Non-Smad Signaling Pathways of the TGF-β Family

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Transforming growth factor β (TGF-β) and structurally related factors use several intracellular signaling pathways in addition to Smad signaling to regulate a wide array of cellular functions. These non-Smad signaling pathways are activated directly by ligand-occupied receptors to reinforce, attenuate, or otherwise modulate downstream cellular responses. This review summarizes the current knowledge of the mechanisms by which non-Smad signaling pathways are directly activated in response to ligand binding, how activation of these pathways impinges on Smads and non-Smad targets, and how final cellular responses are affected in response to these noncanonical signaling modes.

Transforming growth factor β (TGF-β) and structurally related polypeptide growth factors, including bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins, nodal, and Müllerian inhibitory substance, have a diverse array of regulatory functions ranging from specifying tissue pattern formation as morphogens during embryonic development to maintaining physiological homeostasis as cytokines in adult organisms (Wu and Hill 2009; Massagué 2012). The functions of the specific factors within the TGF-β family vary and can even be opposing in different cells and at different developmental stages. A frequently noted example of the context-dependent roles is the dichotomy of TGF-β's roles in tumorigenesis. TGF-β is a tumor suppressor for early-stage tumors and a potent growth inhibitor of cells of epithelial origin, but, in advanced stage of cancers, promotes tumor growth and progression by inducing epithelial-to-mesenchymal transition (EMT) and subsequent tumor invasion and metastasis (Massagué 2012; Katsuno et al. 2013). Understanding the diversity of the signaling activities of the TGF-β family has been a herculean task and a rich ground of important discoveries. Intense studies across different fields over the past three decades have revealed that all TGF-β-related ligands bind a heteromeric complex of type I and type II transmembrane receptors, each equipped with an intracellular kinase domain (Shi and Massagué 2003; Feng and Derynck 2005). In the ligand-bound receptor complex, the type II receptor kinases phosphorylate and thereby activate the type I receptors, which are also known as activin receptor–like kinases (ALKs). Downstream from this focal complex, the main conduit for

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ligand-initiated signaling events is the Smad family of transcription factors, among which Smad2 and Smad3 are activated by type I receptors for TGF-β, activin, or nodal, for example, ActRIB (ALK-4), TβRI (ALK-5), and ALK-7; whereas Smad1, Smad5, and Smad8 are activated by type I receptors of BMP signaling, for example, BMPRIA (ALK-3) and BMPRIB (ALK-6), or by ALK-1 and ALK-2, two type I receptors that mediate both TGF-β and BMP signaling. These receptor-activated Smads (R-Smads) then form a trimeric complex with Smad4, which acts as a common Smad to all ligand-activated Smad pathways, and accumulate in the nucleus to regulate target gene expression. Inhibitory Smads (I-Smads), that is, Smad6 and Smad7 in vertebrates, play a crucial role in repressing Smad-mediated signaling responses. Against this backdrop of Smad-mediated, canonical TGF-β signaling mechanisms, the activated receptors also signal through other signal transducers, for example, the mitogen-activated protein kinase (MAPK) pathways, including the extracellular signal-regulated kinases (Erks), c-Jun amino terminal kinase (JNK), p38 MAPK, as well as the IκB kinase (IKK), phosphatidylinositol-3 kinase (PI3K) and Akt, and Rho family GTPases, which in the context of TGF-β family signaling are collectively known as non-Smad signaling pathways (Moustakas and Heldin 2005; Zhang 2009). These receptor-activated, non-Smad transducers mediate signaling responses either as stand-alone pathways or in conjunction with Smads, and they converge onto Smads to control Smad activities. This review summarizes our current knowledge of the mechanisms by which the non-Smad signaling pathways are directly activated in response to ligand binding, how activation of these pathways impinges on Smads and non-Smad targets, and the final outcomes of cellular responses to these noncanonical signaling pathways. We aim to provide a comprehensive view that incorporates all data reported. Although we focus the discussion mostly on those pathways activated by TGF-β, the paradigms presented may extend to signaling by other ligands of the TGF-β family.

ACTIVATION OF ERK MAPK BY AND TYROSINE PHOSPHORYLATION OF TGF-β FAMILY RECEPTORS

Already, before the discovery of Smads, Erk MAPKs were thought to play a role in TGF-β signaling because it was shown that TGF-β causes a rapid activation of Ras in normal epithelial as well as colon carcinoma cells (Mulder and Morris 1992; Yan et al. 1994). Activation of Erk1/2 by TGF-β was shown to occur within 5–10 min of ligand treatment, a time frame that is comparable to the kinetics shown by mitogenic growth factors, such as epidermal growth factor (EGF), albeit with a lower intensity (Olsson et al. 2001). Rapid activation of Ras and/or Erk1/2 by BMP was also observed in myoblasts, osteoblasts, stem cells, endothelial cells, and cancer cells (Gallea et al. 2001; Lai and Cheng 2002; Zhou et al. 2007; Le Page et al. 2009). The TGF-β- or BMP-induced activation of Erk1/2 is dependent on the cell type and culture conditions, and caution must be taken in observing Erk activation because Erk activation in response to growth factors in the serum, cell–cell contacts, cell interactions with extracellular matrix, or oncogenic activation in cancer cells could easily obscure the much lower Erk1/2 activation in response to TGF-β or BMP. Interestingly, BMP-induced activation of Erk1/2 in osteoblasts and embryonic stem cells is usually followed by repression of Erk activation (Kua et al. 2012; Li et al. 2012). In some other cells, TGF-β or BMP induces a delayed Erk activation, typically with peak phosphorylation after several hours rather than minutes, implying an indirect mechanism that requires de novo protein synthesis (Simeone et al. 2001). Smad-mediated transcription may play roles in both the delayed activation of Erk1/2 and the inhibition of Erk activity (Javelaud and Mauviel 2005; Li et al. 2012).

