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TGF-β Signaling from Receptors to Smads

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Transforming growth factor β (TGF-β) and related growth factors are secreted pleiotropic factors that play critical roles in embryogenesis and adult tissue homeostasis by regulating cell proliferation, differentiation, death, and migration. The TGF-β family members signal via heteromeric complexes of type I and type II receptors, which activate members of the Smad family of signal transducers. The main attribute of the TGF-β signaling pathway is context-dependence. Depending on the concentration and type of ligand, target tissue, and developmental stage, TGF-β family members transmit distinct signals. Deregulation of TGF-β signaling contributes to developmental defects and human diseases. More than a decade of studies have revealed the framework by which TGF-βs encode a context-dependent signal, which includes various positive and negative modifiers of the principal elements of the signaling pathway, the receptors, and the Smad proteins. In this review, we first introduce some basic components of the TGF-β signaling pathways and their actions, and then discuss posttranslational modifications and modulatory partners that modify the outcome of the signaling and contribute to its context-dependence, including small noncoding RNAs.

The transforming growth factor β (TGF-β) family of secreted growth and differentiation factors comprises more than 30 structurally related proteins. These ligands signal through cell-surface receptors, which are dual-specificity kinases, and intracellular Smad signal transducer proteins. On activation of the receptors, Smad proteins are phosphorylated by type I receptor kinase at the two carboxy-terminal serine residues and translocate into the nucleus to regulate gene expression (Fig. 1). In addition to Smad-dependent signaling, TGF-β receptors also activate several signaling pathways that are collectively called Smad-independent signaling or non-Smad signaling (Fig. 1) (Moustakas and Heldin 2009; Massagué 2012). Both Smad-dependent and Smad-independent signaling pathways are finely tuned to generate cell-type-specific or context-dependent signals through cross talk with other signaling pathways (Fig. 1). This review focuses on the Smad signaling pathway, its modes of regulation, and the Smad-mediated control of gene expression.

BASIC COMPONENTS OF THE TGF-β/SMAD SIGNALING PATHWAY

All TGF-βs and TGF-β-related family of secreted factors bind and activate heteromeric cell-surface complexes of receptors that are classified

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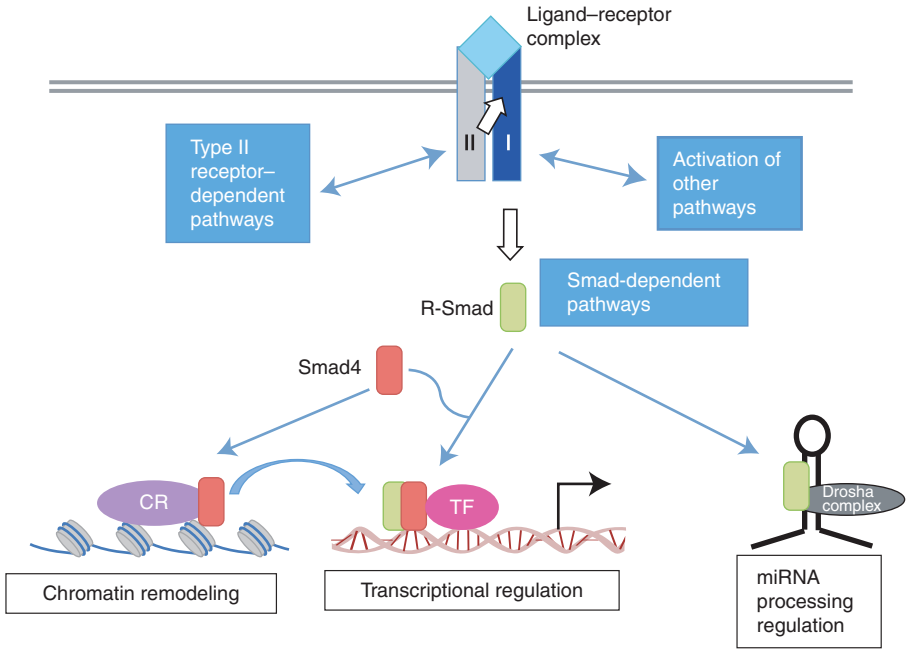

Figure 1. Transforming growth factor β (TGF-β) receptors and signal transducers. TGF-β family ligands, shown in light blue, transmit signals by assembling a heterotetrameric receptor complex with two type I receptors, shown in dark blue, and two type II receptors, shown in gray. Upon ligand binding, signaling is transmitted by a cytoplasmic kinase domain of type I receptors by phosphorylating receptor-regulated Smad proteins (R-Smad proteins, green box). This is considered as the "Smad signaling pathway." Additionally, the receptor complex can activate "non-Smad signaling pathways" through type II receptor– and type I receptor–interacting proteins. TGF-β and bone morphogenetic protein (BMP) receptors can also activate mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) pathways. Activated R-Smad proteins form a complex with the common-Smad, Smad4 (co-Smad, shown in red box), and, as a complex, translocate to the nucleus, where they regulate transcription of target genes together with cofactors (pink circle). R-Smads also form a complex with chromatin remodeling proteins (CR, purple circle) that recognizes certain histone modifications and promotes formation of active chromatin, which is a prerequisite for transcriptional activation by R-Smad/co-Smad complexes. Additionally, R-Smad proteins can participate in microRNA (miRNA) processing by the Drosha microprocessor complex (black circle) for the biogenesis of a subset of primary transcripts of miRNA (pri-miRNAs).

