Non-Smad TGF-β signals

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Journal of Cell Science 118, 3573-3584 Published by The Company of Biologists 2005
doi:10.1242/jcs.02554

Summary

During the past 10 years, it has been firmly established that Smad pathways are central mediators of signals from the receptors for transforming growth factor β (TGF-β) superfamily members to the nucleus. However, growing biochemical and developmental evidence supports the notion that alternative, non-Smad pathways also participate in TGF-β signalling. Non-Smad signalling proteins have three general mechanisms by which they contribute to physiological responses to TGF-β: (1) non-Smad signalling pathways directly modify (e.g. phosphorylate) the Smads and thus modulate the activity of the central effectors; (2) Smads directly interact and modulate the activity of other signalling proteins (e.g. kinases), thus transmitting signals to other pathways; and (3) the TGF-β receptors directly interact with or phosphorylate non-Smad proteins, thus initiating parallel signalling that cooperates with the Smad pathway in eliciting physiological responses. Thus, non-Smad signal transducers under the control of TGF-β provide quantitative regulation of the signalling pathway, and serve as nodes for crosstalk with other major signalling pathways, such as tyrosine kinase, G-protein-coupled or cytokine receptors.

Key words: BMP, MAPK, PI3K, Phosphatase, Ras, Rho, Smad, TGF-β

Introduction

The transforming growth factor β (TGF-β) superfamily encompasses a large variety of signalling proteins, including TGF-β isoforms, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins, inhibins and Müllerian inhibiting substance (Roberts and Sporn, 1993; Piek et al., 1999). TGF-β signalling pathways are important during embryonic development; in adult organisms, they regulate tissue homeostasis and are frequently implicated in diverse pathological conditions (Barolo and Posakony, 2002; Waite and Eng, 2003). TGF-β superfamily members signal through receptor serine/threonine kinases and intracellular Smad proteins (Moustakas et al., 2001; Shi and Massagué, 2003). In addition, several intracellular proteins that mediate signalling by receptor tyrosine kinases, G-protein-coupled receptors or cytokine receptors also participate in the TGF-β signalling network (Derynck and Zhang, 2003; Nohe et al., 2004).

The Smad pathway represents an evolutionarily conserved signalling module that transmits signals to the nucleus and is of paramount importance for the precise execution of tissue- and organ-patterning programmes during animal development. By contrast, the relative weight and evolutionary or developmental significance of non-Smad effectors has been less clear. However, the cellular and genetic evidence for important roles of these effectors downstream of TGF-β ligands is increasing. Here, we discuss non-Smad signalling proteins downstream of the receptors for various TGF-β superfamily members. We classify their modes of action into three distinct but interrelated signalling mechanisms (Fig. 1): (1) non-Smad signalling pathways that directly modify Smad function; (2) non-Smad proteins whose function is directly modulated by Smads and which transmit signals to other pathways; and (3) non-Smad proteins that directly interact with or become phosphorylated by TGF-β receptors and do not necessarily affect the function of Smads. The existence of such pathways raises several important questions. Why are they needed? Do they promote signalling specificity? Are they truly independent of Smads? Are non-Smad proteins points of convergence between signalling by TGF-β and other factors? Below we examine these questions and discuss the evidence for non-Smad signalling mechanisms downstream of serine/threonine kinase receptors.

Overview of the Smad pathway

The dimeric TGF-β superfamily ligands interact with hetero-tetrameric complexes of type II and type I receptors, which leads to phosphorylation-dependent activation of the dormant type I receptor kinase by the constitutively active type II receptor kinase (Shi and Massagué, 2003). Then, the activated type I receptor kinase phosphorylates and activates receptor-activated (R)-Smads (Moustakas et al., 2001). TGF-β superfamily signalling can be classified into two branches: (1) the TGF-β branch, represented by ligands such as TGF-β, activin, nodal or myostatin, which activate Smad2 and Smad3; and (2) the BMP branch, represented by ligands such as BMPs and GDFs, which activate Smad1, Smad5 and Smad8 (Moustakas et al., 2001). The phosphorylated R-Smads form complexes with the common mediator, Smad4, and enter the nucleus, where they bind to DNA and interact with transcription factors to regulate gene expression (Fig. 2A). This basic Smad pathway is conserved throughout evolution and is regulated by diverse phosphorylation events, nucleocytoplasmic shuttling, ubiquitin-mediated proteasomal degradation and, finally, by inhibitory (I)-Smads (Shi and

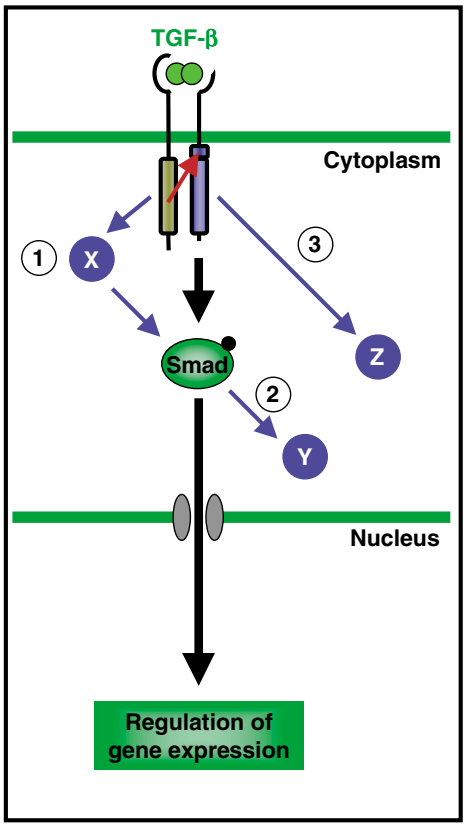

Fig. 1. Non-Smad signalling. The canonical Smad pathway starting from the ligand-receptor complex and ending in the nucleus is illustrated by thick black arrows. Non-Smad signalling mechanisms are shown by thin blue arrows. The receptor complex activates (by interaction and/or phosphorylation) protein X, which then modulates the activity of the Smad (pathway 1). The phosphorylated Smad activates (by interaction) protein Y, which then transmits further signals into the cell (pathway 2). The receptor complex activates (by interaction and/or phosphorylation) protein Z, which then transmits signals without direct crosstalk with the Smad (pathway 3). Proteins X, Y and Z can be enzymes (e.g. protein or lipid kinases) or adaptor proteins.