Akin to the tyrosine receptor signaling, the TGF-β-induced GTP loading on Ras could lead to recruitment of the proto-oncogene product Raf to the plasma membrane, resulting in activation of Erk1/2 through MEKs. Well known as serine-threonine kinases, both type I and type II TGF-β receptors also phosphorylate on tyrosine

and are tyrosine phosphorylated. Three tyrosines of TβRII, Tyr259, Tyr336, and Tyr424, were shown to be autophosphorylated, albeit at a much lower level than the autophosphorylation of TβRII on serines and threonines (Lawler et al. 1997). Tyrosine phosphorylation of TβRII may lead to recruitment of Src homology 2 (SH2) domain–containing (Shc) proteins by analogy to signaling events elicited by receptor tyrosine kinase (RTK) signaling (Schlessinger 2000; McKay and Morrison 2007). In support of this view, Src was shown to phosphorylate TβRII on Tyr284, which serves as a docking site for the recruitment of growth factor receptor-bound protein 2 (Grb2) and Shc, thus linking TβRII to Ras and Erk activation (Galliher and Schiemann 2007). Interestingly, high levels of ectopic TβRII expression preferentially activate Erk1/2 in dermal cells, whereas high levels of ectopic TβRI expression channel the signaling toward the Smad pathway in epidermal cells (Bandyopadhyay et al. 2011), contrasting the difference in substrate preference between type I and type II receptors. Additionally, TβRI was phosphorylated on tyrosine after TGF-β treatment (Lee et al. 2007). Because TβRI is activated by TβRII on ligand binding and forms a tetrameric receptor complex with TβRII, it is not clear whether the tyrosine phosphorylation results from autophosphorylation or phosphorylation in *trans* by TβRII. Nevertheless, the activated TβRI recruits and phosphorylates ShcA on tyrosine and serine residues, and ShcA tyrosine phosphorylation then promotes the formation of ShcA-Grb2-Sos complexes that lead to activation of Ras at the plasma membrane (Lee et al. 2007). The kinase activities of both TβRI and TβRII are required for ShcA phosphorylation, and overexpressed ShcA mutants lacking either its phosphotyrosine binding (PTB) or its SH2 domain blocked the Erk1/2 activation by TGF-β (Lee et al. 2007). Therefore, recruitment of signaling mediators as a result of receptor tyrosine phosphorylation is one of the mechanisms that enables TGF-β to activate non-Smad signaling (Fig. 1). With this scenario, both TβRI and TβRII should be seen as dual-specific kinase receptors that accommodate the less recognized roles resulting from tyrosine phosphorylation.

It is not known whether the BMPRII receptor is tyrosine phosphorylated, although c-Src was shown to interact with a carboxy terminal sequence of BMPRII (Wong et al. 2005). The BMPRIA receptor is phosphorylated on tyrosine residues by c-Abl, a non-RTK (Kua et al. 2012). However, c-Abl-mediated tyrosine phosphorylation of BMPRI inhibits BMP-induced Erk activation, and enhances Smad1 and Smad5 activity (Kua et al. 2012). Although the role of tyrosine phosphorylation of BMP receptors remains elusive, Shc is required for BMP-induced Erk activation. BMP-4 stimulation induces rapid dissociation of β_3-integrin from BMPRIA and BMPRIB, and association of β_3-integrin with focal adhesion kinase (FAK) and Shc, and ultimately, Erk activation (Chang et al. 2009).

A key biological function of TGF-β is the induction of EMT, which normally occurs in embryonic development and is pathologically associated with tumor invasion and dissemination and fibrosis (Moustakas and Heldin 2012; Lamouille et al. 2014). During EMT, cells lose epithelial characteristics and acquire properties of mesenchymal cells, including down-regulation of adherens junctions and associated proteins (e.g., E-cadherin), increased matrix metalloproteinase (MMP) activity, induction of actin stress fibers, and acquisition of motile and invasive properties. In advanced tumors, TGF-β promotes tumorigenesis by inducing EMT through a combination of Smad-dependent and -independent mechanisms. Erk activation was shown to be essential in TGF-β-induced EMT, and is required for disassembly of adherens junctions and cell motility (Zavadil et al. 2001; Xie et al. 2004). Target genes that mediate Erk's role in TGF-β-induced EMT have been identified (Zavadil et al. 2001), and their functions relate to remodeling integrin-based cell–matrix interactions, and promoting endocytosis, as well as cell motility.

Like TGF-β, BMPs can also induce EMT, cell migration, and invasion in certain cancer cells. Erk activity has been shown to contribute to BMP-7-mediated morphologic conversion in prostate cancer cells (Lim et al. 2011). Blocking Erk activity using chemical inhibitors significantly inhibits BMP-2-induced motility and