as type I and type II based on their sequence similarities. Both types of receptor contain a cytoplasmic kinase domain that has both serine/threonine kinase activity and tyrosine kinase activity and are, hence, classified as dual-specificity kinases (Fig. 1) (ten Dijke and Heldin 2006). Below we summarize the various steps of receptors activation and the different regulatory factors that are critical modifiers of the signal received by the cytoplasmic transducers, the Smad proteins.

Upon binding to the dimeric ligands, two type I and two type II receptors assemble into heteromeric complexes that allow the type II receptors, which are constitutively active kinases, to phosphorylate the juxtamembrane regions of the cytoplasmic domains of the type I receptors, activating the type I receptor kinases (Moustakas and Heldin 2009; Massagué 2012; Xu et al. 2012; Weiss and Attisano 2013). Subsequently, the type I receptor kinases phosphorylate two serine (Ser) residues in the Ser–Ser–X–Ser sequence (known as "SSXS motif") at the carboxy-terminal end of the receptor-regulated Smads (R-Smads). This event activates the R-Smads and enables the formation of heteromeric complexes between two R-Smads and one common-Smad (co-Smad), Smad4, and their

translocation to the nucleus. Seven type I and five type II receptors exist in humans; based on the R-Smads that they phosphorylate, type I receptors can be further divided into two subgroups, those that activate Smad2 and Smad3 in response to TGF-β-like proteins, and those that activate Smad1, Smad5, and Smad8 in response to bone morphogenetic proteins (BMPs). Their specificities are determined by the L45 loop of the type I receptors and the L3 loop of Smads (Feng and Derynck 1997; Chen et al. 1998; Lo et al. 1998). In addition, the inhibitory Smads (I-Smads), Smad6 and Smad7, antagonize the signaling mediated by R-Smads and co-Smad. TGF-βs, in particular TGF-β1 and TGF-β3, appear to bind with the high affinity type II receptor, which then recruits the lower affinity type I receptor, whereas BMPs bind both type I and type II receptor with equal affinity and greater flexibility (Groppe et al. 2008; Huang et al. 2011).

The duration and intensity of the signals transmitted to the Smad proteins depend on the abundance and availability of ligands and their inhibitors, such as extracellular ligand-trapping proteins (e.g., noggin and Gremlin1, which trap BMP ligands) or antagonistic ligands (e.g., Lefty, which inhibits nodal binding to receptors) (Moustakas and Heldin 2009; Massagué 2012; Weiss and Attisano 2013). They also depend on the level of expression and cell-surface localization of type I and type II receptors, and on the posttranslational modifications of the receptors that modulate their kinase activities and substrate recognition.

RECEPTORS: ACTIVATION AND REGULATION OF THEIR ACTIVITIES

Receptor complex formation is essential for TGF-β signaling initiation. Upon ligand binding, TGF-β receptors form a hetero-oligomer, most likely containing two type I and two type II receptor molecules (Wrana et al. 1992, 1994; Yamashita et al. 1994; Massagué 1998; Massagué and Chen 2000; Feng and Derynck 2005). Biochemical and immunofluorescence copatching studies revealed that both type I (TβRI) and type II (TβRII) TGF-β receptors form ligand-independent homomeric complexes, and the binding of TGF-β to preformed homomeric TβRII leads to the formation of a heterotetrameric TβRI–TβRII complex (Chen and Derynck 1994; Henis et al. 1994; Gilboa et al. 1998). However, single-molecule imaging studies showed that, when receptors were expressed at amounts close to the endogenous levels, most TβRI and TβRII molecules are monomers, and TGF-β treatment causes dimerization of TβRII and then recruitment of TβRI, forming a heterotetrameric TβRI–TβRII complex (Zhang et al. 2009, 2010). As these studies are mainly based on ectopic expression systems due to lack of good antibodies for endogenous receptors, these discrepancies could be a result of the different expression levels of the receptors. It will be important to investigate this issue with other approaches, such as clustered regularly interspaced short palindromic repeat (CRISPR)-mediated knockin of tags to follow endogenous proteins. Although the formation of a multimeric complex between TβRI and TβRII is thought to be required for TGF-β signaling under physiological conditions, a study using a synthetic TGF-β3 dimer, consisting of one wild-type and one receptor-binding-deficient mutant, showed that the each pair of TβRI:TβRII heterodimers is sufficient for signaling (Huang et al. 2011).