Massagué, 2003). The latter are induced by Smad signalling, accumulate in the nucleus and are exported from the nucleus after stimulation of cells with TGF-β or BMP. They then bind to type I receptors and exert negative feedback by blocking R-Smad phosphorylation and R-Smad–Smad4 complex formation, stimulating receptor dephosphorylation by recruiting phosphatases, and promoting receptor ubiquitylation and lysosomal degradation (Fig. 2B).

Non-Smad TGF-β signalling

The identification of non-Smad signalling proteins that participate in TGF-β signal transduction pre-dates the discovery of Smads (reviewed by Yue and Mulder, 2000). Most of the experiments were performed in cell models in vitro and implicated the small GTPase Ras and the mitogen-activated protein kinases (MAPKs) ERKs, p38 and c-Jun N-terminal kinases (JNKs) in TGF-β signalling (reviewed by Yue and Mulder, 2000). However, many in vitro studies of non-Smad signalling pathways have relied primarily on pharmacological inhibitors of various intracellular protein or lipid kinases. The specificity and the effective doses of such inhibitors must be evaluated with great caution because, for example, 'specific' inhibitors of p38 can also lead to inhibition of the TGF-β receptor kinases (Yakymovych et al., 2001; Fu et al., 2003).

In many cases where a role for a 'Smad-independent' pathway downstream of TGF-β, activin or BMP receptors has been proposed, the link between the activated receptor complex and the cytoplasmic effector molecule remains to be elucidated. Below, we review the most prominent examples of non-Smad signalling whose physical links to the receptor complex are at least partially understood. We also list some additional cases where the physical links to the receptors are more ambiguous, but the potential physiological role of such non-Smad proteins makes them interesting targets for future investigation.

Apoptosis

A set of genes regulated by Smads is proposed to mediate the pro-apoptotic effects of TGF-β. These include those encoding the phospholipid phosphatase SHIP, death-associated protein kinase (DAPK) and TGF-β-inducible early response gene 1 (TIEG1) (Fig. 3A) (reviewed by ten Dijke et al., 2002; Siegel and Massagué, 2003). In addition, through Smad3, TGF-β induces expression and activation of the Fas receptor, leading to caspase-8 activation and apoptosis of gastric carcinoma cells (Kim et al., 2004). However, the most consistent observation in apoptotic mechanisms downstream of TGF-β ligands is the involvement of MAPKs, such as p38 and JNK, as outlined below.

The type II receptor for TGF-β interacts with the pro-apoptotic adaptor protein Daxx, which leads to activation of JNK and induction of apoptosis in epithelial cells and hepatocytes (Perlman et al., 2001). The Daxx-JNK pathway also involves homeodomain-interacting protein kinase 2 (HIPK2), which interacts with and phosphorylates Daxx; this activates the MAPK kinases MKK4 and MKK7, which ultimately activate JNK and induce apoptosis (Fig. 3B) (Hofmann et al., 2003). Genetic evidence in normal mammary epithelial cells stably transfected with a mutant TGF-β type I receptor that cannot bind and thus activate R-Smads, also implicates the p38 and JNK pathways in mammary cell apoptosis (Yu et al., 2002; Itoh et al., 2003). This work shows that the type I receptor kinase activates p38 and JNK independently of factors (including Smads) that bind to the L45 loop of the receptor. Whether the two distinct TGF-β receptors signal towards different non-Smad proteins in order to mobilize the p38 and JNK pathways remains an interesting open question.

A second direct link between receptor complexes and intracellular kinases involves the TGF-β-activated kinase 1 (TAK1), which can form a complex with the BMP receptors through its binding partner TAB1 and the inhibitor of apoptotic caspases XIAP, an E3 ubiquitin ligase (Fig. 3B) (Yamaguchi et al., 1999). The complex was reported to promote BMP signalling during *Xenopus* embryogenesis. Whether BMP receptors activate Smad versus non-Smad (e.g. TAK1-p38) pathways seems to be at least partially regulated by the mode of oligomerization of the cell-surface receptor (Nohe et al.,

