled by other pathways, as we discuss below, through the mechanisms that control Smad function in different cell compartments.

TGFβ receptor regulation and endocytosis

Receptor phosphorylation is important for TGFβ family signal transduction, and thus receptor dephosphorylation might also be important. New evidence shows that TGFβ/nodal receptors are reciprocally regulated by Bα and Bδ, two isoforms of regulatory subunit B of the protein phosphatase 2A (PP2A). PP2A that contains the Bα subunit positively, whereas PP2A that contains the Bδ subunit negatively, regulates receptor signaling in *Xenopus* embryos and in mammalian cells (Batut et al., 2008). The direct molecular targets of PP2A and the serine or threonine residues that they dephosphorylate await further analysis.

In addition, TβRI can be sumoylated by an as yet unknown sumo-ligase in mammalian cells (Fig. 3A), which enhances TGFβ signaling by facilitating SMAD3 recruitment to the receptor for phosphorylation (Kang et al., 2008). TβRI sumoylation might provide a docking site for an adaptor that mediates SMAD3 binding to the receptor, might induce a conformational change in the receptor that is required for SMAD3 binding, or might be coupled to the internalization mechanism.

The activated TGFβ receptors are internalized via clathrin-coated pits into early endosomes, where the receptors encounter Smad anchor for receptor activation (SARA; ZFYVE9), which facilitates the recruitment of SMAD2 and SMAD3 to TβRI and their subsequent phosphorylation (Fig. 3A,B) (Tsukazaki et al., 1998). SARA binds SMAD2 and SMAD3, but not BMP R-Smads, and also contacts the TβRI receptor. A homolog of SARA, endofin (ZFYVE16), plays a similar role in BMP pathways (Fig. 3D,E) (Shi et al., 2007). However, endofin can also operate in TGFβ pathways by scaffolding SMAD4 and by mediating the formation of complexes of SMAD2, SMAD3 and SMAD4 in association with the type I receptor (Chen et al., 2007).

In mammals, SARA cooperates with the cytoplasmic promyelocytic leukemia (cPML) tumor suppressor protein, which stabilizes the SARA-Smad complex (Fig. 3B) (Lin et al., 2004). This process also involves another adaptor, the PML competitor for TGIF association (PCTA), which binds to the nuclear homeodomain repressor protein TGIF (5′TG3′-interacting factor) and retains cPML in the nucleus (Faresse et al., 2008). In response to TGFβ, PCTA releases cPML to translocate to the cytoplasm, reach SARA and promote TGFβ signaling.

The *Drosophila* ortholog, dSARA, plays a similar role to mammalian SARA during Dpp/Mad signaling (Bökel et al., 2006). Since dSARA has no other homolog in *Drosophila*, it might mediate both Mad and dSmad2 signaling, although this remains to be established. In the epithelial cells of the *Drosophila* wing, which undergo apical and basolateral differentiation, Dpp receptor-dSARA complexes reside in apically located endosomes and are precisely segregated during cell division (Bökel et al., 2006). In this way, the daughter cells receive equal numbers of signaling complexes, which ensures the conservation of signaling strength from mother to daughter cells.

Many additional cytoplasmic regulators of early TGFβ receptor signaling have been described recently (Fig. 3B). Most notably, the small GTPase RAP2 inhibits activin/nodal receptor recycling, thus controlling receptor levels on the surface of *Xenopus* embryonic cells (Choi et al., 2008). During signaling, RAP2 antagonizes the negative effects of SMAD7, thus positively contributing to nodal signal propagation and the onset of gastrulation. In addition, RIN1, a RAB5 GEF, promotes TGFβ receptor endocytosis and overall signaling, which contributes to the pro-tumorigenic action of TGFβ in breast epithelial cells (Hu et al., 2008). The PDZ-containing protein erbin (ERBB2IP) binds to phosphorylated SMAD2, SMAD3, prevents their association with SMAD4 in mammalian cells, and produces opposite effects on signaling to SARA or endofin (Dai, F. et al., 2007). The protein Dapper 2 (DACT2) contributes to TGFβ receptor downregulation, which modulates nodal signaling in *Xenopus*, zebrafish, and mice (Su et al., 2007), although its partners and mechanism of action require further exploration. It would be interesting to elucidate the mechanism that regulates the