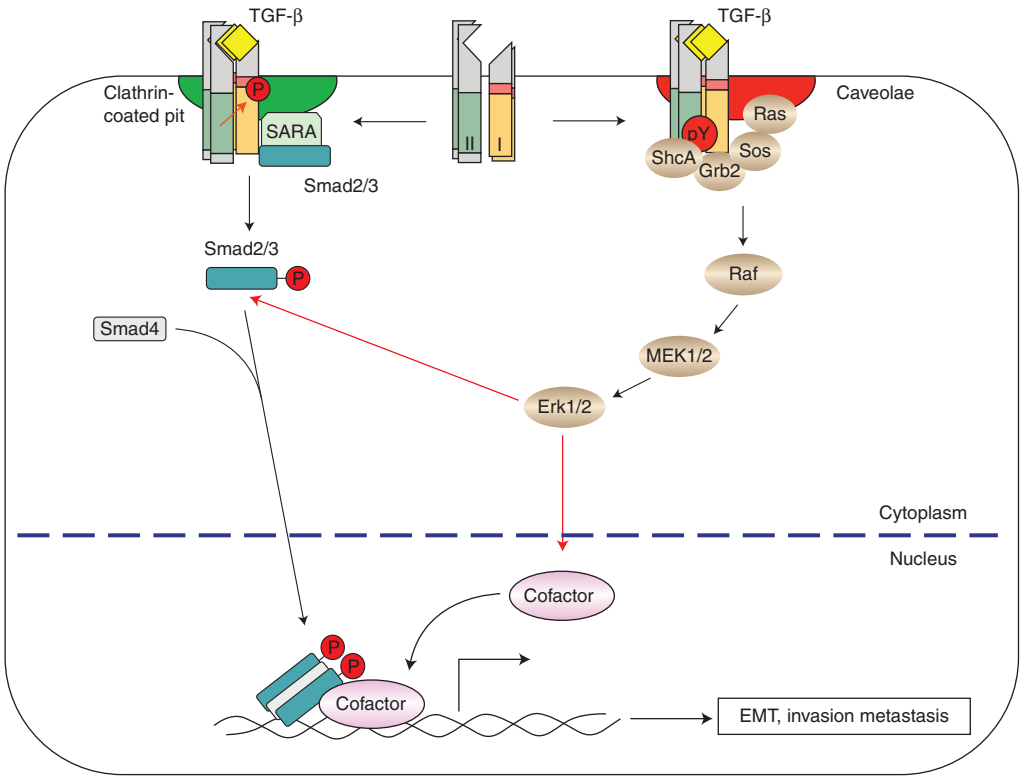

Figure 1. Transforming growth factor β (TGF-β)-induced activation of the extracellular signal-regulated kinase (Erk) mitogen-activated protein kinase (MAPK) pathway. On binding of TGF-β to its receptor complex, the constitutively active type II receptors phosphorylate the type I receptors at Ser/Thr residues, and induce tyrosine phosphorylation of both the type I and II receptors and ShcA. The phosphorylated tyrosines then recruit Grb2/Sos to activate Erk1/2 MAPK through activation of Ras, Raf, and MEK1/2 in a Smad-independent manner. ShcA directs the TGF-β receptors to caveolin-1-containing lipid raft for Erk MAPK activation. Clathrin-dependent internalization of TGF-β receptors into the Smad anchor for receptor activation (SARA)-containing early endosome is required for Smad activation. Activated Erk MAPK contributes to epithelial-to-mesenchymal transition (EMT) by phosphorylating targeted transcription factors, which in turn control transcription of EMT-related genes without or in cooperation with activated Smad complexes. Erk MAPK also directly phosphorylates R-Smads, thus controlling their activity.

invasiveness in lung and gastric cancer cells (Hsu et al. 2011; Kang et al. 2011). In addition, Erk activity was shown to be essential for BMP-induced cell differentiation by regulating BMP target genes, such as alkaline phosphatase (ALP), collagen 1, fibronectin, osteopontin, osteocalcin, and Runx2 (Gallea et al. 2001; Lai and Cheng 2002).

One of the mechanisms by which Erk regulates the TGF-β- and BMP-induced biological functions is through phosphorylation of its substrates, such as AP-1 family members, p53, and other transcription factors (Yoon and Seger 2006). Many of these Erk-regulated transcription factors interact and cooperate with Smads to regulate gene expression. In addition, Erk can also regulate the activities of R-Smads, including Smad1, Smad2, and Smad3, through direct phosphorylation (Kretzschmar et al. 1997, 1999; Funaba et al. 2002). In cell culture systems, phosphorylation of Smads by Erk often inhibits the transcription activity of R-Smads, and this regulation has been invoked to explain how oncogenic Ras overrides TGF-β-mediated growth arrest in cancer cells (Kretzschmar et al. 1999). However, others reached different con-

clusions, in part based on differences in cell type and the response examined (Lehmann et al. 2000; Javelaud and Mauviel 2005). Furthermore, ShcA, although critical for TGF-β-induced Erk activation, can also repress Smad activation by competing with Smad2 or Smad3 for TβRI binding and directing TβRI to caveolin-1-containing lipid raft, a membrane domain that differs from the early endocytic compartment in which Smads are activated (Muthusamy et al. 2015).

ACTIVATION OF TGF-β-ACTIVATED KINASE 1 (TAK1) AND DOWNSTREAM JNK, P38 MAPK, AND IKK

Ligand binding also induces TGF-β receptors to activate the JNK and p38 MAPK signaling pathways (Fig. 2). These two MAPKs are specifically activated by MAP kinase kinases (MKKs) MKK4 or MKK3/6, respectively, in response to cytokines and environmental stress (Weston and Davis 2007). Similarly to cytokine stimulation, TGF-β induces activation of JNK through MKK4 (Frey and Mulder 1997; Engel et al. 1999; Hocevar et al. 1999) and p38 MAPK through MKK3 or MKK6 in various cell lines (Hanafusa et al. 1999; Sano et al. 1999; Bhowmick et al. 2001b; Yu et al. 2002). Depending on cell contexts, the activation of JNK and p38 MAPK by TGF-β or BMP can be either rapid or relatively delayed. The delayed activation usually requires Smad signaling, for example, resulting from Smad-mediated transcription activation of Gadd45β, an upstream activator of MKK4, as seen in pancreatic cancer cells, hepatocytes, and osteoblasts (Takekawa et al. 2002). In contrast, rapid and direct transient activation of JNK and p38 MAPK, independent of Smad activation, is revealed in *Smad3*$^{-/-}$ cells, *Smad4*$^{-/-}$ cells, and cells that express a dominant-negative form of Smad3 (Engel et al. 1999; Hocevar et al. 1999). Smad-independent activation of JNK and p38 MAPK is also observed using a mutant TβRI receptor with an altered L45 loop, which renders the receptor defective in Smad binding and phosphorylation, but still retains intact kinase activity (Yu et al. 2002; Itoh et al. 2003).

MAP kinase kinase kinases (MAPKKKs) act upstream of and directly activate MKK3/6 and MKK4. Among these, the MAPKKK named TAK1 was identified using a mouse cDNA screen, based on its ability to substitute a latent MAPKKK of *Saccharomyces cerevisiae* in the yeast-mating pheromone response, and found to be capable of activating TGF-β signaling and to be activated in response to TGF-β (Yamaguchi et al. 1995). In *Xenopus*, TAK1 is required for BMP-induced mesoderm induction and patterning during embryonic development (Shibuya et al. 1998), whereas in mice, TAK1 is required for the proper development of the vasculature in both embryo and yolk sac, a process that also depends on the type I receptor ALK-1 and the type III receptor endoglin (Shim et al. 2005; Jadrich et al. 2006). The requirement of TAK1 for TGF-β-induced JNK and NF-κB activation was also shown in *Tak1*-deficient mouse embryonic fibroblasts (MEFs) (Shim et al. 2005).

In contrast to the activation of Smad and Erk pathways by phosphorylation, TGF-β receptors enlist an elaborate mechanism that centers on ubiquitylation by tumor necrosis factor receptor-associated factor 6 (TRAF6), a RING-domain E3 ligase, to activate this branch of non-Smad signaling. Known to control the activation of TAK1 that is induced by the interleukin-1 receptor (IL-1R) and Toll-like receptors (TLRs) (Wu and Arron 2003), TRAF6 was also found to mediate the TGF-β-induced activation of TAK1 and, subsequently, JNK and p38 MAPK (Sorrentino et al. 2008; Yamashita et al. 2008). Mammalian genomes encode six TRAF proteins, identified by a conserved carboxy-terminal TRAF domain, a RING E3 ligase domain, and several Zn fingers in the variable amino terminus (Inoue et al. 2007). Analogous to the mechanism associated with IL-1R or TLR receptor signaling, TRAF6 associates with the activated type II and type I TGF-β receptor complex through its TRAF domain. This binding activates the E3 ligase activity of the RING domain and leads to intramolecular polyubiquitylation of TRAF6 at Lys63 (Yamashita et al. 2008). Unlike ubiquitylation at Lys48, which targets the protein for proteasomal degradation, polyubiq-