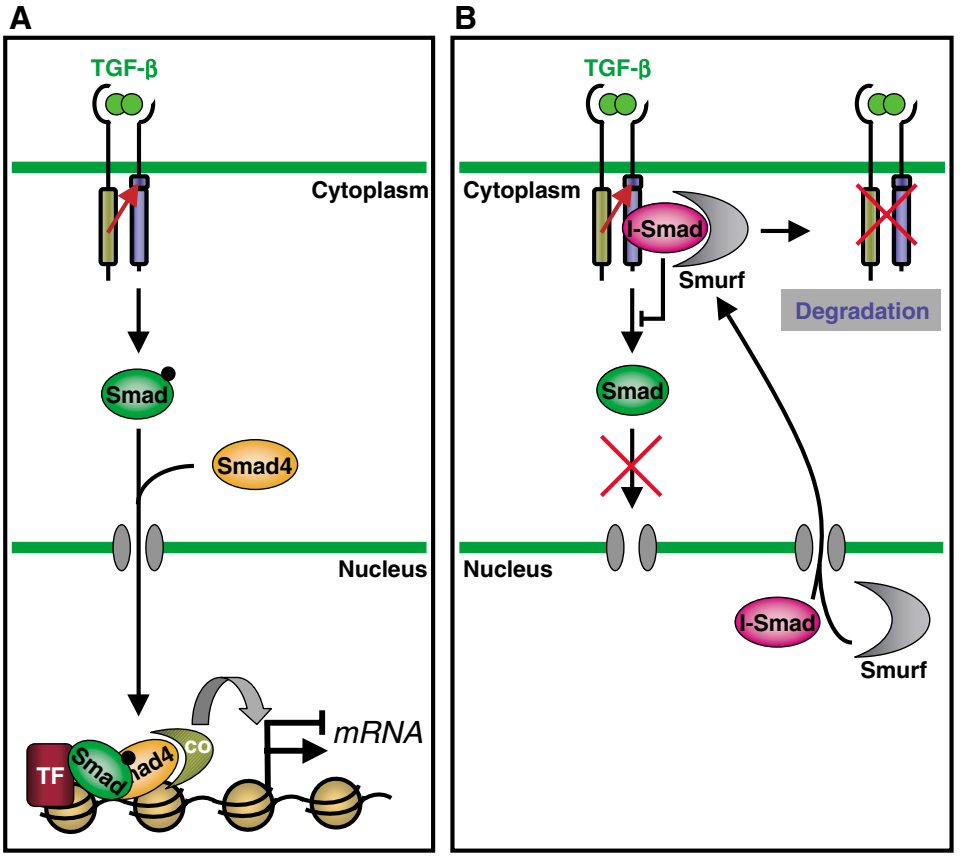

Fig. 2. (A) The canonical Smad pathway. TGF-β bound to the two receptor serine/threonine kinases (type II, light green; type I, blue) initiates signalling by trans-phosphorylation of the type I receptor juxtamembrane domain (red arrow). Signalling ensues with R-Smad phosphorylation (Smad with black dot), oligomerization of R-Smad with Smad4, nuclear translocation and formation of complexes between transcription factors (TF) and co-activators/co-repressors (co) on chromatin. This leads to positive or negative regulation of mRNA synthesis (grey arrow). (B) The inhibitory I-Smad, together with the E3 ligase Smurf, exit the nucleus in response to the incoming TGF-β signal and bind the receptor complex, leading to shut-down of R-Smad phosphorylation and receptor downregulation.

2002). In this model, pre-assembled type-I–type-II receptor complexes activate Smads in response to BMP, whereas ligand-induced receptor complexes activate the TAK1-p38 module (reviewed by Nohe et al., 2004).

TAK1 can also act downstream of TGF-β by initiating a kinase cascade that leads to Stat3 activation during mesoderm induction in *Xenopus* (Ohkawara et al., 2004). TAK1, despite its original name, has also been firmly placed as a crucial signalling intermediate in many pro-inflammatory cytokine and Toll-like receptor signalling pathways (reviewed by Moustakas and Heldin, 2003). In these pathways, TAK1 cooperates with TRAF-mediated signalling to regulate inhibitor of nuclear factor κB (IκB) function. XIAP was also found to interact with multiple type I receptors of the TGF-β superfamily, enhancing their signalling output (Birkey Reffey et al., 2001). However, downstream of TGF-β receptors, XIAP seems to activate JNK and nuclear factor κB (NF-κB) through cooperation with Smad4, leading to regulation of gene expression but not apoptosis (Birkey Reffey et al., 2001). These observations underscore two general principles: there is a high degree of cell-type specificity, and many non-Smad signalling proteins modulate the activity of the Smad pathway. Thus, the delineation of true Smad-independent signals is rather complicated, as we discuss throughout this review.

Another mechanism in which XIAP is implicated in TGF-β-mediated apoptosis involves the mobilization of mitochondrial components by TGF-β signalling. TGF-β induces export of the mitochondrial septin family member ARTS to the cytoplasm and promotes its association with, and inactivation of, XIAP (Fig. 3A) (Larisch et al., 2000); XIAP inactivation leads to caspase-3 activation and apoptosis (Gottfried et al., 2004).

An alternative mode of crosstalk is between kinases such as TAK1 and inhibitory Smads. Thus, BMPs can cause apoptosis of various cell types through TAK1 and p38, and Smad6 and Smad7 efficiently block such responses (Yanagisawa et al., 2001). However, TGF-β induces apoptosis of prostate carcinoma cells by promoting cooperation between Smad7 and the TAK1-p38 signalling module (Fig. 3B) (Edlund et al., 2003). These complex functions of the I-Smads may well depend on their emerging role as adaptor proteins, whereby they not only inhibit R-Smad phosphorylation by TGF-β receptors, but also mediate recruitment of phosphatases and ubiquitin ligases to the receptor complex (Shi and Massagué, 2003). The link between TAK1 signalling and Smad7 also includes effects on transcription: the TAK1-p38 module activates *Smad7* expression by regulation of the transcription factor ER81, a member of the ETS family (Dowdy et al., 2003). It is therefore possible that even more complex mechanisms linking TGF-β superfamily receptors to non-Smad signal transducers through inhibitory Smads will surface in the near future.

Finally, TGF-β might also antagonize pro-survival signals. The relative levels of Smad3 and the pro-survival kinase Akt are proposed to define whether a cell undergoes apoptosis in response to TGF-β (Fig. 3A) (Conery et al., 2004; Remy et al., 2004). This model is based on the physical interaction between Smad3 and Akt and, because of its strictly quantitative nature, requires further validation in a large set of cell types. The adaptor molecule CD2-associated protein (CD2AP) activates