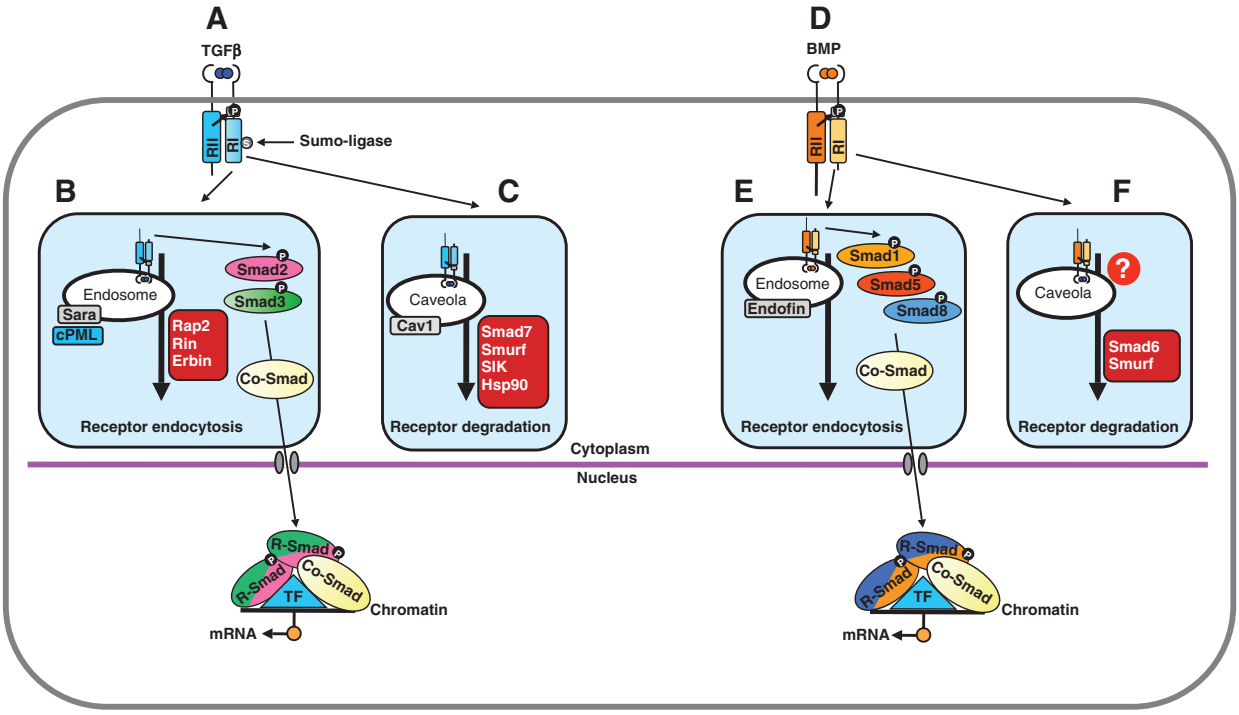

Fig. 3. TGFβ receptor endocytosis and downregulation. (**A**,**D**) The (A) TGFβ and (D) BMP receptor complexes are shown at the plasma membrane. Type II receptors are labeled as RII, type I receptors as RI. Sumoylation (S) of the TβRI that positively influences Smad signaling is highlighted. (**B**) TGFβ receptors are internalized in endosomes characterized by the presence of the endocytic protein SARA and the regulatory adaptor cPML. The receptors can signal by SMAD2 and SMAD3 phosphorylation and activation of the Co-Smad and by accumulation of nuclear Smad complexes on chromatin. Key regulatory proteins involved in the process of receptor endocytosis are listed on the side of the arrow that indicates the flow of endosomal trafficking. (**C**) The TGFβ receptor degradation pathway via caveolae, which are characterized by the presence of caveolin 1 (CAV1), and the key regulatory proteins involved as shown within the box. (**E**) The corresponding endocytic pathway for BMP receptors with the endocytic protein endofin is less well understood. (**F**) The corresponding caveolae-based degradation pathway for the BMP receptors remains unexplored (?). Key established regulatory proteins are shown in the box. BMP (bone morphogenetic protein), cPML (cytoplasmic promyelocytic leukemia protein), HSP90 (heat-shock protein of 90 kDa), RAP2 (Ras-related protein 2), RIN (Ras-like protein expressed in neurons), SARA (Smad anchor for receptor activation), SIK (salt-inducible kinase), Smurf (Smad ubiquitylation regulatory factor), TGFβ (transforming growth factor β).

recruitment of positive regulators, such as SARA, endofin and RIN1, and of negative regulators, such as RAP2, erbin and Dapper 2, to the TGFβ receptor complex to ensure appropriate R-Smad phosphorylation and SMAD4 association.

Receptor endocytosis both controls the flow of signaling and regulates the availability of TGFβ ligand on the cell surface. An in vitro kinetic analysis of TGFβ ligand bioavailability has shown that constitutive endocytosis of TβRII depletes excess ligand (Clarke et al., 2009). This mechanism enables a cell to fine-tune the level of signaling growth factor on the cell surface.

The regulatory proteins described above highlight the link between TGFβ signaling and the regulation of receptor internalization. However, the developmental relevance of many of these factors awaits further analysis.

TGFβ receptor downregulation and the role of I-Smads

TGFβ ligand-receptor complexes can be additionally internalized via lipid rafts into caveolae, and then into lysosomes, where the ligand-receptor complex is degraded (Di Guglielmo et al., 2003) (Fig. 3C). TGFβ receptor trafficking via caveolae is marked by their association with I-Smads and SMURF1 or SMURF2 ubiquitin ligases, which negatively regulate the signaling cascade.