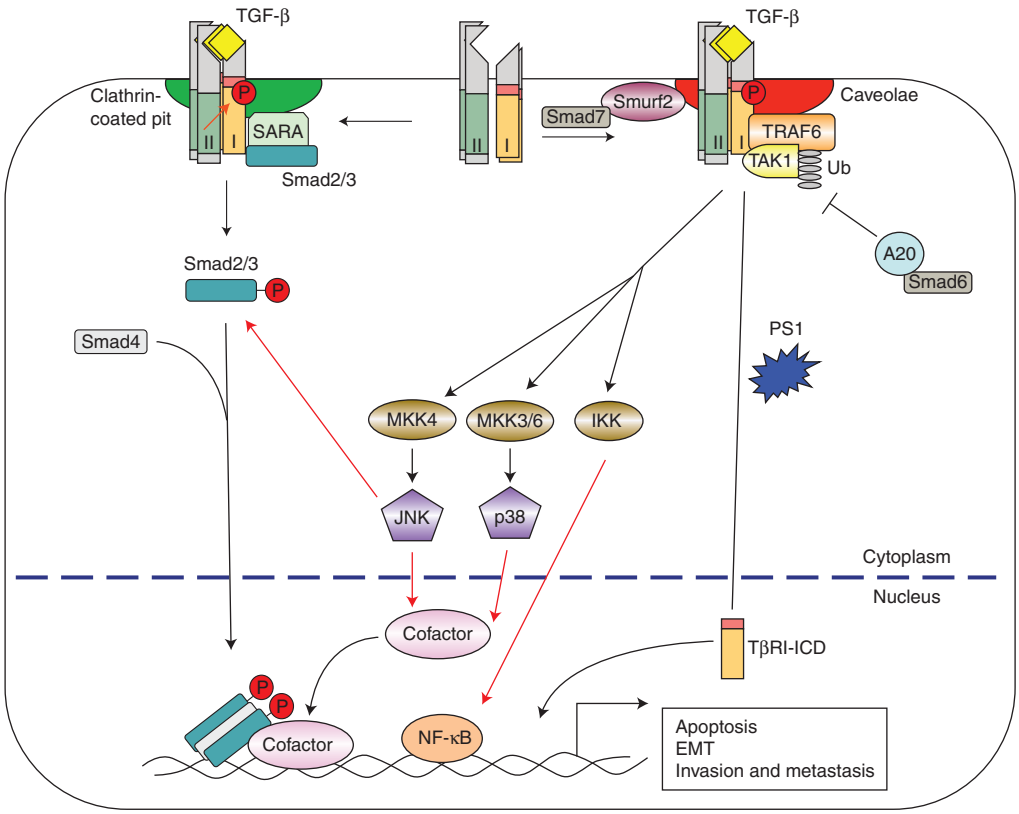

Figure 2. Transforming growth factor β (TGF-β)-induced activation of c-Jun amino terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signaling, and the IκB kinase (IKK) pathway. Ligand-bound TGF-β receptors interact with TRAF6 and TGF-β-activated kinase 1 (TAK1), and induce Lys63-linked polyubiquitylation of TRAF6, which then activates TAK1 and downstream kinases, such as JNK, p38 MAPK, and IKK. Smad6 inhibits the TGF-β-induced JNK and p38 MAPK activation by recruiting a deubiquitylase A20 to deubiquitylate TRAF6, whereas Smad7 promotes TGF-β-induced JNK and p38 MAPK activation by directing the TGF-β receptors to caveolin-1-containing lipid rafts through interaction with Smurf2. Activated JNK and p38 MAPK then phosphorylate their targeted transcription factors, and IKK phosphorylates nuclear factor-κB (NF-κB), and these transcription factors cooperate with activated Smads to regulate apoptosis and epithelial-to-mesenchymal transition (EMT). In addition, JNK directly phosphorylates receptor-activated Smad (R-Smad) to regulate Smad activity. TRAF6 can also stimulate proteolytic cleavage of TβRI dependent on presenillin 1 and Lys63 ubiquitylation of TRAF6 to generate the intracellular domain (ICD) of TβRI, which then translocates into the nucleus to regulate cell invasion. SARA, Smad anchor for receptor activation; MKK, MAP kinase kinase; TRAF6, tumor necrosis factor receptor–associated factor 6.

uitylation of TRAF6 at Lys63 provides a scaffold for the assembly and activation of protein kinase complexes (Haglund and Dikic 2005). Polyubiquitylated TRAF6 thus recruits TAK1 through association and induces TAK1 activation, allowing TAK1 to activate JNK and p38 MAPK (Fig. 2) (Wang et al. 2001). Recent studies showed that TRAF4 also interacts with TβRI, resulting in Lys63-linked polyubiquitylation of TRAF4 and subsequent activation of TAK1 (Zhang et al. 2013). It will be interesting to determine whether TRAF4 and TRAF6 function in a common complex to activate TAK1, and whether TRAF6 and TRAF4 are required for BMP-mediated TAK1 activation. In addition, other MAPKKKs, such as MEKK1, MEKK4, MLK2, MLK3, and ASK1, were also reported to be involved in TGF-β-mediated activation

of JNK or p38 MAPK (Atfi et al. 1997; Brown et al. 1999; Zhang et al. 2003; Kim et al. 2004; Sapkota 2013). It remains to be determined whether they can be recruited to TGF-β receptors in the same manner as the TAK1.