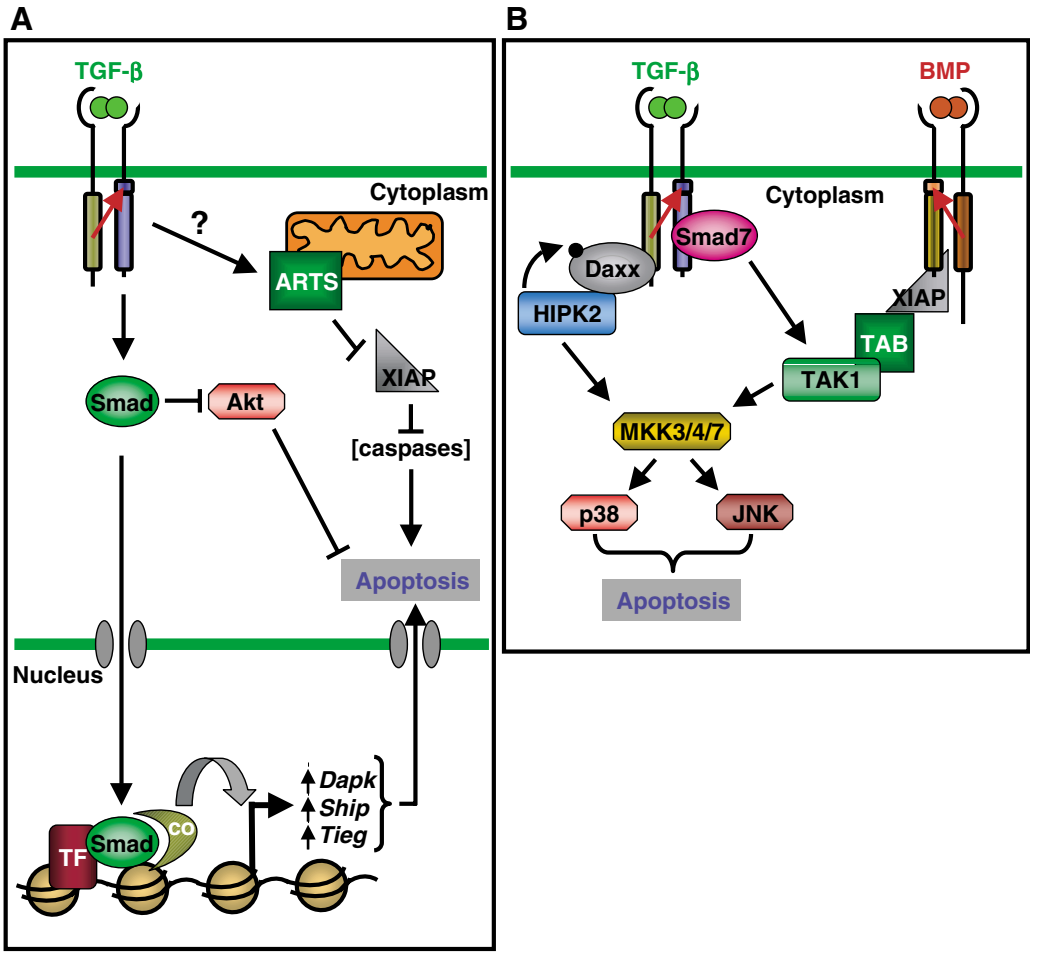

Fig. 3. (A) Smads in apoptosis. TGF-β receptors induce through Smads the expression of *DAPK*, *SHIP* and *TIEG* pro-apoptotic genes. Smads also bind and inactivate the survival kinase Akt, thus promoting apoptosis. TGF-β can also mobilize the mitochondrial serpin ARTS to the nucleus, which blocks XIAPs, the inhibitors of caspases, thus leading to apoptotic events. (B) The TAK1 pathway leading to apoptosis. The TGF-β type I receptor binds Smad7, the type II receptor binds the pro-apoptotic protein Daxx, whereas BMP receptors bind XIAP and its interacting partners, TAB and TAK1. Both TGF-β and BMP receptors activate TAK1, leading to MKK3, MKK4 or MKK7 activation. This activates JNK or p38, both of which lead to apoptosis of various cell types. In the Daxx pathway, HIPK2 phosphorylates Daxx (curved arrow), which then activates MKK4 and MKK7.

the phosphoinositide 3-kinase (PI3K)/Akt pathway, enhancing cell survival and protecting from TGF-β-induced apoptosis in kidney podocytes (Schiffer et al., 2004). Interestingly, the regulatory subunit of PI3K, p85, indirectly associates with type I and type II TGF-β receptors and the former activates PI3K in a ligand-dependent manner (Yi et al., 2005). Whether the bridge between the receptor complex and p85 of PI3K is CD2AP or another protein remains unknown.

Overall, we can conclude that the apoptotic response of normal or tumour cells to TGF-β family members involves the interplay between Smad and non-Smad pathways and, in many cases, both pro-survival and pro-apoptotic pathways are activated. Thus, the end result may well depend on other signalling inputs the cell receives.

Epithelial-mesenchymal transition and migratory responses

Morphogenetic responses to TGF-β members include cell migration and epithelial/endothelial-mesenchymal transitions (EMTs), which are crucial during embryogenesis, fibrotic diseases and advanced carcinoma spreading (Tosh and Slack, 2002; Condeelis and Segall, 2003; Gotzmann et al., 2004). The EMT is a characteristic change in polarized epithelia in which the cell-cell and cell-matrix adhesion is disrupted, the surrounding matrix is degraded, and the cell phenotype is changed by rearranging its actin cytoskeleton to become more motile and invasive. Since the Smad pathway primarily leads to regulation of gene expression, it was originally thought that non-Smad effectors signal the rapid or direct effects of TGF-β on the actin cytoskeleton. Today, however, we appreciate that Smads are crucial mediators of this process downstream of TGF-β, because they induce dramatic changes in gene expression in epithelial cells (Fig. 4A) (Zavadil et al., 2001; Kowanetz et al., 2004; Valcourt et al., 2005). However, the EMT also depends on ERK activity, and pathways activated by oncogenic Ras or physiological Notch receptor signalling synergize with TGF-β (Oft et al., 1996; Oft et al., 1998; Zavadil et al., 2001; Jechlinger et al., 2003; Zavadil et al., 2004). The Smads, especially Smad3 and Smad4, are critical for the EMT response not only in vitro (Valcourt et al., 2005), but also in vivo, for aggressive carcinoma metastasis and lens EMT (Oft et al., 2002; Li et al., 2003; Tian et al., 2003; Saika et al., 2004; Tian et al., 2004). Mutant TGF-β type I receptors that lack the Smad-docking site fail to induce EMT but can activate endogenous p38 or JNK signalling (Yu et al., 2002; Itoh et al., 2003). These mutant type I receptors also inhibit endogenous autocrine TGF-β signalling and thus induce a stronger epithelial phenotype in vitro, and suppress breast carcinoma metastasis to the lung in vivo (Tian et al., 2004; Valcourt et al., 2005).