The inhibitory Smads, SMAD6 and SMAD7, bind to type I receptors, thereby competitively inhibiting R-Smad phosphorylation and recruiting phosphatases and Smurf ubiquitin ligases to downregulate receptor levels and function (reviewed by Itoh and ten Dijke, 2007). Whereas SMAD7 inhibits both TGFβ and BMP pathways, SMAD6 more selectively inhibits BMP pathways (Fig. 1) and shows greater selectivity for the BMP type I receptors ALK1, ALK2, ALK3 and ALK6, as demonstrated recently in mammalian cells. Furthermore, SMAD6 binds with even higher affinity to specific amino acid residues in the BMPRIA (ALK3) and BMPRIB (ALK6) kinase domains, than to ALK1 and ALK2 domains (Goto et al., 2007). By contrast, SMAD7 shows broader specificity as it binds to all type I receptors via specific lysine residues in its MH2 domain (Mochizuki et al., 2004).

Two regulatory mechanisms that mediate the SMAD7-dependent ubiquitylation and downregulation of the TGFβ receptor have recently been uncovered in mammalian cells (Fig. 3C). The chaperone protein HSP90 binds to TβRII and to TβRI and protects them from ubiquitylation by SMURF2, positively contributing to TGFβ signaling (Wrighton et al., 2008). Conversely, the AMP-regulated kinase member salt-inducible kinase (SIK) is induced at the mRNA and protein levels by TGFβ

signaling, concomitantly with the induction of SMAD7 and SMURF2 (Kowanetz et al., 2008). SIK binds to SMAD7 and to TβRI to promote receptor downregulation. The *C. elegans* SIK ortholog, KIN-29, exhibits a conserved function by regulating body size in the Sma/Mab pathway; however, the molecular mechanism of KIN-29 action in worms remains unexplored (Maduzia et al., 2005).

Although SMAD7 primarily acts at the type I receptor level, it also resides in the nucleus, and new evidence suggests that it can bind to DNA and to nuclear complexes of SMAD2, SMAD3 and SMAD4, disrupting their complexes and inhibiting their transcriptional activity (Fig. 4) (Zhang et al., 2007).

Based on the importance of the mechanisms of TGFβ receptor endocytosis and downregulation, and the links of such mechanisms to the nucleocytoplasmic shuttling of Smads (see below), future studies into the biology of I-Smads promise to reveal interesting and novel findings.

Regulation of Smad trafficking by motor proteins

In parallel to TGFβ receptor endocytosis, R-Smads become phosphorylated by the type I receptors and accumulate in the nucleus. However, Smads, like the receptors, show dynamic mobility and shuttle in and out of the nucleus even when they are not activated by receptors (reviewed by Moustakas and Heldin, 2008). The cytoplasmic trafficking of both TGFβ receptors and Smads is often mediated by motor proteins that are associated with microtubules. Motor proteins are important both before and after R-Smad C-terminal phosphorylation.

Accordingly, Smads interact with kinesin 1, which mediates the recruitment of SMAD2 to the receptor complex in *Xenopus* and mammalian cells (Batut et al., 2007). Smads are sequestered away from the receptors when bound to microtubules, from where they can be released, for example, by connexin 43 (GJα1), which competes with microtubules to bind Smads (Dai, P. et al., 2007). Additional motor proteins, such as the dynein light chain km23-1 (DYNLRB1), also promote Smad traffic towards the nucleus of mammalian cells (Jin et al., 2007). The developmental roles of the connexin 43 and km23-1 mechanisms have not yet been specifically addressed.

Microtubules also transport specialized pools of Smad proteins. For example, the pool of SMAD1 that has been subjected to inhibitory phosphorylation in its linker domain by Erk MAPKs (see below) moves towards the centrosome via microtubules, where it is degraded by proteasomes (Fuentealba et al., 2008). Interestingly, when mammalian embryonic and adult cells, or *Drosophila* blastoderm cells, complete mitosis, phosphorylated SMAD1 and the centrosomal degrading apparatus segregate to only one of the two daughter cells (Fuentealba et al., 2008). Thus, Smad trafficking and segregation to daughter cells is regulated developmentally in a stage-specific manner. Additional studies in *Xenopus* embryos show that SMAD2-SMAD4 complexes are recruited to chromatin during every cell division, when mitosis dissolves the nuclear envelope (Saka et al., 2007). Again, this event is regulated developmentally as it does not occur prior to the mid-blastula transition. Moreover, SMAD3 regulates the activity of the anaphase-promoting complex, a primary initiator of mitosis during the mammalian cell cycle (Fujita et al., 2008). Whether these three mechanisms of Smad regulation during embryonic cell mitosis constitute one and the same process remains to be elucidated.