Although independent from R-Smad activation, the activation of the JNK and p38 MAPK through TAK1 by TGF-β receptors is regulated by the inhibitory Smad6 and Smad7. TGF-β and BMP induce expression of Smad6 and Smad7, which in turn inhibit canonical, Smad-dependent TGF-β/BMP signaling (Afrakhte et al. 1998; Ishida et al. 2000; Miyazono 2008). Smad6 and Smad7 also inhibit BMP-induced TAK1 and p38 MAPK activation, possibly through interaction with TAK1 or the TAK1-binding protein, TAB1 (Kimura et al. 2000; Yanagisawa et al. 2001). Smad6 also inhibits the TGF-β-induced activation of TAK1, JNK, and p38 MAPK. This occurs through a different mechanism in which Smad6 recruits the deubiquitylase A20 to TRAF6, thus blocking Lys63-linked polyubiquitylation of TRAF6 (Jung et al. 2013). In contrast, Smad7 was shown to facilitate the TGF-β-induced activation of JNK and p38 MAPK (Mazars et al. 2001; Edlund et al. 2003). The possible function of Smad7 as a scaffold to support the interactions among TAK1, MKK3, and p38 MAPK in TGF-β signaling (Edlund et al. 2003) does not explain how the interaction of Smad7 and TAK1 in BMP signaling inhibits p38 MAPK activation. Additionally, Smad7 may act as an adaptor that bridges TβRI to Smurf2, an HECT-domain E3 ligase, thereby routing TβRI to caveolin-1-containing lipid rafts for turnover by lysosomes (Kavsak et al. 2000; Ebisawa et al. 2001; Di Guglielmo et al. 2003). Endogenous TRAF6 is substantially associated with lipid rafts (Ha et al. 2003a), and sequestration of TRAF6 in the lipid rafts was shown to be required for TRAF6-mediated activation of NF-κB in response to TLR activation or IL-1β signaling (Ha et al. 2003b; Soong et al. 2004; Oakley et al. 2009). It is, therefore, possible that Smad7 enhances TGF-β-induced activation of JNK and p38 MAPK by routing the TGF-β receptors to lipid rafts where TRAF6 is localized. Indeed, TβRI localization in lipid rafts is essential for TGF-β-mediated activation of Erk, JNK, and p38 MAPK (Zuo and Chen 2009; Shapira et al. 2014). The localization of BMP receptors in distinct plasma membrane domains was also shown to have a major impact on BMP signaling specificity (Hartung et al. 2006). In contrast to TGF-β signaling, association of lipid raft with BMP receptors is important for canonical BMP-induced R-Smad phosphorylation but not p38 MAPK activation (Zhou et al. 2010). This could explain why Smad7 enhances TGF-β- but inhibits BMP-induced p38 MAPK activation.

One of the important consequences of JNK or p38 MAPK activation is apoptosis, which is recognized as an activity of BMP in development and a mechanism of tumor suppression by TGF-β. Consistent with this notion, overexpression of TAK1 causes cells or *Xenopus* embryos to undergo apoptosis, whereas cells that ectopically express a kinase-inactive mutant TAK1 are protected from TGF-β- or BMP-induced apoptosis (Shibuya et al. 1998; Kimura et al. 2000; Edlund et al. 2003). Moreover, silencing the expression of TRAF6 using siRNAs or treating the cells with a pharmacological inhibitor of p38 MAPK efficiently blocks TGF-β-induced apoptosis (Yu et al. 2002; Sorrentino et al. 2008; Yamashita et al. 2008; Jung et al. 2013). Because Smad3 also plays an essential role in the proapoptotic function of TGF-β (Yamamura et al. 2000; Jang et al. 2002; Valderrama-Carvajal et al. 2002; Yang et al. 2006), therefore, the JNK and p38 MAPK pathway cooperates with the Smad pathway in promoting apoptosis.

Because TAK1 is capable of activating IKKs, which in turn activates NF-κB (Takaesu et al. 2003), TGF-β and BMPs may cross talk with the NF-κB pathway. Indeed, activation of TAK1 by TGF-β has been linked to activation of NF-κB signaling in hepatocytes, fibroblasts, osteoclasts, and hepatocellular carcinomas (Arsura et al. 2003; Gingery et al. 2008). Consistent with this notion, Lys63-linked polyubiquitylation of TAK1 by TRAF6 is required for activation of NF-κB signaling by TGF-β in HepG2 hepatoma cells (Hamidi et al. 2012). Future studies are necessary to determine the cellular context or environmental cues that lead to either the

proapoptotic activity of TGF-β or the prosurvival activity of NF-κB by activating TAK1.

In addition to induction of apoptosis, TGF-β-induced JNK and/or p38 MAPK signaling also contributes to TGF-β-induced EMT. Blocking p38 MAPK activity using small molecular inhibitors or by expressing a dominant-negative MKK3 mutant blocks the changes in cell shape and reorganization of actin cytoskeleton that are associated with EMT (Bakin et al. 2002; Yoo et al. 2003). In addition, silencing TRAF6 expression also causes inhibition of TGF-β-induced EMT (Yamashita et al. 2008). The role of TRAF6 in promoting EMT and cancer cell invasion may result from its ability to stimulate proteolytic cleavage of TβRI in a presenillin 1 and Lys63 ubiquitylation-dependent manner to generate an intracellular domain (ICD) of TβRI, which was surprisingly found in the nucleus to promote cell invasion in certain cancer cells (Mu et al. 2011; Gudey et al. 2014). Moreover, TRAF4 was shown to be required for migration, EMT, and metastatic dissemination in response to TGF-β (Zhang et al. 2013). It is likely that non-Smad signaling through TRAF4 or TRAF6 and TAK1 leading to activation of JNK and p38 MAPK signaling is an obligatory step in TGF-β-induced EMT and cancer cell invasion.

THE PI3K-AKT PATHWAY IN TGF-β FAMILY SIGNALING

The serine/threonine kinase protein kinase B, more commonly known as Akt, regulates many biological processes, including cell survival, proliferation, increase in cell size, and metabolism (Carnero et al. 2008; Fruman and Rommel 2014). Growth factors, hormones, and cytokines are known to activate Akt through PI3K. TGF-β and BMP were also shown to activate Akt through PI3K, and Smad-independent or -dependent mechanisms have been reported (Fig. 3) (Bakin et al. 2000; Vinals and Pouysségur 2001; Ghosh-Choudhury et al. 2002; Lamouille and Derynck 2007; Gamell et al. 2008; Boergermann et al. 2010). By immunoprecipitation, p85, the regulatory subunit of PI3K was found constitutively associated with TβRII, but its association with TβRI was shown only in the presence of TGF-β (Yi et al. 2005). Although the interaction between TGF-β receptors and p85 may not be direct, the receptor kinase activities are essential for activation of PI3K, and TβRI kinase inhibitors block the TGF-β-induced activation of Akt by PI3K (Bakin et al. 2000; Lamouille and Derynck 2007). TGF-β-induced PI3K and Akt signaling was also shown to be activated through receptor-mediated Lys63-ubiquitylation of TRAF6, which in this way not only activates TAK1, but also induces ubiquitylation, membrane recruitment, and activation of Akt (Yang et al. 2009). Besides these direct, Smad-independent mechanisms, TGF-β was also shown to activate the PI3K-Akt pathway by inducing the expression of the miR-216a/217 microRNA cluster in kidney glomerular mesangial carcinoma and liver hepatocellular carcinoma cells (Kato et al. 2009; Xia et al. 2013). MiR-216a/217 is capable of inducing hyperactivation of the PI3K-Akt pathway by repressing the expression of Smad7 and PTEN (phosphatase and tensin homolog), an inhibitor of Akt. TGF-β was also reported to activate Akt signaling in mesangial cells by inducing the expression of another microRNA, miR-21, that represses PTEN expression (Dey et al. 2012). Finally, TGF-β can also indirectly activate PI3K-Akt signaling by inducing the expression of TGF-α that activates EGF receptor signaling and, thus, PI3K-Akt signaling (Vinals and Pouysségur 2001). Conversely, the TGF-β-induced, Smad-dependent expression of the lipid phosphatase SHIP (SH2-containing inositol 5′-phosphatase) can down-regulate PI3K-Akt signaling (Valderrama-Carvajal et al. 2002), and this mechanism may account for or contribute to the transient nature of TGF-β-induced Akt phosphorylation.