A direct link between the TGF-β receptors and the Rho

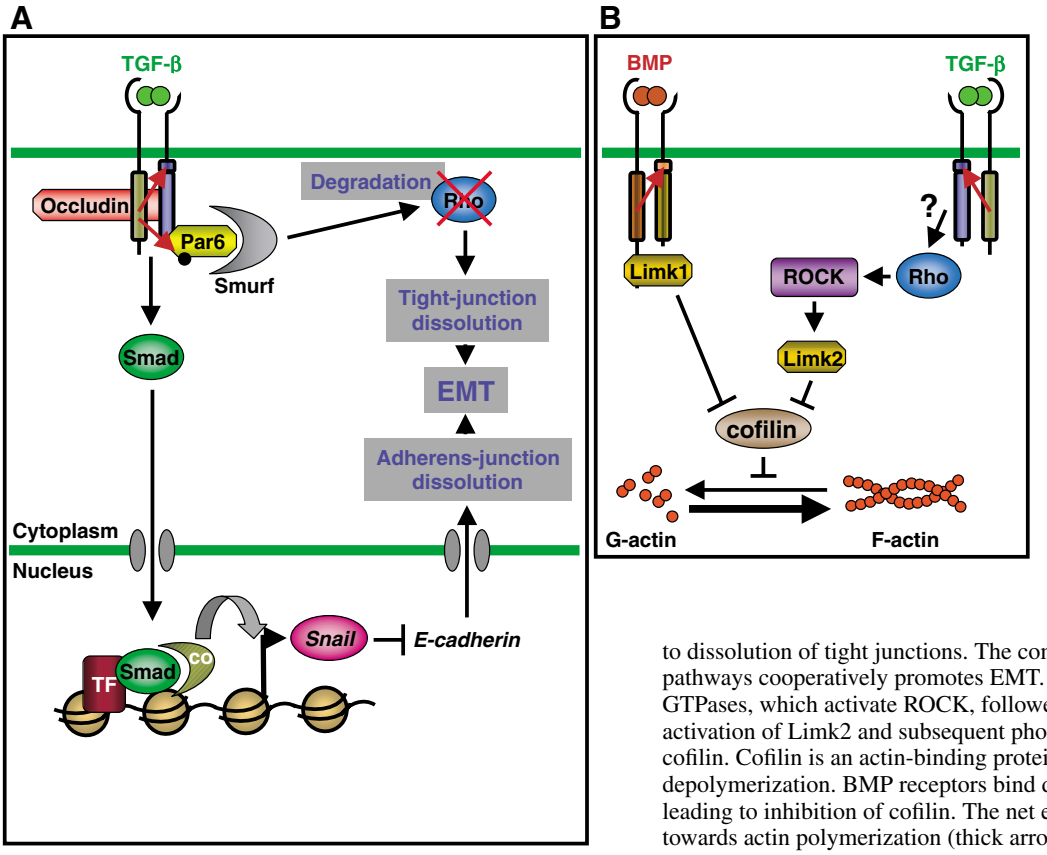

Fig. 4. Regulation of the actin cytoskeleton and the EMT response. (A) TGF-β induces Smads, which regulate genes such as that encoding Snail, the transcriptional repressor of E-cadherin gene expression that leads to the dissolution of adherens junctions. Alternatively, the receptors constitutively associate with occludin and the polarity protein Par6. Upon ligand stimulation, the type II receptor phosphorylates Par6 directly. This then recruits the ubiquitin ligase Smurf1, which ubiquitylates and degrades RhoA, thus leading to dissolution of tight junctions. The combined outcome of the two pathways cooperatively promotes EMT. (B) TGF-β activates Rho GTPases, which activate ROCK, followed by phosphorylation and activation of Limk2 and subsequent phosphorylation and inhibition of cofilin. Cofilin is an actin-binding protein that leads to actin depolymerization. BMP receptors bind directly to Limk1 and activate it, leading to inhibition of cofilin. The net effect of both pathways is a shift towards actin polymerization (thick arrow).

GTPase was recently uncovered and provides a novel mechanism by which TGF-β promotes EMT, at least in vitro (Barrios-Rodiles et al., 2005; Ozdamar et al., 2005). In polarized epithelial cells, the TGF-β receptor complex is recruited to tight junctions by the structural protein occludin, where it also interacts with the polarity protein Par6 (Fig. 4A). Upon TGF-β signalling, the type II receptor phosphorylates not only the type I receptor but also the type-I-receptor-tethered Par6, leading to recruitment of the ubiquitin ligase Smurf1 and subsequent ubiquitylation and degradation of RhoA (Ozdamar et al., 2005). This leads to local disassembly of the actin cytoskeleton and dissolution of tight junctions, which is one of the hallmarks of EMT (Thiery, 2003). Simultaneously, activation of Smads by the type I receptor leads to transcriptional induction of genes involved in EMT such as that encoding Snail (Peinado et al., 2003), which is a transcriptional repressor of the gene encoding E-cadherin (Fig. 4A) (Nieto, 2002). When E-cadherin levels drop inside the cell, the adherens junctions of polarized epithelial cells also dissolve, which is a second hallmark of EMT (Thiery, 2003). Direct phosphorylation of Par6 by the type II receptor kinase is the first example of a non-TGF-β receptor protein substrate for this receptor kinase. This opens the exciting possibility that a diverse set of proteins, in addition to the type I receptor of TGF-β, are phosphorylated and regulated by the type II receptor serine/threonine kinase.

Two recent reports similarly directly link the cytoplasmic protein kinase Limk1 to the long cytoplasmic tail of the BMP type II receptor (BMPR-II) (Foletta et al., 2003; Lee-Hoeflich et al., 2004). Since Limk1 is a well-studied kinase that signals downstream of Rho GTPases and regulates reorganization of the actin cytoskeleton (Raftopoulou and Hall, 2004), this observation may be relevant to the mechanism by which BMPs regulate the actin cytoskeleton during neuronal dendrite morphogenesis (Fig. 4B) (Foletta et al., 2003; Lee-Hoeflich et al., 2004). Possible misregulation of this pathway during primary pulmonary hypertension has also been proposed (Foletta et al., 2003; Lee-Hoeflich et al., 2004). The small GTPase Cdc42 has also been shown to be required for activation of Limk1 after BMP stimulation (Foletta et al., 2003; Lee-Hoeflich et al., 2004). In contrast to BMPR-II, the TGF-β or activin type II receptors lack the critical motifs of the long tail of BMPR-II, which suggests a pathway-specific link between the BMP receptor and Limk1. However, the TGF-β type I receptor can activate the related Limk2 indirectly through Rho and its downstream effector ROCK1 (Vardouli et al., 2005). Whether Limk2 can directly associate with TGF-β receptors has not yet been examined. Thus, both direct physical links and multi-effector signalling cascades might regulate the activation of Limk isoforms by TGF-β family members (Fig. 4B).

In addition to the activation of Limk isoforms, Smad3-Smad4 cooperates with Rho and p38 signalling to drive expression of NET1 and tropomyosins, respectively, which are important for long-term establishment of actin stress fibres (Shen et al., 2001; Bakin et al., 2004). In prostate cancer cells, TGF-β mobilizes the small GTPases RhoA and Cdc42, as well as the downstream effector p38, to induce membrane ruffling (Edlund et al., 2002). Interestingly, the inhibitory Smad7 mediates activation of Cdc42 by TGF-β receptors during the

actin response (Edlund et al., 2004), which mirrors its role in induction of apoptosis in the same system (Edlund et al., 2003).