Mechanisms of Smad shuttling through the nuclear envelope

Smad nuclear import via nuclear pores is mediated by nucleoporins, which are integral constituents of the pores, and by importins, carrier proteins that bind to both cargo proteins and nucleoporins and that catalyze their nuclear translocation in an energy-dependent manner (reviewed by Moustakas and Heldin, 2008). All Smads have a conserved NLS in their MH1 domain (Fig. 2), which binds to specific importins, such as importin β (in

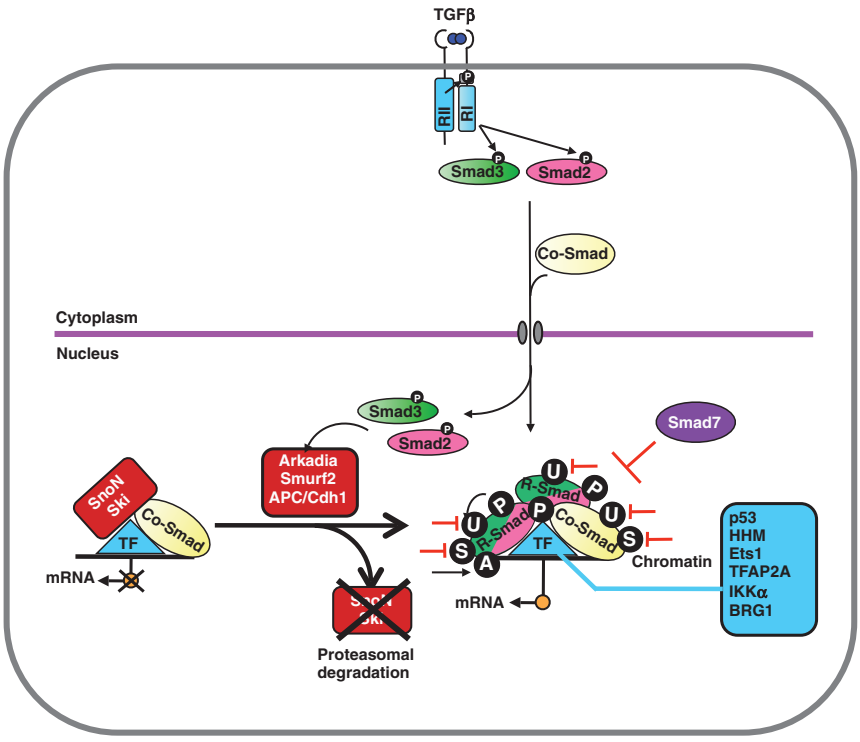

Fig. 4. Regulation of the nuclear Smad complex. Following TGFβ receptor activation, the activated Smad complex translocates to the nucleus and associates with various Smad partners [represented by the blue, triangular transcription factor (TF) and by examples discussed in the text] and undergoes post-translational modifications (A, acetylation; U, ubiquitylation; S, sumoylation; P, phosphorylation). Black arrows indicate a positive effect of a post-translational modification on Smad transcriptional function, red T-bars indicate a negative effect. (Left) Derepression of Smad target genes when the SNON/SKI co-repressors are proteasomally degraded after being ubiquitylated by the ubiquitin ligases arkadia, SMURF2 or APC/CDH1. APC/CDH1 (anaphase-promoting complex and its ubiquitin ligase subunit CDH1), BRG1 (Brahma-related gene 1), ETS1 (v-ets erythroblastosis virus E26 oncogene homolog 1), HHM (human homolog of Maid), IKKα (IκB kinase α), SMURF2 (Smad ubiquitylation regulatory factor 2), TFAP2A (transcription factor activating enhancer-binding protein 2α), TGFβ (transforming growth factor β).

the case of SMAD1 and SMAD3) and importin α (in the case of SMAD4). A recent genome-wide study in *Drosophila* S2 cells reported the roles of additional importins, such as Msk, which imports Mad, and of its mammalian orthologs, importin 7 and importin 8, which import SMAD2, SMAD3 and SMAD4 (Xu et al., 2007; Yao et al., 2008).

Smad proteins have characterized nuclear export signals (NESs) in their MH2 (SMAD1, SMAD3) or linker (SMAD4) domains (Fig. 2). The NESs bind specific exportins – exportin 4 for SMAD3 and exportin 1 for SMAD1 and SMAD4 – and export is catalyzed by the small GTPase RAN. Recently, a new exportin was described for SMAD2 and SMAD3, the RAN-binding protein 3 (RANBP3), which is a known exportin family member (Dai et al., 2009). RANBP3 was shown to recognize dephosphorylated nuclear SMAD2 and SMAD3 proteins and export them to the cytoplasm. Importins and exportins for SMAD5 and SMAD8 or for the I-Smads remain to be characterized. However, as we discuss below, our understanding of how Smads are transported into the nucleus has been significantly advanced by recent findings.