Activation of the PI3K-Akt pathway also contributes to TGF-β-induced EMT. Pharmacological inhibitors have implicated PI3K activation in the actin filament reorganization and cell migration during EMT induced by TGF-β. However, caution must be taken when interpreting results with PI3K inhibitors because the dosage routinely applied to block PI3K-Akt-mTOR (mammalian target of rapamycin)

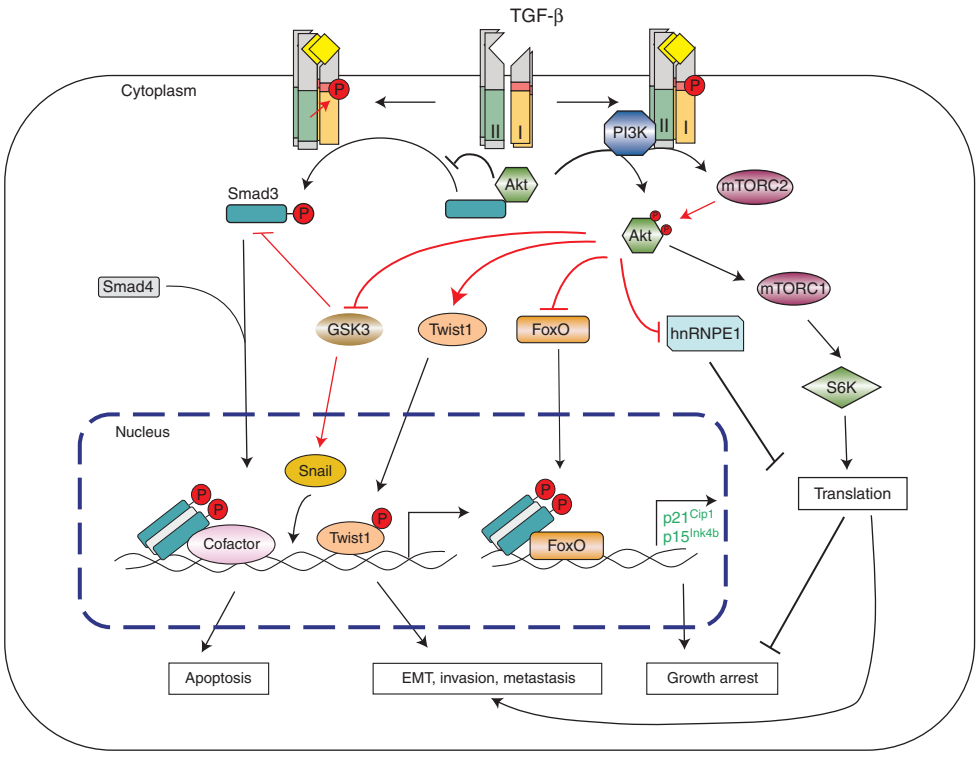

Figure 3. Transforming growth factor β (TGF-β)-induced activation of the PI3K-Akt pathway. TGF-β can induce PI3K and Akt activation, possibly through interaction of the p85 subunit of PI3K with the TGF-β receptors. Activated Akt then controls translational responses through mTOR1 and S6K, or directly acts on the translational responses by inducing phosphorylation of hnRNPE. TGF-β also induces activation of mTORC2, which contributes to enhanced Akt activation and forms a reinforcing feedback loop in PI3K-Akt activation. These non-Smad-mediated translational responses collaborate with Smad-mediated transcriptional responses during epithelial-to-mesenchymal transition (EMT), but can antagonize Smad-mediated transcription responses during growth arrest or apoptosis. Akt can regulate Smad3 activity by sequestering Smad3 in the cytoplasm or inhibiting GSK3β-mediated Smad3 phosphorylation and degradation. Akt can also regulate apoptosis or EMT by directly phosphorylating FoxO or Twist1 or inducing Snail phosphorylation by GSK3β.

signaling also reduces the Smad2 and Smad3 activation (Bakin et al. 2000; Edlund et al. 2004), which may result from interference with the SARA (Smad anchor for receptor activation)-dependent association of Smad2 and/or Smad3 to the TGF-β receptors (Tsukazaki et al. 1998; Hayes et al. 2002).

mTOR, a target of the Akt kinase, plays important roles in the contribution of the PI3K-Akt pathway to TGF-β-induced EMT. In mammary epithelial cells and keratinocytes, TGF-β induces rapid activation of mTOR complex 1 (mTORC1) and S6 kinase (S6K), leading to increased protein synthesis, cell size, motility, and invasion (Lamouille and Derynck 2007). TGF-β also induces activation of mTOR complex 2 (mTORC2), which promotes cytoskeletal reorganization, RhoA activation, and cell migration (Lamouille et al. 2012). In addition, mTORC2 contributes to enhanced Akt activation at a late stage of EMT (Lamouille et al. 2012), thus forming a positive feedback loop in the PI3K-Akt pathway activation. Besides mTOR, Akt also regulates key EMT transcription factors. By phosphorylating glycogen synthase kinase (GSK) 3β, Akt stabilizes NF-κB and the EMT transcription factor Snail, thus enhancing Snail-dependent transcription in EMT (Zhou et al.

2004; Julien et al. 2007). Akt also phosphorylates the EMT transcription factor Twist1, enhancing its activity to induce expression of TGF-β2, which in turn promotes TGF-β receptor signaling, PI3K-Akt pathway activity, and EMT (Xue et al. 2012). Furthermore, TGF-β-activated Akt2 was shown to phosphorylate hnRNP E1 (heterogeneous nuclear ribonucleoprotein E1) and disrupt the hnRNP E1–eEF1A1 (eukaryotic translation elongation factor 1 α1) interaction, thereby releasing translational inhibition of several genes required for EMT (Hussey et al. 2011). Besides its role in EMT, PI3K also affects the TGF-β-induced fibroblast proliferation and morphological transformation (Wilkes et al. 2005).