In systems where cell migration is a clear-cut response, the role of Smads remains unclear. Migratory metastatic breast cancer cells, which produce large amounts of autocrine TGF-β, activate the PI3K/Akt and ERK pathways to drive their motility (Dumont et al., 2003). A similar mechanism, which also involves Rac1, is activated by TGF-β in mammary epithelial cells transformed by overexpression of the epidermal growth factor receptor 2 (HER2) (Ueda et al., 2004). Genetic evidence from MEKK1-knockout mice strongly implicates MEKK1 and the downstream MAPK JNK in the migratory properties of the eyelid epithelium and the underlying effects on the actin cytoskeleton induced by TGF-β or activin (Zhang et al., 2003). In the same fashion, keratinocytes migrate in response to activin by using the RhoA-ROCK-MEKK1-JNK/p38 pathway (Zhang et al., 2005).

The above evidence indicates that a combination of Smad and non-Smad signals is important for morphogenic responses of cells to TGF-β. Thus, whereas specific inhibitors of Smad3 have been emphasized for the treatment of tumour metastasis (Flanders, 2004), a combination of Smad inhibitors and kinase or GTPase inhibitors may be more efficacious.

Cell proliferation

TGF-β was discovered as a factor that induces anchorage-independent growth of fibroblasts (Roberts et al., 1980; Moses et al., 1981). Thus, initially, it was thought to stimulate cell proliferation. Subsequently, TGF-β was shown to inhibit proliferation of epithelial cells and lymphocytes (Tucker et al., 1984; Pietenpol et al., 1990). Today, it is widely accepted that TGF-β inhibits the growth of non-transformed epithelial, endothelial and haematopoetic cells, and also primary fibroblasts of embryonic origin (reviewed by ten Dijke et al., 2002). Nevertheless, TGF-β has mitogenic activity in certain transformed cells and in immortalized fibroblasts (Alexandrow and Moses, 1995).

The growth inhibitory pathway induced by TGF-β includes critical regulators of the G1 phase of the cell cycle, whose gene expression is modulated by Smads (Massagué, 2004). These include the cell-cycle inhibitors p15, p21 and p57, which are induced by Smad signals, the proto-oncogene product Myc, and the inhibitors of differentiation (Id1, Id2 and Id3), which are repressed by Smads (Fig. 5). p21 is induced rapidly by all TGF-β superfamily receptor complexes (Pardali et al., 2005). Smads, together with FoxO, p53 and Sp1, form large transcriptional complexes on the *p21* promoter enhancer (Fig. 5) (Datto et al., 1995; Moustakas and Kardassis, 1998; Pardali et al., 2000; Cordenonsi et al., 2003; Seoane et al., 2004). However, *p21* is also induced by TGF-β through mechanisms that involve Ras, MEKK1 and ERK (Hu et al., 1999; Kivinen and Laiho, 1999). Furthermore, *p21* can be induced in tumour cells that lack Smad4 (Ijichi et al., 2004), which indicates that the role of Smad4 in the regulation of this gene is not exclusive. In human keratinocytes and hepatoma cells, TGF-β activates protein kinase Cα (PKCα) (Miyazaki et al., 2004; Sakaguchi et al., 2004). PKCα phosphorylates the regulatory protein S100C/A11, which translocates to the nucleus, binds to transcription factor Sp1 and recruits it to the promoters of the *p15* and *p21* genes. This pathway is very similar to the parallel

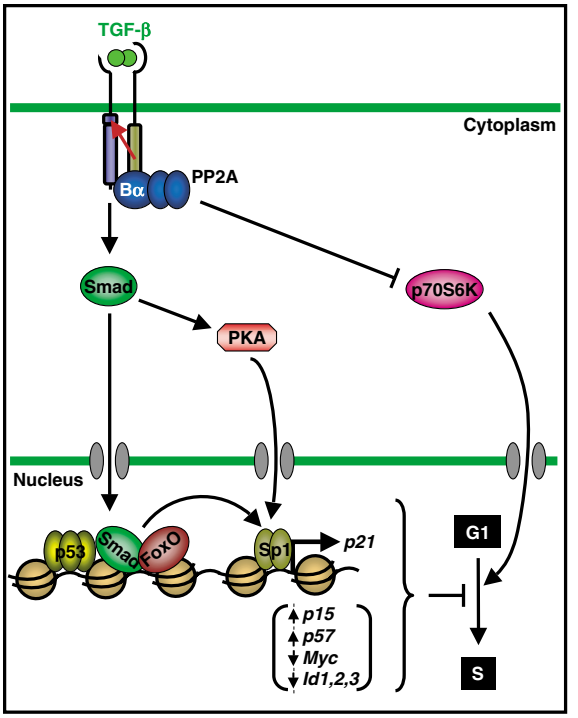

Fig. 5. Epithelial growth suppression induced by TGF-β. The TGF-β receptors activate Smads, which induce *p21* expression in cooperation with the transcription factors FoxO, p53 and Sp1. The Smad pathway also induces transcription of *p15* or *p57* and represses expression of *Myc* and *Id* genes. The net result of all these transcriptional events is the arrest of the cell cycle in early G1 phase. Smads can also activate PKA, which leads to Sp1 phosphorylation and induction of *p21* expression. The TGF-β receptor also binds the regulatory subunit Ba of PP2A phosphatase, leading to inactivation of p70 S6K kinase, thus indirectly inhibiting cell-cycle progression.

Smad pathway, which also induces these genes through interactions between Smad proteins and Sp1 (Fig. 5) (Feng et al., 2000; Pardali et al., 2000). Simultaneously, PKCα can phosphorylate Smad3 close to its DNA-binding domain and thereby block its transcriptional activity (Yakymovych et al., 2001). Thus, the two mechanisms might constitute a single integrated network for quantitative regulation of *p21* and deserve further analysis.

Recent reports shed light on a novel mechanism by which the Smads themselves can contribute to the activation of non-Smad signalling proteins (Lee et al., 2004; Zhang et al., 2004). Smad2 can activate ERK in carcinoma cells growing in suspension, and Smad3 binds to the regulatory subunit of protein kinase A (PKA) and activates the enzyme independently of cAMP levels (Fig. 5). This effect is linked to the transcriptional activation of *p21* and subsequent inhibition of cell growth.