Regulation of Smad nuclear shuttling

The dynamic movement of Smads in and out of the nucleus is highly regulated. A recent mathematical model of SMAD2 and SMAD4 trafficking reported that their nuclear accumulation in response to TGFβ reflects a shift in the equilibrium between the cytoplasmic and nuclear pools of Smads that is brought about by a decrease in nuclear export (Schmierer et al., 2008). During signaling, however, low-level R-Smad dephosphorylation by nuclear phosphatases, among other factors, continues to ensure their subsequent nuclear export (Schmierer et al., 2008).

R-Smad shuttling is regulated not only by cycles of receptor-mediated C-terminal phosphorylation and dephosphorylation by nuclear phosphatases (reviewed by Wrighton et al., 2009), but also by sumoylation and ubiquitylation. Sumoylation of SMAD3 by the protein inhibitor of activated Stat y (PIASy; PIAS4) sumo-ligase promotes its nuclear export in mammalian cells (Imoto et al., 2008). Sumoylation of Medea, by an as yet unidentified sumo-ligase, also promotes its nuclear export, providing negative regulation that restricts the competence of early *Drosophila* embryonic cells to respond to Dpp (Miles et al., 2008). This mechanism resembles the previously established role of mammalian SMAD4 sumoylation by PIAS ligases (reviewed by Lönn et al., 2009). It is possible that in vivo Smad sumoylation might not negatively regulate TGFβ signaling; rather, it might promote continuous Smad shuttling. However, under overexpression conditions, sumo-ligases may shift the shuttling equilibrium by pushing Smads to the cytoplasm, thus reducing their time in the nucleus.

SMAD4 can also be monoubiquitylated (Morén et al., 2003) by the nuclear ubiquitin ligase TIF1γ (ectodermin; TRIM33), which promotes its nuclear export and inhibits the formation of nuclear complexes of SMAD2, SMAD3 and SMAD4 (Dupont et al., 2009). Once monoubiquitylated, SMAD4 is exported from the nucleus (Wang et al., 2008), whereupon fat facets in mouse (FAM; USP9X) deubiquitylates it, recharging it for subsequent cycles of shuttling, as demonstrated in *Drosophila*, *Xenopus* and human cells (Dupont et al., 2009).

Other mechanisms also regulate the nuclear residence and function of Smad complexes. Heteromeric complexes of SMAD2, SMAD3 and SMAD4 bind to the shuttling protein transcriptional co-activator with PDZ-binding motif (TAZ) in the nucleus of human cells, and are then recruited to chromatin via factors such as the activator-recruited co-factor (ARC) protein ARC105 (MED15), a member of the Mediator complex that ensures the progression of gene transcription (Varelas et al., 2008). TAZ is regulated by phosphorylation and by interaction with 14-3-3 family adaptors that control its timely residence in the nucleus. Furthermore, the *Drosophila* nuclear lamin Otefin interacts with Medea and tethers Smad complexes to the nuclear envelope (Jiang et al., 2008). The Otefin-Medea complexes bind to specific gene-regulatory elements that control germline stem cell development (Jiang et al., 2008). Future research into Smad shuttling and the regulation of Smad compartmentalization will bring to light additional regulatory mechanisms of TGFβ signaling.

Negative regulation of Smad signaling by phosphorylation and ubiquitylation

In addition to regulating Smad nucleocytoplasmic shuttling, C-terminal tail dephosphorylation, linker domain phosphorylation and ubiquitylation are implicated in the negative regulation of Smad signaling (Figs 2 and 4).

The developmental importance of such post-translational modifications has been recognized during BMP-dependent neurogenesis in *Xenopus* and mouse C_2C_{12} osteoblast differentiation. Fibroblast growth factor (FGF) signaling via Ras-Erk MAPK negatively regulates BMP signaling, as Erk (and GSK3β kinase) directly phosphorylates the SMAD1 linker, leading to recruitment of SMURF1 and to the proteasomal degradation of SMAD1 in perinuclear centrosomes (Fuentealba et al., 2007; Sapkota et al., 2007). During *Xenopus* neurogenesis, SMAD1 degradation is triggered in three ways: by chordin antagonising BMP activity extracellularly; by the Wnt antagonist Dickkopf 1 blocking Wnt activity extracellularly; and by Erk MAPK pathway activation via FGF and insulin-like growth factor (IGF) signaling. Thus, FGF/IGF signaling provides negative feedback to BMP signaling in the developing nervous system. Conversely, Wnt signaling induces GSK3β degradation, leading to decreased SMAD1 linker phosphorylation and to its prolonged signaling, an event that is required for *Xenopus* epidermal differentiation (Fuentealba et al., 2007). This is a good example of positive cross-talk between Wnt and BMP signaling during skin differentiation.

GSK3β also negatively controls TGFβ signaling, as it directly phosphorylates SMAD3 in its MH1 domain in mammalian cells (Guo et al., 2008a). This phosphorylation is followed by the ubiquitylation and proteasomal degradation of SMAD3, which regulate its steady-state levels. By contrast, upon TGFβ receptor phosphorylation, SMAD3 can be further phosphorylated in its MH2 domain by casein kinase 1 γ2, which leads to the specific ubiquitylation and degradation of the activated form of SMAD3 (Guo et al., 2008b).