Receptor Activation

The activities of type I and type II receptors are controlled by phosphorylation at multiple residues (Table 1). TβRII is thought to be constitutively active (Lin and Wang 1992; Lin et al. 1992), and its activity is influenced by phosphorylation. For instance, autophosphorylation at Ser213 and Ser409 is essential for signaling, whereas Ser416 phosphorylation exerts an inhibitory effect (Luo and Lodish 1997). In addition, TβRII can autophosphorylate tyrosine (Tyr) residues Tyr259, Tyr336, and Tyr424, and substitution of these three residues with phenylalanine blocks the receptor kinase activity (Lawler et al. 1997). Thus, TβRII, as well as TβRI, is a dual specificity kinase that can phosphorylate both serine/threonine and tyrosine residues (Lee et al. 2007). Additional phosphorylation sites have been identified in TβRII

Table 1. Posttranslational modifications of transforming growth factor β (TGF-β) receptors

	Receptors	Sites	Mediators	Other regulators	Functions	References
Phosphorylation	TβRI	GS domain	TβRII		TβRI activation	Wrana et al. 1994; Wieser et al. 1995
		S165	TβRII		Required for TGF-β-mediated apoptosis, but attenuating growth inhibition and extracellular matrix (ECM) production	Souchelnytskyi et al. 1996
		Tyrosine	Unknown		Extracellular signal-regulated kinase (ERK) activation	Lee et al. 2007
	TβRII	S213, S409	Autophosphorylation		TβRII activation	Luo and Lodish 1997
		S416	Autophosphorylation		TβRII inhibition	Luo and Lodish 1997
		Y259, Y336, Y424	Autophosphorylation		TβRII activation	Lawler et al. 1997
Dephosphorylation	TβRI		Protein phosphatase 1	Smad7, SARA, GADD34	Inhibition of TGF-β signaling	Bennett and Alphey 2002; Shi et al. 2004
Ubiquitylation	TβRI		Smurf1/2, WWP1, NEDD4-2	Smad7	Receptor degradation	Kavsak et al. 2000; Ebisawa et al. 2001; Komuro et al. 2004; Kuratomi et al. 2005
	TβRII		Unknown		Receptor degradation	Atfi et al. 2007; Zuo et al. 2013
	BMPRI, BMPRII		Smurf1	Smad7	Receptor degradation	Murakami et al. 2010
	BMPRII		Itch			Durrington et al. 2010
Deubiquitylation	TβRI		UCH37	Smad7	Receptor stabilization	Wicks et al. 2005
			USP4		Receptor stabilization	Zhang et al. 2012
			USP15	TRAF4	Receptor stabilization	Zhang et al. 2013a
			USP11	Smad7?	Receptor stabilization	Al-Salihi et al. 2012
Sumoylation	TβRI	K389	Unknown		Receptor activation	Kang et al. 2008
Neddylation	TβRII	K556, K567	c-Cbl		Receptor stabilization	Zuo et al. 2013

GADD34, Growth arrest and DNA damage protein; SARA, Smad anchor for receptor activation; TRAF4, tumor necrosis factor receptor-associated factor 4; USP, ubiquitin-specific protease.

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(Souchelnytskyi et al. 1996); however, the responsible kinase(s) and functional significance of this phosphorylation are yet to be elucidated.

TGF-β ligand binding brings the type I receptor to the type II receptor at the cell surface and promotes phosphorylation of TβRI by TβRII, which is essential for TβRI activation (Cárcamo et al. 1995). Major sites of TβRI phosphorylation by TβRII are serine and threonine residues in a region that precedes the kinase domain and is enriched in glycine and serine residues, hence its name "GS domain" (Wrana et al. 1994). Phosphorylation of any four of the five serine or threonine residues in the GS domain (TTSGSGSG) appears sufficient for TβRI activation and signal transduction (Wieser et al. 1995). A similar mechanism involving GS domain phosphorylation activates other TGF-β family type I receptors, including those for activins and BMPs (Willis et al. 1996; Massagué 1998). TGF-β can also induce tyrosine phosphorylation of TβRI by yet-to-be-identified kinases, which contributes to TGF-β-induced extracellular signal-regulated kinase (ERK) activation (Lee et al. 2007). As phosphorylation is necessary for TβRI activation, dephosphorylation should turn off its activity. Indeed, protein phosphatase 1 (PP1) has been shown to dephosphorylate TβRI and antagonize TGF-β signaling (Bennett and Alphey 2002; Shi et al. 2004), an effect that is mediated by the membrane-associated protein Smad anchor for receptor activation (SARA) and by recruitment of the catalytic subunit of PP1 (PP1c) to the receptor (Bennett and Alphey 2002). The I-Smad Smad7 interacts with GADD34 (growth arrest and DNA damage-inducible protein 34), a regulatory subunit of the PP1 holoenzyme, within the receptor complex, and, thus, cooperates with SARA to enforce the phosphatase activity of PP1 toward TβRI (Shi et al. 2004). The regulatory subunit Bα of PP2A (Ser/Thr protein phosphatase 2A) has also been shown to interact with TβRI through its WD40 domain in response to TGF-β treatment and enhance TGF-β-induced growth inhibition, presumably via the kinase p70S6K (p70 S6 kinase) (Griswold-Prenner et al. 1998; Petritsch et al. 2000), but it is unclear whether PP2A modulates receptor phosphorylation. No phosphatases have been identified for TβRII so far.