Akt also directly affects Smad activities in response to TGF-β and, thus, Smad-mediated transcription responses. For example, the association of Akt with unphosphorylated Smad3 restricts TβRI-induced activation and nuclear localization of Smad3 and, thus, attenuates the Smad3-mediated transcription (Conery et al. 2004; Remy et al. 2004). Consistent with this, activation of PI3K or Akt can protect cells from TGF-β-induced apoptosis and growth inhibition (Chen et al. 1998; Shin et al. 2001; Song et al. 2006), whereas TGF-β stimulation inhibits the association of Akt with Smad3, allowing Smad3 to escape Akt-mediated cytoplasmic sequestration (Conery et al. 2004; Remy et al. 2004). Akt can also regulate Smad-mediated transcription by phosphorylating the forkhead transcription factor FoxO, which is required in several Smad-mediated responses important in growth arrest, for example, the induction of $p15^{INK4B}$ and $p21^{CIP1}$ expressions (Seoane et al. 2004; Gomis et al. 2006). Phosphorylation of FoxO proteins by Akt hampers their nuclear localization, and prevents their participation in Smad-mediated transcription regulation. Akt activation directly promotes TβRI stability by phosphorylating and promoting membrane localization of a deubiquitylating enzyme, USP4 (ubiquitin-specific peptidase 4), which deubiquitylates and thus stabilizes TβRI and promotes non-Smad pathways (Zhang et al. 2012). On the other hand, activation of Akt was shown to enhance TGF-β and BMP-induced transcription responses by stabilizing Smad1 and Smad3. This increased stability results from inactivating GSK3β, because phosphorylation of Smad1 and Smad3 by GSK3β leads to ubiquitylation and degradation (Sapkota et al. 2007; Guo et al. 2008).

ACTIVATION OF RHO-LIKE GTPases IN RESPONSE TO TGF-β PROTEINS

The Rho-like GTPases, RhoA, RhoB, Rac, and Cdc42, control the dynamics of cytoskeletal organization, cell motility, and gene expression through a variety of effectors (Jaffe and Hall 2005). TGF-β and BMP can activate Rho-like GTPases in a cell-type-dependent manner (Fig. 4). For example, in epithelial cells and primary keratinocytes, TGF-β activates RhoA in its GTP-bound state within 5 min, and this activation is followed by a rapid attenuation by 15 min (Bhowmick et al. 2001a; Edlund et al. 2002). In mesenchymal stem cells, BMP induces a rapid activation of RhoA and Rho-associated protein kinase (ROCK) after cell spreading (Wang et al. 2012). Activation of RhoA by TGF-β or BMP is likely independent of Smad2 or Smad3, as suggested by the rapid onset and the inability of a dominant-negative Smad3 mutant or Smad4 deficiency to block RhoA activities (Bhowmick et al. 2001a; Voorneveld et al. 2014). Interestingly, JNK and p38 MAPK can be activated by direct association with these Rho GTPases, as an alternative to the mode of activation by MAPKKKs (Coso et al. 1995; Minden et al. 1995); however, it is not clear whether this mechanism plays a role in TGF-β- or BMP-induced JNK and p38 MAPK activation.

Paradoxically, TGF-β was found to downregulate RhoA protein levels through Par6 (Ozdamar et al. 2005), a scaffold protein that binds TβRI at tight junctions and regulates cell polarity in polarized epithelial cells. Engagement with TGF-β causes the receptor complex to accumulate at tight junctions, which then leads to phosphorylation of Par6 by TβRII and, subsequently, recruitment of the ubiquitin E3 ligase Smurf1 to the activated receptor complex. The Par6–Smurf1 complex then mediates localized ubiquitylation and turnover of RhoA in a pro-

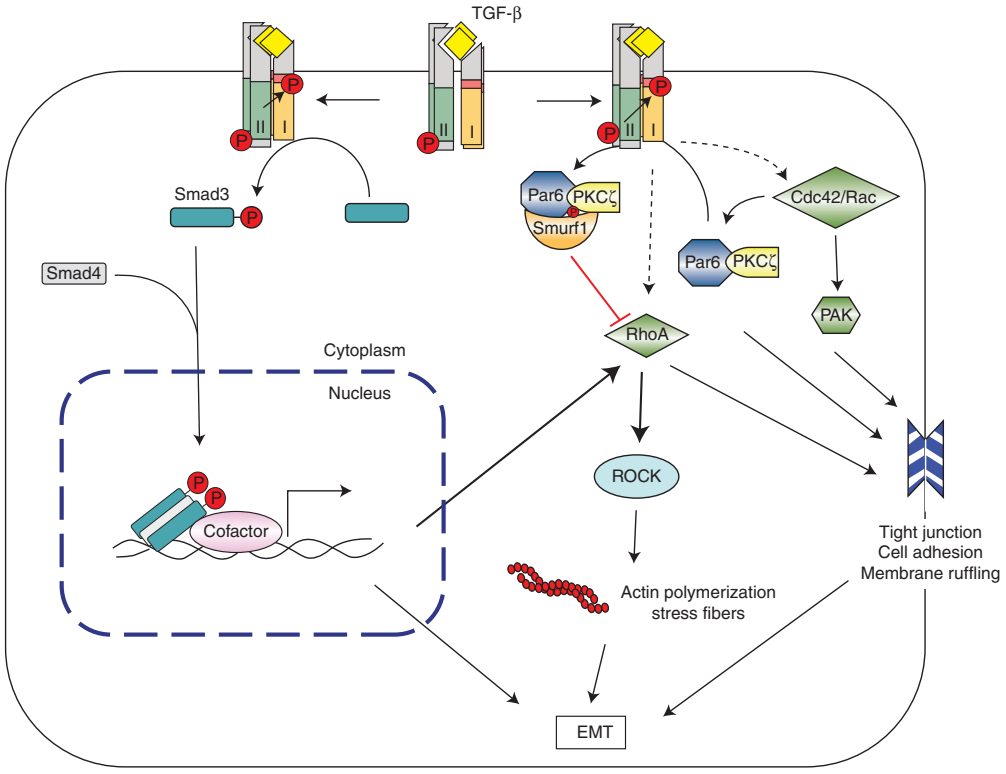

Figure 4. Transforming growth factor β (TGF-β)-induced regulation of the Rho family of small GTPases. RhoA and Rho-associated protein kinase (ROCK) can be activated by TGF-β by either Smad-dependent or -independent mechanisms to induce actin polymerization and stress fiber formation during epithelial-to-mesenchymal transition (EMT). Additionally, the TβRII receptor can directly phosphorylate Par6 by recruiting Smurf1, thus targeting RhoA for degradation, which leads to tight junction dissociation. TGF-β can also induce tight junction dissociation and cell migration during EMT by recruiting Cdc42 and/or Rac1 to the receptor complex, and activate p21-activated kinase (PAK) signaling. PKC, Protein kinase C.

tein kinase Cζ (PKCζ)-dependent manner, which enables TGF-β-dependent dissolution of tight junctions, a prerequisite for EMT. It is therefore possible that TGF-β regulates RhoA activity in two different phases and in two different cell compartments. Rapid activation of RhoA in the early phase of signaling may be complemented by localized down-regulation of RhoA levels at tight junctions at a later stage. Both phases of this regulation of RhoA in response to TGF-β appear to be essential for the TGF-β-induced EMT.