A recent report has shed more light on the complexity of Smad regulation through the phosphorylation of its linker domain (Wang et al., 2009). After SMAD3 C-terminal phosphorylation by TβRI in mammalian cells, nuclear GSK3β and cyclin-dependent kinases phosphorylate three distinct SMAD3 linker residues, downregulating its transcriptional activity. Thus, TGFβ signaling tightly controls the activity of one of its main transducers through highly regulated phosphorylation events.

Recent evidence also shows that during mitosis of mammalian cells in culture, the kinase MPS1 (TTK) can directly C-terminally phosphorylate SMAD2 and SMAD3, thus activating their nuclear activities in the absence of TGFβ receptor activation (Zhu et al.,

2007). This is one of the first clear TGFβ-independent mechanisms that engages Smads and mimics the action of TGFβ. Thus, whereas R-Smad C-terminal phosphorylation by type I receptor kinases is a positive regulator of TGFβ family signaling, Smad phosphorylation by other kinases can negatively affect TGFβ family signaling in a cell cycle-, developmental- or tissue-specific manner.

Transcriptional regulation by Smads

The list of transcription factors to which Smads bind to regulate gene expression continues to grow (see Table S1 in the supplementary material) (reviewed by Feng and Derynck, 2005). Nuclear Smad complexes bind with weak affinity to Smad-binding elements (SBEs) on DNA (reviewed by Schmierer and Hill, 2007). Notably, the most common isoform of SMAD2 fails to bind to SBEs owing to an insertion within its DNA-binding domain, which resides in the MH1 domain of all Smads (see Fig. 2). SMAD3 recognizes 5′-GTCTG-3′ as its SBE. By contrast, the BMP Smads and SMAD4 recognize GC-rich sequences that have less conserved motifs, which are sometimes in close proximity to an SBE. In general, recruitment of Smad complexes to chromatin is dependent on their direct interaction with transcription factors that bind to DNA with higher affinity (Fig. 4).

Upon binding to DNA and to their transcriptional partners, Smads recruit co-activators and histone acetyltransferases, such as p300, C/EBP-binding protein (CBP) and p300/CBP-associated factor (P/CAF), facilitating the initiation of transcription (reviewed by Schmierer and Hill, 2007). Recent evidence has shown that p300/CBP also acetylates SMAD2/3, enhancing their DNA-binding activity in mammalian cells (Simonsson et al., 2006; Tu and Luo, 2007). Conversely, histone deacetylases inhibit SMAD1 transcriptional activity during neuronal differentiation in the mouse embryonic brain (Shakéd et al., 2008). However, direct acetylation or deacetylation of BMP-specific Smads has yet to be demonstrated.

The negative regulation of Smad signaling by Smad ubiquitylation was summarized above. Positive regulation of nuclear Smad signaling by ubiquitylation has more recently come to light from studies in mouse embryos and in mammalian cells (Mavrakis et al., 2007). Nuclear Smad complexes associate with the ubiquitin ligase arkadia (RNF111) in a ligand-dependent manner and promote the ubiquitylation and degradation of their interacting co-repressors SKI and SNON (SKIL) (Le Scolan et al., 2008; Levy et al., 2007; Nagano et al., 2007). This mechanism brings about the derepression and transcriptional induction of target genes by nuclear Smads (Fig. 4). Interestingly, the proteasomal degradation of SNON depends on its phosphorylation by TAK1, the non-Smad effector of TGFβ signaling (see Fig. 1C) (Kajino et al., 2007). However, it is unclear whether SNON ubiquitylation by arkadia requires its prior phosphorylation by TAK1. Arkadia also ubiquitylates the inhibitory SMAD7 (Koinuma et al., 2003), but whether this process takes place in the nucleus or in the cytoplasm awaits clarification.

Genome-wide screens have revealed an association between Smads and the SWI/SNF family chromatin remodeling protein Brahma-related gene 1 (BRG1; SMARCA4) and the DNA-binding proteins ETS1 and transcription factor activating enhancer-binding protein 2α (TFAP2α) (Koinuma et al., 2009; Xi et al., 2008). A current model suggests that chromatin-bound Smads cannot perform transcriptional work in the absence of essential chromatin remodeling factors, such as BRG1 and the mediator component ARC105 (reviewed by Schmierer and Hill, 2007). Interestingly, ARC105 localization in distinct chromatin domains is regulated by TAZ, the nuclear Smad-tethering factor (Varelas et al., 2008). These early reports open the door to future studies that might demonstrate how TGFβ alters the dynamic architecture of chromatin, leading to gene-specific transcriptional induction or repression. Such research might, for the first time, establish links between the epigenetic regulation of chromatin and the function of the TGFβ pathways.