Regulation of Receptor Activity by Other Posttranslational Modifications

In addition to phosphorylation, the receptor activity is regulated by a variety of posttranslational modifications (Table 1) (Kang et al. 2009; Huang and Chen 2012; Xu et al. 2012). Both TβRI and TβRII are polyubiquitylated, leading to receptor degradation (Atfi et al. 2007; Kang et al. 2009; Imamura et al. 2013; Zuo et al. 2013). Little is known about TβRII ubiquitylation (Fukasawa et al. 2010), although much attention has been devoted to TβRI ubiquitylation. Several E3 ubiquitin ligases, such as HECT (homologous with E6-associated protein carboxyl terminus) domain-containing Smurf1 (Smad-specific E3 ubiquitin protein ligase 1), Smurf2, WWP1 (WW domain containing E3 ubiquitin protein ligase 1, also known as TGIF-interacting ubiquitin ligase 1 or Tiul1), and NEDD4-2 (neural precursor cell expressed, developmentally down-regulated protein 4-2, also known as NEDD4L), have been shown to mediate TβRI ubiquitylation, all by interaction with Smad7 as an adaptor to TβRI (Kavsak et al. 2000; Ebisawa et al. 2001; Komuro et al. 2004; Kuratomi et al. 2005).

Like phosphorylation, ubiquitylation is a reversible process. Several deubiquitylating enzymes have been reported to remove ubiquitin from TβRI, leading to receptor stabilization and enhanced TGF-β signaling, including UCH37 (ubiquitin carboxy-terminal hydrolase 37), USP4 (ubiquitin-specific protease 4), USP11, and USP15 (Wicks et al. 2005; Al-Salihi et al. 2012; Zhang et al. 2012, 2013a). As both UCH37 and USP11 require Smad7 to bind TβRI, a question to be addressed is how Smad7 regulates the level of TβRI ubiquitylation by recruiting both E3 ubiquitin ligases and deubiquitylating enzymes (Wicks et al. 2005; Al-Salihi et al. 2012). Additional regulators could be involved; for instance, Hsp90 (heat shock protein 90) interacts with and stabilizes both TβRI and TβRII by blocking Smurf2-mediated ubiquitylation (Wrighton et al. 2008).

Table 2. Transforming growth factor β (TGF-β)-receptor-interacting proteins

Interacting partners	Functions	References
TβRI		
14-3-3ε	Enhance TGF-β signaling	McGonigle et al. 2001
BAMBI	Interfere with receptor activation and Smad phosphorylation	Onichtchouk et al. 1999; Yan et al. 2009
Caveolin-1	Promote TβRI degradation	Razani et al. 2001; Nohe et al. 2005; Hartung et al. 2006
c-Ski	Block Smad2 release from TβRI	Ferrand et al. 2010
Dab2	Enhance the clathrin-mediated endocytosis of TβRI and inhibit TGF-β-induced JNK activation	Shapira et al. 2014
Dapper2	Target receptors for lysosomal degradation	Zhang et al. 2004; Su et al. 2007
DRAK2	Interfere with the recruitment of Smad2 and 3 to TβRI	Yang et al. 2012
Endofin	Facilitate Smad activation	Chen et al. 2007
FKBP12	Block receptor complex formation and inhibit the basal TGF-β signaling	Chen et al. 1997
FKBP12	Attenuate TβRI internalization	Yao et al. 2000
Hsp90	Stabilize both TβRI and TβRII by blocking Smurf2-mediated ubiquitylation	Wrighton et al. 2008
Hrs/Hgs	Facilitate activation of Smad2 and Smad3	Miura et al. 2000
PICK1	Enhance its ubiquitylation and degradation in lipid rafts/caveolae	Zhao et al. 2012
Regulatory subunit Bα of PP2A	Enhance TGF-β-induced growth inhibition	Griswold-Prenner et al. 1998; Petritsch et al. 2000
SARA	Recruit the catalytic subunit of PP1 to TβRI to inactivate the receptor	Bennett and Alphey 2002
SARA	Facilitate activation of Smad2 and Smad3	Tsukazaki et al. 1998
ShcA	Promote the caveolar localization of TβRI, attenuate Smad3 signaling and activate Erk MAP kinase	Lee et al. 2007; Muthusamy et al. 2015
Smad7-UCH37	Induce TβRI deubiquitylation and stabilization	Wicks et al. 2005
Smad7-GADD34	Recruit PP1 to TβRI to inactivate the receptor	Shi et al. 2004
Smad7-NEDD4-2	Induce TβRI ubiquitylation and degradation	Kuratomi et al. 2005
Smad7-SIK	Induce degradation of the activated TβRI	Kowanetz et al. 2008
Smad7-Smurf1/2	Induce TβRI ubiquitylation and degradation	Kavsak et al. 2000; Ebisawa et al. 2001
Smad7-USP11	Induce TβRI deubiquitylation and stabilization	Al-Salihi et al. 2012
Smad7-WWP1	Induce TβRI ubiquitylation and degradation	Komuro et al. 2004
STRAP	Promote Smad7 binding to the activated TβRI, leading to inhibition of TGF-β signaling	Datta and Moses 2000
Tollip	Interact with both Smad7 and ubiquitylated TβRI and promote TβRI degradation	Zhu et al. 2012
TRAF4-USP15	Induce TβRI deubiquitylation and stabilization	Zhang et al. 2013a
TRAF6	Mediate TβRI cleavage by TACE and γ-secretase	Gudey et al. 2014
TRAF6	Mediate p38 MAP kinase activation	Yamashita et al. 2008
TSC-22	Stabilize TβRI by impairing the association of Smad7/Smurfs to the receptor	Yan et al. 2011
VEPH1	Block Smad2 release from TβRI	Shathasivam et al. 2015