In addition to RhoA, TGF-β also activates the Cdc42 GTPase. Similarly to activation of RhoA, Cdc42 activation occurs independently of Smads, and blocking both Smad2 and Smad3 phosphorylation does not affect the activation of p21-activated kinase 2 (PAK2), downstream from Rac and Cdc42 activation (Wilkes et al. 2003). Association of Cdc42 with TGF-β receptor complexes at the cell surface has been shown (Barrios-Rodiles et al. 2005), and a cluster of proteins involved in Cdc42 and PAK signaling was found in the TGF-β receptor–associated protein complex. This complex includes the PAK-interacting Cdc42 GTPase, the Rac1 exchange factors α-PIX and β-PIX, PAK1 itself, a PAK1-interacting partner, oxidative stress-responsive kinase-1 (OSR1), and occludin, a tight-junction accessory protein (Barrios-Rodiles et al. 2005). Additionally, LIM kinase 1 (LIMK1), an effector of the PAK network, asso-

ciates with BMPRII and this interaction synergizes with Cdc42 and activates the catalytic activity of LIMK1, increases the phosphocofilin level, and induces changes in the actin cytoskeleton (Foletta et al. 2003; Lee-Hoeflich et al. 2004). As in the TGF-β pathway, BMP-induced activation of Rho, ROCK, and LIMK is also important for cancer cell dissemination (Voorneveld et al. 2014). It is noteworthy that a delayed peak of RhoA and Cdc42 activation, dependent on new protein synthesis, is observed in certain cells. Induction of this peak of GTPase activity by TGF-β may require expression of NET1, a RhoA-specific guanine exchange factor that activates RhoA through Smad-mediated transcription (Shen et al. 2001).

ACTIVATION OF OTHER NON-Smad SIGNALING PATHWAYS

In some mesenchymal cell lines, but not in epithelial cell lines, TGF-β was shown to activate the c-Abl tyrosine kinase, likely independent of Smad2 or Smad3 activation (Daniels et al. 2004). Moreover, inhibition or loss of c-Abl prevents TGF-β-induced morphological changes, extracellular matrix gene expression, and cell proliferation in fibroblasts (Daniels et al. 2004; Wang et al. 2005). In BMP signaling, c-Abl was found to associate with and phosphorylate BMPRIA, thus skewing BMP signaling toward activation of the Erk1/2 MAPK pathway instead of the Smad pathway, to regulate osteoblast expansion (Kua et al. 2012). It is not clear whether BMP can induce c-Abl activation, nor is the mechanism known that enables TGF-β-induced c-Abl activation. Given the similarity in activation kinetics and promoting TGF-β-mediated morphological changes in the same cell types (Wilkes et al. 2003; Daniels et al. 2004), it is possible that c-Abl functions downstream from the Cdc42-PAK2 pathway. Furthermore, Akt activation in response to TGF-β or BMP may also lead to c-Abl activation.

Activation of Jak-Stat signaling has also been seen in response to TGF-β in several cell types. TGF-β can activate Jak1-Stat3 signaling in hepatic stellate cells, and Jak2 in fibroblasts, and involves Jak-Stat signaling in its ability to promote fibrosis (Dees et al. 2012; Liu et al. 2013). Furthermore, Jak-Stat3 signaling cooperates with TGF-β or BMP pathways in neural progenitor cells and hepatoma cells (Nakashima et al. 1999; Yamamoto et al. 2001). However, conflicting reports suggest that TGF-β inhibits IL-6-induced Stat3 activation in acute myeloid leukemia blast cells and hepatocellular carcinoma and IL-12-induced Jak-Stat signaling in T lymphocytes (Bright and Sriram 1998; Wierenga et al. 2002; Tang et al. 2008). The molecular mechanisms underlying these regulations remain to be determined.

Finally, protein kinase A (PKA) was reported to be activated in response to TGF-β (Wang et al. 1998). Unlike activation of MAPK or GTPase signaling, TGF-β-induced activation of PKA requires the formation of Smad3–Smad4 complexes. However, rather than participating in transcriptional regulation, the Smad3–Smad4 complex interacts with the regulatory subunit of PKA, thereby releasing the catalytic subunit from the PKA holoenzyme and causing its activation (Zhang et al. 2004). Activation of PKA may play an important role in the TGF-β-induced phosphorylation of transcription factor CREB (cAMP-response element-binding protein) and expression of fibronectin (Wang et al. 1998).

CONCLUDING REMARKS

As apparent from this overview, many signaling pathways are controlled by TGF-β and BMP receptors, and the heteromeric receptor complexes that are activated by TGF-β family proteins act as nodal points for multiprotein assemblies that activate different signaling pathways. Future characterization of receptor-associated proteins using proteomics and/or phosphoproteomics analyses may reveal other previously unappreciated non-Smad pathways that are activated in response to TGF-β proteins. The differential activation of non-Smad pathways is often highly context dependent, yet plays important roles in a variety of cellular functions. It is of special importance to determine how TGF-β can direct membrane-bound receptors at the cell surface to recruit and acti-

vate multiple effectors, and how the selectivity in downstream signaling is achieved. It is likely that different proteins that associate with the receptor complexes at different activation states or subcellular locales regulate the receptor functions, routing, and pathway activation. A combination of in vivo imaging and manipulation of gene expression through RNA interference, CRISPR/Cas9-mediated genomic editing or traditional gene silencing approaches will be powerful in addressing the roles and mechanisms of TGF-β receptors, their regulators, and their effectors in eliciting the many signaling responses through Smad-dependent and -independent signaling pathways.

As we continue to advance our understanding of TGF-β family signaling networks, the complexity of signaling cross talk with other pathways becomes increasingly apparent, and we start appreciating how subtle perturbations can result in pathological dysregulation. A major challenge is to identify the targets in different context for treatment of different diseases to truly benefit individual patients.

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