Regulatory mechanisms of Smad transcriptional co-factors

From the numerous Smad-transcription factor complexes and their resulting mechanisms of target gene regulation (see Table S1 in the supplementary material) (Feng and Derynck, 2005), we highlight here a few selected examples that are of demonstrated or potential developmental relevance.

Xenopus mesoderm specification is driven by the concerted action of TGFβ/activin and FGF-Ras-Erk MAPK signaling (Cordenonsi et al., 2007). The FGF-Ras-Erk MAPK pathway acts in distinct regions of the developing *Xenopus* embryo, such as in the marginal zone, and induces, via phosphorylation, the activity of casein kinases, which then phosphorylate serines 6 and 9 of the tumor suppressor p53, contributing to mesoderm development. This phosphorylation activates the transcriptional activity of p53, making it competent to pair with Smads. This interaction leads to the transcriptional induction of mesoderm-defining genes, such as the transcription factors *Snail*, *Xbra* (*brachyury*) and *Mix.2*. Conversely, during *Xenopus* ectoderm specification, p53 is inhibited by the zinc-finger protein XFDL156 (Sasai et al., 2008). This mechanism is essential for preventing the aberrant activation of nodal signaling in the ectoderm, the developmental fate of which depends primarily on the activity of BMP pathways. Although the above example emphasizes positive cross-talk between FGF and activin signaling during *Xenopus* mesoderm specification, this should not be interpreted as the only developmental FGF signaling mechanism during frog mesoderm induction. The FGF response is multifactorial and multigenic, as revealed by recent genome-wide transcriptomic screens (Branney et al., 2009). Interestingly, although Smads cooperate with wild-type p53 to promote developmental processes, they also cooperate with mutant p53, which often accumulates in human cancers (Adorno et al., 2009; Kalo et al., 2007). The Smad-mutant p53 complex represses *TGFBR2* transcription, leading to the induction of pro-metastatic genes.

An unexpected transcriptional partner of SMAD2 and SMAD3 is the well-characterized IκB kinase α (IKKα; CHUK), which participates in the nuclear factor κ B (NFκB) pathway (Descargues et al., 2008). In the epidermis of *Smad4*-null mice, a complex of SMAD2, SMAD3 and IKKα forms in the absence of SMAD4 and regulates mammalian keratinocyte differentiation by binding to the regulatory sequences of the transcription factor genes *Mad1* (*Mxd1* – Mouse Genome Informatics) and *Ovol1*, which induce epidermal differentiation. Transcriptional induction mediated by the complex of SMAD2, SMAD3 and IKKα is independent of the kinase activity of IKKα. Interestingly, invasive squamous cell carcinomas that are resistant to the tumor suppressor action of TGFβ have defective IKKα that cannot enter the nucleus and act as a Smad co-factor (Marinari et al., 2008).

Genes that are either transcriptionally co-regulated at the same developmental time, or in the same tissue, are referred to as synexpression groups (Niehrs and Pollet, 1999). One such group is the inhibitor of differentiation (Id) family of genes, which like other TGFβ-responsive synexpression groups, respond to TGFβ family members via specific regulatory sequences present in their genes (see Table S1 in the supplementary material) (Karaulanov et al., 2004). Synexpression groups also require specific Smad-interacting transcriptional co-factors for their expression. For example, the

mammalian FoxO transcription factors bind to Smads and coordinate the regulation of 11 genes that define the cytostatic, apoptotic and adaptive signaling responses of keratinocytes to TGFβ (see Table S1 in the supplementary material) (Gomis et al., 2006a). The helix-loop-helix HHM (human homolog of Maid; CCNDBP1) protein regulates a specific synexpression group of cell cycle and cell migration regulators (see Table S1 in the supplementary material), and, accordingly, regulates growth inhibition and migration in mammalian epithelial cells in response to TGFβ, while mediating other responses in different cells (Ikushima et al., 2008).

In the *Drosophila* wing imaginal disc, the BMP ligand Dpp activates Mad (an R-Smad) and Medea (its Co-Smad) (see Table 1), which then directly repress certain transcription factor genes, including the transcriptional repressor *brinker* (*brk*). This repression of *brk* by Mad-Medea leads to the derepression of *optomotor blind* (*omb*; *bifid* – FlyBase), *zerknüllt* (*zen*) and *spalt* (*sal*), which encode transcription factors that regulate the expression of other transcription factors, to provide patterning and morphogenetic information to the developing wing (de Celis and Barrio, 2000; Shen et al., 2008). *sal*, however, additionally requires direct binding and transactivation by the Mad-Medea complex.