Continued

Table 2. *Continued*

Interacting partners	Functions	References
TβRII		
ADAM12	Block TβRII internalization into caveolin1-positive vesicles and stabilize receptor	Atfi et al. 2007
c-Cbl	Induce TβRII neddylation and stabilization	Zuo et al. 2013
eIF2α	Inhibit TGF-β signaling	McGonigle et al. 2002
eIF3/TRIP-1	Inhibit TGF-β signaling	Chen et al. 1995; Choy and Derynck 1998
Par6	Mediate TGF-β-induced EMT	Ozdamar et al. 2005

ADAM12, A disintegrin and metalloproteinase 12; BAMBI, BMP and activin membrane-bound inhibitor; Endofin, endosome-associated FYVE-domain protein; GADD34, growth arrest and DNA damage protein; EMT, epithelial to mesenchymal transition; PP1, protein phosphatase 1, PP2A: protein phosphatase 2A; SARA, Smad anchor for receptor activation; SIK, salt-inducible kinase; STRAP, serine-threonine kinase receptor-associated protein; TRAF, tumor necrosis factor receptor-associated factor; TRIP-1, TGF-β receptor-interacting protein-1; TSC-22, TGF-β-stimulated clone 22; USP, ubiquitin-specific protease; WWP1, WW domain-containing protein 1; TACE, tumor necrosis factor-α converting enzyme.

Sumoylation is a ubiquitylation-like post-translational modification, regulating protein activity and subcellular localization (Flotho and Melchior 2013). TβRI, but not other type I receptors, can be sumoylated (Kang et al. 2008). The sumoylation at Lys389 in the kinase domain of TβRI is important for TGF-β signaling, but its mediator is unknown. As TβRI sumoylation is induced by TGF-β and requires the kinase activities of TβRI and TβRII (Kang et al. 2008), receptor phosphorylation may control this process.

TβRII can be "neddylated" (i.e., linked to the ubiquitin-like protein NEDD8) (Zuo et al. 2013), a modification that regulates protein activity, subcellular localization, and stability (Rabut and Peter 2008; Watson et al. 2011). TβRII neddylation is mediated by the proto-oncogene c-Cbl, an E3 ligase for both ubiquitin and NEDD8 (Thien and Langdon 2001; Oved et al. 2006), and stabilizes TβRII by antagonizing its ubiquitylation and lipid raft-mediated endocytosis (Zuo et al. 2013).

Regulation of Receptors by Their Interacting Proteins

Numerous proteins have been reported to interact with TGF-β receptors (Table 2), many of them as negative regulators (reviewed in Kang et al. 2009; Lönn et al. 2009). The best-characterized protein is the I-Smad, Smad7, which associates with activated type I receptors (Hayashi et al. 1997; Nakao et al. 1997). Smad7 antagonizes TGF-β signaling through multiple mechanisms, including interfering with R-Smad recruitment, promoting receptor dephosphorylation, recruiting E3 ubiquitin ligases to induce receptor degradation, and blocking the functional Smad complex from interacting with DNA in the nucleus (reviewed in Yan and Chen 2011). The pseudoreceptor BAMBI (BMP and activin membrane-bound inhibitor) inhibits TGF-β family signaling by directly forming a complex with TGF-β, activin, and/or BMP receptors to generate an inactive receptor complex (Onichtchouk et al. 1999; Sekiya et al. 2004a), or by binding and enforcing the inhibitory effect of Smad7 on TGF-β signaling (Yan et al. 2009). BAMBI expression is induced by Wnt/β-catenin signaling and repressed by Toll-like receptor 4 (TLR4) signaling, thus allowing for cross talk of these pathways with TGF-β signaling (Sekiya et al. 2004b; Seki et al. 2007). Serine-threonine kinase receptor-associated protein (STRAP), a WD domain-containing protein, can interact with both TβRI and TβRII (Datta et al. 1998) and promote Smad7 binding to the activated TβRI, leading to inhibition of TGF-β signaling (Datta et al. 1998; Datta and Moses 2000). Tollip, which contains both ubiquitin-associated domains and an endosome-targeting domain, can interact with both Smad7 and ubiquitylated TβRI to promote TβRI degradation via the en-