Alternative pathways in the mechanism of cell growth control by TGF-β include inhibition of p70 S6 kinase through dephosphorylation by protein phosphatase PP2A (Petritsch et al., 2000). The TGF-β receptor complex binds directly to its regulatory subunit PP2A-Bα (Griswold-Prenner et al., 1998; Petritsch et al., 2000). This leads to assembly of the tri-subunit (PP2A-Bα, Aβ, Cα) phosphatase and its association with p70

S6 kinase (Fig. 5). Alternatively, TGF-β activates the small GTPase RhoA, which activates ROCK1, ultimately leading to phosphorylation and inactivation of the phosphatase Cdc25A in epithelial cells (Bhowmick et al., 2003). Cdc25A dephosphorylates cyclin-dependent kinases and thus promotes cell-cycle progression, whereas inactivation of Cdc25A contributes to cell-cycle arrest in early G1 phase. Similarly, Rho and p38 activation in cooperation with the Smad pathway elicits growth arrest of mammary epithelial cells (Kamaraju and Roberts, 2005).

The mitogenic response of fibroblasts to TGF-β involves activation of the cytoplasmic kinase PAK2 (Wilkes et al., 2003). Small GTPases, such as Rac1 or Cdc42, link TGF-β receptors to PAK2; Smad2 and Smad3 are possibly dispensable for this pathway, which seems to operate in fibroblasts but not in epithelial cells. TGF-β also induces mitogenesis of carcinoma cells, especially those from advanced, invasive tumours that harbour mutations in Ras. In such cells, signalling by the Ras pathway appears to be crucial and, in them, TGF-β can either induce degradation of the cell cycle inhibitor p21 or fail to induce *p15* and *p21*, thus ultimately leading to cell-cycle stimulation (Yan et al., 2002). However, in other tumour cells, TGF-β can stabilize p21 levels independently of its Smad-mediated transcriptional effects (Gong et al., 2003). Finally, TGF-β superfamily members stimulate the proliferation and self-renewal of stem cells. For example, BMP-4 signals through ERK and p38 to support such self-renewal of embryonic stem cells (Qi et al., 2004).

The growth inhibitory response of epithelial cells to TGF-β thus appears to be governed by gene expression programs regulated by combinations of Smad and non-Smad signalling molecules. More rigorous analysis of the mitogenic effects of TGF-β are warranted, and even more critical is the need to decipher whether the above models apply to the relevant tissues in vivo.

Matrix regulation

TGF-β induces several genes that encode major constituents of the extracellular matrix and matrix regulatory enzymes (reviewed by Siegel and Massagué, 2003; Schiller et al., 2004), including plasminogen activator inhibitor 1 (PAI-1), collagenase I and the collagens (Dennler et al., 1998; Qing et al., 2000; Javelaud et al., 2003). Smads regulate PAI-1 expression in cooperation with transcription factors, such as Sp1 and TFE3 (Dennler et al., 1998; Hua et al., 1998; Song et al., 1998; Stroschein et al., 1999; Datta et al., 2000), but signals from ERK and Rac1 are also required (Mucsi et al., 1996; Kutz et al., 2001). Fibronectin gene expression was originally shown to depend on JNK signals activated by TGF-β in a Smad-independent manner (Hocevar et al., 1999), but subsequently a role for Smads was recognized (Itoh et al., 2003; Dai and Liu, 2004; Kowanetz et al., 2004). By contrast, regulation of the urokinase-type plasminogen activator receptor gene by TGF-β seems to require exclusively the Ras/MKK4/JNK1 pathway (Yue et al., 2004).

Other non-Smad effectors that regulate expression of genes encoding matrix components include the calcium-dependent phosphatase calcineurin and its downstream partner NFATc, and the tyrosine kinase Abl (Daniels et al., 2004; Gooch et al., 2004). The latter has clinical importance because an Abl inhibitor, Imatinib/Glivec, successfully blocks TGF-β-induced lung and kidney fibrosis. This suggests that also targeting non-Smad TGF-β pathways can be beneficial for treatment of TGF-β-induced disease (Daniels et al., 2004; Wang et al., 2005).

Cell differentiation

Regulation of gene expression by TGF-β superfamily members is often critically linked to cell differentiation. Osteoblasts, for example, require BMP inputs to differentiate from pluripotent progenitor cells, and Smads together with transcription factors of the Runx family and Id proteins contribute greatly to this process (reviewed by ten Dijke et al., 2003; Miyazono et al., 2004). In addition, accumulating evidence implicates the p38, ERK and JNK pathways in osteoblast differentiation in response to BMP-2, BMP-4 or BMP-7 (Gallea et al., 2001; Lai and Cheng, 2002; Vinals et al., 2002; Xiao et al., 2002; Guicheux et al., 2003). Moreover, regulation of *Runx2* expression by TGF-β and BMPs involves both Smad and p38 inputs (Lee et al., 2002). In a parallel scenario, chondrocyte differentiation involves Smads and non-Smad effectors such as PKA, p38 and ERK (Lee and Chuong, 1997; Valcourt et al., 2002; Noth et al., 2003; Seto et al., 2004).

An alternative mode of BMP receptor signalling during differentiation has been revealed by studies of the inherited disorder brachydactyly (Sammar et al., 2004). Here, GDF-5, a member of the BMP family, signals through a receptor complex that involves the known BMP type I receptor BMPR-IB and the receptor tyrosine kinase Ror2. BMPR-IB phosphorylates and activates Ror2, which blocks the BMPR-IB-induced Smad pathway; these effects are critical for chondrogenesis, and BMPR-IB and Ror2 are mutated in alternative forms of brachydactyly. Thus, like cell proliferation, apoptosis and cell migration, cell differentiation induced by TGF-β family members frequently utilizes the abundant MAPK modules and occasionally alternative non-Smad effectors.

Evidence from developmental studies

Most of the studies described so far have used in vitro cell culture models, which are often indispensable for the elucidation of signalling and gene regulatory mechanisms, but have obvious limitations. Below, we briefly summarize a few developmental studies that provide in vivo evidence for non-Smad signalling mechanisms.

During migration of embryonic epithelia in *Drosophila*, the JNK pathway and the small GTPases Dcdc42 and Drac1 are important regulators of decapentaplegic (Dpp) expression and secretion, and eventually synergize with Dpp signalling (the *Drosophila* BMP pathway) (Glise and Noselli, 1997). During this migratory process, cytoskeletal regulation is very important, and the JNK and Drac1 pathways cooperate with Dpp-activated Dcdc42 and DPAK, which become the key players (Ricos et al., 1999). This scenario is similar to that discussed earlier in the context of the MEKK1 knockout (Zhang et al., 2003). Furthermore, the critical role of *Drosophila* JNK in secretion of Dpp resembles that of JNK in autocrine TGF-β secretion that has been revealed in studies of JNK1-JNK2 double-knockout mice (Ventura et al., 2004).