In *C. elegans*, the *sma-9* gene is involved in neuronal specification within a restricted group of rays in the tail of the developing worm and is expressed during early larval stages (Liang et al., 2003). SMA-9 is the ortholog of the *Drosophila* zinc-finger transcription factor Schnurri, a co-factor of the Mad-Medea complex that represses *brk* expression (Marty et al., 2000). By analogy with *Drosophila*, SMA-9 might mediate BMP-like DBL-1 signaling by complexing with SMA-2, SMA-3 or SMA-4. Indeed, SMA-9 acts as both a transcriptional repressor and an activator downstream of DBL-1. Newly identified targets of this pathway are orthologs of transcription factors that are already implicated in mammalian BMP signaling, such as Runx and Fos, or orthologs of Hedgehog signaling proteins (Liang et al., 2007). Interestingly, mouse Schnurri-2 (HIVEP2 – Mouse Genome Informatics) also regulates TGFβ/BMP-dependent gene expression. It binds to SMAD1-SMAD4 and to the transcriptional co-factor C/EBPα (CCAAT/enhancer-binding protein α) to induce the *PPARγ2* (peroxisome proliferator-activated receptor γ2; *Pparg*) gene that regulates adipocyte differentiation (Jin et al., 2006). Thus, mice that lack the *Schnurri-2* gene have reduced fat.

Finally, as we discuss in Box 3, TGFβ family transcriptional regulation in development also occurs via the regulation of micro-RNA (miRNA) genes, and via the reciprocal regulation of TGFβ signaling by miRNAs.

Box 3. TGFβ/BMP signaling and microRNAs

TGFβ regulates micro-RNA (miRNA) gene expression in mammalian cells (Zavadil et al., 2007). TGFβ promotes the epithelial-to-mesenchymal transition (EMT) by repressing transcription of the *miR-200* family (Burk et al., 2008; Gregory et al., 2008; Korpal et al., 2008), which downregulates the pro-EMT transcription factors zinc-finger E-box-binding homeobox 1 (ZEB1) and ZEB2. Transcriptional repression of *miR-24*, a positive regulator of myogenesis, in part explains the anti-myogenic effects of TGFβ (Sun et al., 2008). BMP induces osteoblast differentiation from mesenchymal progenitors and concomitantly regulates expression of 22 miRNAs (Li et al., 2008). Among these, *miR-133* targets the transcription factor RUNX2, a known target of BMP/Smad signaling that promotes osteoblast differentiation, whereas *miR-135* targets SMAD5. These miRNAs progressively inhibit BMP signaling to fine-tune bone development.

In addition, SMAD1, SMAD3 and SMAD5, but not SMAD4, directly bind to and regulate components of the DROSHA microprocessor complex, which regulates miRNA biogenesis (Davis et al., 2008). This post-transcriptional mechanism selectively regulates only certain miRNAs during vascular smooth muscle differentiation. Understanding the mechanism of this selectivity and its developmental relevance is important.

miRNAs also target TGFβ signaling components. In *Xenopus*, *miR-15* and *miR-16* downregulate *ActRIIA*, which restricts receptor expression to specific cells, thus defining the embryonic territory that responds to activin/nodal (Martello et al., 2007). In zebrafish, *miR-430* downregulates the nodal-like ligand *squint* (*ndr1* – Zebrafish Information Network) and its extracellular antagonist *lefty*, which is required for proper patterning of the early fish embryo (Choi et al., 2007).

Conclusions

As TGFβ research continues with ever increasing speed, we foresee important novel findings regarding the mechanisms of TGFβ receptor regulation and specificity of signaling, cytoplasmic trafficking of receptors and Smads, nuclear dynamics of Smad-chromatin associations and their relationship to developmental processes. The functional implications of 'promiscuous' signaling by TGFβ family receptor kinases that simultaneously activate TGFβ- and BMP-like Smad pathways, as well as MAPK and other pathways, needs to be analyzed carefully and with quantitative methods. The area of post-translational modifications of TGFβ receptors and Smads will continue its prolific expansion. More sensitive proteomic approaches will be useful in dissecting all the components of signaling complexes and their dynamic nature, especially if coupled to multi-protein imaging in real time. Progress in the modeling of signaling dynamics and of the protein networks that participate in the TGFβ family cascades should provide fresh ideas about new regulatory nodes in the network, and should also define more quantitatively critical parameters that govern the behavior of the network. A major challenge is to decipher the roles of the TGFβ pathways during late stages of embryogenesis and during neonatal life by conditional activation and inactivation of TGFβ signaling components in model organisms. The importance of cross-talk during different developmental stages between TGFβ and Wnt, Hedgehog, FGF or other pathways should be another focus for future research. Finally, in the context of development, more complete circuits of target genes, and their corresponding protein or RNA regulators, will need to be delineated in the global effort to provide a systems-level description of TGFβ pathways in every tissue and organ.

Acknowledgements

We acknowledge funding by the Ludwig Institute for Cancer Research (LICR), the Atlantic Philanthropies/LICR Clinical Discovery Program, the Swedish Cancer Society, the Swedish Research Council, the European Union Marie Curie Research Training Network 'EpiPlastCarcinoma' and the European Union Network of Excellence 'ENFIN'. We acknowledge the contribution of all past and present members of the TGFβ signaling group to the scientific work emanating from our laboratory.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/22/3699/DC1

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