docytic pathway (Zhu et al. 2012). The salt-inducible kinase (SIK), a TGF-β-inducible gene, was reported to cooperate with Smad7 to induce degradation of the activated TβRI, creating another negative feedback regulation of TGF-β signaling (Kowanetz et al. 2008). In addition, the basal activities of the TGF-β family type I receptors are controlled by FKBP12 (Wang et al. 1996; Chen et al. 1997; Huse et al. 1999; Spiekerkoetter et al. 2013), possibly by preventing the spontaneous formation of type I and type II receptor complex (Chen et al. 1997) or by forming a complex with Smad7 and Smurf1 and promoting ubiquitylation and degradation of type I receptors (Yamaguchi et al. 2006). Two WD-repeat proteins that regulate translation initiation factors (eIFs), eIF2α and eIF3 (also called TRIP-1 for TGF-β receptor-interacting protein-1), associate with and are phosphorylated by TβRII. Both of them exert an inhibitory effect on TGF-β signaling (Chen et al. 1995; Choy and Derynck 1998; McGonigle et al. 2002).

In contrast to the above-mentioned proteins that negatively regulate receptor activity, a few proteins that facilitate TGF-β signaling have been identified. TSC-22 (TGF-β-stimulated clone 22) is a TGF-β target that suppresses cell proliferation and promotes differentiation (Kawamata et al. 2004). It may promote TGF-β signaling by interacting with Smad4 and enhancing the transcriptional activities of Smad proteins (Choi et al. 2005) or by interacting with TβRI and Smad7 in a mutually exclusive manner to impair the association of Smad7 and Smurfs with TβRI, thereby preventing receptor degradation (Yan et al. 2011). 14-3-3ε has also been shown to interact with TβRI and enhance TGF-β signaling (McGonigle et al. 2001), but the underlying mechanism for this enhancement is unclear.

Proteolytic Cleavage of TβRI

Many cell-surface receptors are proteolytically cleaved to release their extracellular or intracellular fragments. Two groups have reported proteolytic cleavage of TβRI. Liu et al. (2009) found that on Erk mitogen-activated protein kinase (MAPK) activation, the metalloprotease tumor necrosis factor-α converting enzyme (TACE, also known as ADAM17) cleaves TβRI, but not TβRII. This cleavage decreases the cell-surface level of TβRI and down-regulates TGF-β-mediated Smad3 activation, antiproliferation and epithelial-mesenchymal transition. It has also been reported that, following TACE-mediated cleavage of TβRI at Gly120 and Leu121, the released intracellular fragment translocates to the nucleus, interacts with the p300 acetyltransferase, activates invasion-related genes and promotes TGF-β-mediated invasiveness of cancer cells (Mu et al. 2011). The binding of tumor necrosis factor receptor-associated factor 6 (TRAF6) to TβRI is required for this process as TRAF6 recruits protein kinase C ζ (PKC-ζ), which regulates the subcellular localization of TβRI and promotes TACE-mediated TβRI cleavage (Mu et al. 2011). Defining the physiological role of the cleaved TβRI intracellular fragment requires further investigation. TACE can also cleave the transmembrane protein vasorin to release a soluble form that binds and inhibits TGF-β (Malapeira et al. 2011). TACE expression can be induced by TGF-β (Lu et al. 2011). In addition, genetic polymorphism analysis revealed that TACE functions in angiogenesis by acting as a modifier of TGF-β signaling in mice and humans (Kawasaki et al. 2014). These observations add multiple layers of complexity to the TACE/TGF-β relationship and shed light on the critical role of TACE as a modifier of the TGF-β signaling pathway. TRAF6 can also elicit TβRI proteolytic cleavage by recruiting presenilin 1, a catalytic subunit of the γ-secretase complex (Gudey et al. 2014). γ-Secretase can cleave numerous transmembrane proteins, including amyloid protein precursor and Notch (De Strooper et al. 2012). The presenilin 1–mediated cleavage occurs between Val129 and Ile130 in the transmembrane domain of TβRI after the TACE cleavage and is promoted by TGF-β stimulation (Gudey et al. 2014). Like the TACE-cleaved TβRI intracellular fragment, the presenilin 1–released fragment stimulates the expression of cell-invasion-related genes, such as *Snai1* (encoding Snail) and *Jag1* (Jagged1) (Gudey et al. 2014). A recent study reported that TRAF6-mediated TβRI

cleavage is regulated by the scaffold protein Ran-binding protein M (RanBPM), which interacts with TβRI and prevents its association with TRAF6 and its subsequent cleavage and nuclear accumulation of the intracellular fragment (Zhang et al. 2014). As this fragment was observed in the nuclei of malignant tumors (Mu et al. 2011; Gudey et al. 2014), these results underscore the role of the finely regulated proteolytic cleavage of TβRI in tumorigenesis.