During *Xenopus* development, the XrhoA protein regulates

formation of the body axis and head in response to BMP signalling (Wunnenberg-Stapleton et al., 1999). In chicken neural crest development, BMP signalling similarly induces RhoB expression, which is necessary for neural crest delamination (Liu and Jessell, 1998). During chicken limb development, BMP-5 induces expression of many genes that drive the process, and both Smads and cooperating p38 pathways mediate this effect (Zuzarte-Luis et al., 2004). Finally, in a rat model of blood-testis barrier development, TGF-β3 signals in Sertoli cells through p38 to downregulate occludin expression (Lui et al., 2003).

The above studies provide some in vivo evidence that non-Smad signalling contributes to crucial developmental processes downstream of TGF-β factors. However, the plethora of non-Smad proteins discussed above remains largely untested by rigorous in vivo developmental or genetic studies.

Modulation of Smad activity by non-Smad effectors

An interesting feature of non-Smad signalling proteins is their ability to modulate Smad activity, most frequently negatively. One of the first clearly demonstrated examples of such regulation was the discovery that the ERK pathway can lead to direct phosphorylation of specific serine residues in the linker domain of R-Smads, blocking their nuclear translocation and transcriptional output (Kretzschmar et al., 1997). This finding has been corroborated by in vivo studies in *Xenopus* (Pera et al., 2003). In addition, the p38 substrate MSK1 kinase regulates the transcriptional activity of Smad3 by promoting its association with the co-activator p300 (Abecassis et al., 2004). TGF-β-activated JNK can phosphorylate Smad3 and induce its nuclear translocation and transcriptional activity (Engel et al., 1999). JNK also phosphorylates Jun, which enhances formation of complexes by Smad2 and its co-repressor protein TGIF and thus inhibits Smad2-dependent transcription (Pessah et al., 2001). A similar effect is evident in TGF-β activation of the PI3K pathway and the downstream phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates Smad3 and enhances its transcriptional activation of the collagen I gene in mesangial cells (Runyan et al., 2004). The same mechanism seems to operate downstream of BMPs during osteoblast differentiation (Ghosh-Choudhury et al., 2002): BMP-2 activates PI3K/Akt, which enhances the transcriptional activity of the BMP-specific Smad5. Thus, the crosstalk between Smads and kinase effectors whose activity is stimulated by TGF-β ligands can be either positive or negative and seems to permeate many physiological processes.

Concluding remarks

A commonly used term in the TGF-β field is the discrimination between Smad-dependent and Smad-independent signalling pathways. An important tool for the delineation of so-called ‘Smad-independent’ pathways is a collection of mammalian cells that lack the gene encoding Smad4 because of a deletion that occurred either in knockout mice or in various tumour cells (Hocevar et al., 1999; Sirard et al., 2000). For example, embryonic fibroblasts from the Smad4-knockout mouse were used to demonstrate that these cells maintain a number of cellular and gene responses to TGF-β (Sirard et al., 2000). Comparative gene profiling screens in the Smad4-null human mammary carcinoma cell line MDA-MB-468 comparing TGF-β and BMP signalling demonstrated that essentially all measurable gene expression responses to TGF-β1 and BMP-7 require reconstitution of wild-type Smad4 (Kowanetz et al., 2004). Smad4-null cellular tools offer the great advantage that signalling downstream of TGF-β, activin, BMP or other ligands is in principle disrupted. However, Smad4-null cells often express higher levels of R-Smads than normal cells (Maurice et al., 2001). Moreover, conditionally knocking out Smad4 in the mouse epiblast showed that only a subset of TGF-β/nodal and BMP responses require Smad4 (Chu et al., 2004). Thus, data supporting ‘Smad-independent’ signalling solely on the basis of studies performed in Smad4-deficient cells must be interpreted with caution. More recent approaches in which mice lacking Smad2 or Smad3 were used are more indicative because R-Smads are the primary effectors activated by TGF-β receptors (Piek et al., 2001; Yang et al., 2003). In addition, the use of RNA interference (RNAi) technology promises a potentially more reliable tool that can examine the role of all Smads downstream of TGF-β members (Kretschmer et al., 2003; Imamura et al., 2004). Tissue-specific multi-Smad-knockout experiments in mice or multi-Smad RNAi approaches are warranted to address this fundamental problem.

Nuclear signals transmitted by non-Smad proteins could regulate transcription independently or synergize with Smads. The latter seems to apply for most genes that receive signalling inputs from the TGF-β superfamily (Massagué, 2000). Nevertheless, many recent efforts have searched for specific Smad-dependent and non-Smad transcriptional modules by gene expression profiling. The pioneering work of Zavadil and colleagues (Zavadil et al., 2001) has provided an example: they measured the contribution of ERK signalling to the profile of gene expression downstream of TGF-β. Similar systematic approaches should soon shed further light on this area.

Whereas a classical view of TGF-β signalling is based on a relatively simple and linear Smad pathway that links receptor complexes on the plasma membrane to gene regulation in the nucleus, increasing evidence indicates that these signalling pathways are more complex networks. Multiple effector proteins can participate and transmit signals to specific cellular compartments, such as the actin cytoskeleton or the apoptotic machinery. In addition, non-Smad proteins can affect the activity of Smads and thus integrate with Smad signalling. A central feature of all these networks is the cell-type- and context-dependent mode of activation, which is a principal characteristic of TGF-β biology. Future studies, aimed at identification of additional components in such networks, need to focus more closely on the receptor complexes and identify novel cytoplasmic proteins whose function is directly regulated by the receptors. Analysis of such nodal points in the signalling networks in vitro and in vivo in model organisms should place non-Smad effectors closer to the heart of TGF-β signalling.

Owing to space limitations, selected literature is cited in this commentary. Funding of the authors’ work is provided by the Ludwig Institute for Cancer Research, the Swedish Cancer Society, the Swedish Research Council and the European Commission FP6 programme. We thank all current and previous members of our laboratories for their contributions to the scientific work. Special thanks to K. Miyazono (Tokyo University) and P. ten Dijke (Leiden University) for continuous collaborations and encouragement.

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