THE SMAD FAMILY OF SIGNALING MEDIATORS

TGF-β family ligands exhibit context-dependent activities mainly by regulating gene expression through receptor-mediated activation of Smad proteins. As critical mediators of TGF-β signaling, various modes of regulation feed into the Smad proteins to modulate signal intensity, duration, and specificity (Massagué 2012). Both R-Smad and co-Smad proteins comprise two highly conserved domains known as Mad homology 1 (MH1) and MH2 domains (Fig. 2). The amino-terminal MH1 domain contains nuclear localization signals and a β-hairpin structure that is critical for DNA binding (Fig. 2) and association with a subset of microRNA (miRNA) primary transcripts (pri-miRNAs). The carboxyl MH2 domain encompasses the L3 loop structure that specifies the interaction of the R-Smads with type I receptors (Lo et al. 1998), leading to the phosphorylation of the carboxy-terminal SSXS motif of R-Smads by the type I receptor kinases. The MH2 domain of Smad4 contains an L3 loop (Lo et al. 1998), but lacks the carboxy-terminal SSXS motif, and is not phosphorylated by the type I receptor (Fig. 2). The type I receptor–mediated phosphorylation of the carboxy end of R-Smads triggers its association with the MH2 domain of Smad4. The heteromeric Smad complex, which is a trimer of two R-Smads and one Smad4, translocates to the nucleus and binds to DNA through the MH1 domain (Massagué 2012). In the nucleus, the MH2 domain interacts with various nuclear factors and controls gene expression by modulating transcription or the epigenetic landscape (Moustakas and Heldin 2009; Massagué 2012; Weiss and Attisano 2013).

The MH1 and MH2 domains are connected by a linker region, which is not conserved among Smad proteins. The linker regions of R-Smads contain multiple serine and threonine residues that are phosphorylated by kinases, such as cyclin-dependent kinases (CDKs), MAPKs, and glycogen synthase kinase 3 (GSK3) (Massagué 2012). Phosphorylation of R-Smads in the linker region controls their nuclear residency and creates docking sites for positive and negative modulators of nuclear R-Smad (Massagué 2012). Smad linker phosphorylation is discussed in detail below (Fig. 2).

The third class of Smad proteins represents the I-Smads, which antagonize the TGF-β signaling pathway by R-Smads and co-Smad (Fig. 2). Unlike R-Smads, I-Smads, which include Smad6 and Smad7, lack an MH1 domain and the SSXS motif, but retain a conserved MH2 domain and negatively regulate signaling (Fig. 2). Both TGF-β- and BMP-specific Smad complexes induce the expression of I-Smads to form a negative feedback loop. I-Smads antagonize the Smad signaling pathway at multiple levels by (1) associating with the type I receptor, (2) recruiting Smurf1 or Smurf2 E3 ubiquitin ligases, (3) binding the receptor-phosphorylated R-Smads and interfering with the association with co-Smad, or (4) interacting with DNA and nuclear Smad complexes. Smad7 acts as a general inhibitor of all TGF-βs, whereas Smad6 preferentially blocks BMP signaling (Moustakas and Heldin 2009; Massagué 2012; Weiss and Attisano 2013).

ACTIVATION OF Smads BY THE RECEPTOR COMPLEXES

Upon ligand binding, TβRIi-mediated phosphorylation of the GS domain of TβRI induces a conformational change that activates the TβRI kinase and enhances the binding affinity of the receptor for Smad2 and Smad3 (Huse et al. 1999, 2001), by creating a binding site for a highly basic surface patch in the MH2 domain of R-Smads (Wu et al. 2000). Phosphorylation of the last two serine residues (SXS) at the carboxy end of R-Smads (Abdollah et al. 1997;

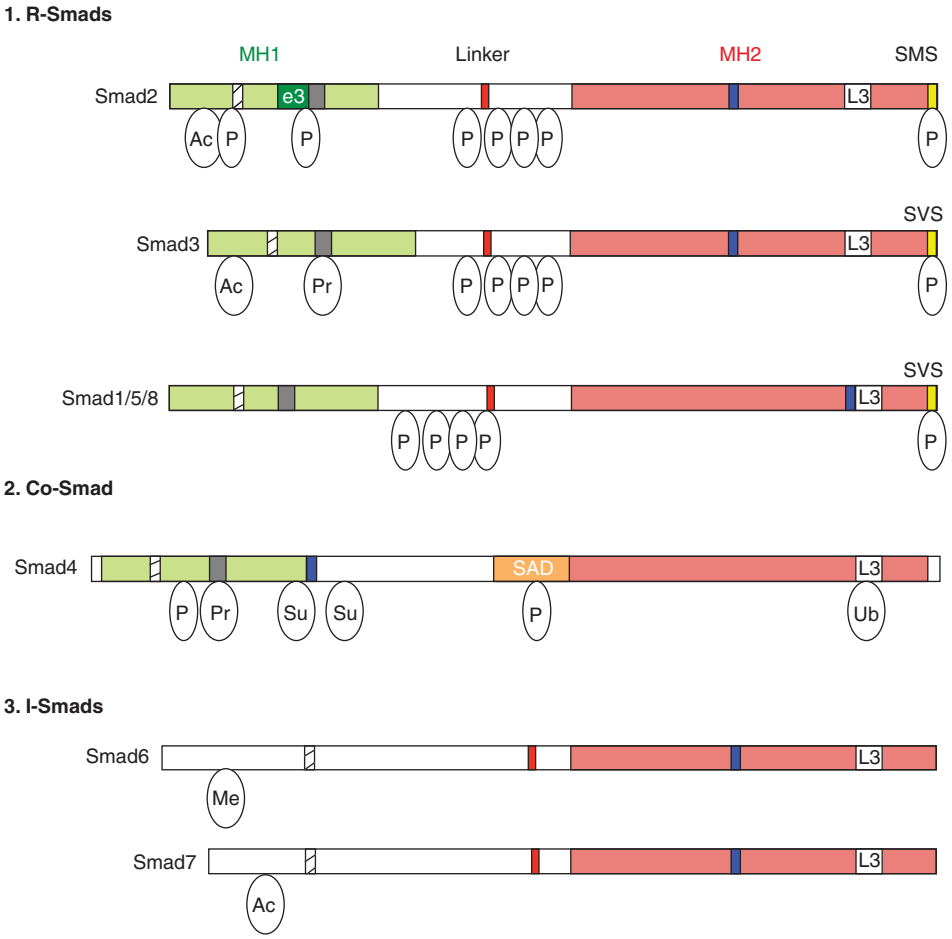

Figure 2. The Smad family. Schematic representations of the eight human Smad proteins divided into (*1*) receptor-regulated Smads (R-Smads), (*2*) common-Smad (co-Smad), and (*3*) inhibitory-Smads (I-Smads). The conserved amino-terminal Mad-homology 1 (MH1) and carboxy-terminal MH2 domains are indicated as green and red boxes, respectively. Highlighted are the nuclear localization signal (NLS, hatched box), the unique insert in Smad2-MH1 domain, which corresponds to exon 3 (e3, dark green box), the β-hairpin in the MH1 domain that binds DNA or the stem region of a subset of primary microRNAs (pri-miRNAs) (black box), the proline-tyrosine (PPXY) motif (red box) in the linker domain that is recognized by the WW domain of Smurf family proteins, the Smad activation domain (SAD, orange box) at the linker-MH2 border of Smad4, the nuclear export signal (NES, blue box), and the L3 loop of the MH2 domain (white box). The carboxy-terminal serine residues in the SXS motif that is phosphorylated by the type I receptor kinases are shown in the yellow box. Relative locations of different posttranslational modifications identified in Smad proteins are indicated. Ac, Acetylation; Ub, ubiquitylation; Pr, poly(ADP)ribosylation; Su, sumoylation; P, Ser or Thr phosphorylation.

Kretzschmar et al. 1997; Liu et al. 1997; Souchelnytskyi et al. 1997) by the type I receptor enables the association of R-Smads with co-Smad (Smad4) and, consequently, the interaction of the Smad complex with other factors to regulate gene expression in the nucleus (Shi and Massagué 2003; Feng and Derynck 2005).

Presentation of Smads to Receptors

Several receptor- or Smad-interacting proteins regulate the activation of Smad2 and Smad3. The FYVE domain-containing protein SARA has been described earlier. It promotes the activation of Smad2 and Smad3 by binding and

recruiting to the receptor, and facilitating phosphorylation by the receptor kinase (Tsukazaki et al. 1998; Wu et al. 2000). The FYVE domain associates with phosphatidylinositol-3-phosphate, which is enriched in the early endosome (Schink et al. 2013), where SARA facilitates TGF-β/Smad signaling (Itoh et al. 2002) with the help of the promyelocytic leukemia (PML) tumor suppressor (Lin et al. 2004). Another FYVE domain-containing protein, hepatic growth factor-regulated tyrosine kinase substrate (Hrs/Hgs), has also been shown to interact with Smad2, and may play a role similar to SARA to promote TGF-β/activin signaling (Miura et al. 2000). Transmembrane prostate androgen-induced (TMEPAI) interacts with Smad2 and Smad3 and competes with SARA for Smad binding, thereby attenuating Smad activation (Watanabe et al. 2010). As TMEPAI expression is induced by TGF-β stimulation, it generates a negative feedback loop to control TGF-β signaling (Watanabe et al. 2010). Like TMEPAI, its homolog C18 ORF1 can also interfere with the interaction of Smad2 and Smad3 with SARA and, thus, attenuates Smad recruitment to TβRI (Nakano et al. 2014). Similarly, ERBIN (ERBB2/HER2-interacting protein), a SARA-interacting protein, inhibits TGF-β signaling by competing with SARA for the association with Smad2 and Smad3 and inhibiting their activation (Sflomos et al. 2011). Although all of these studies support the role of SARA in promoting TGF-β signaling, it was reported that silencing SARA expression using small interfering RNA (siRNA) has no effect on TGF-β signaling in HeLa cells (Bakkebo et al. 2012), which might be caused by incomplete down-regulation of SARA expression and/or a redundant functions of Hrs/Hgs. Nonetheless, more genetic evidence, such as targeted inactivation of SARA expression, is required to clarify the role of SARA in TGF-β signaling. Endofin (endosome-associated FYVE-domain protein), a FYVE domain-containing and early endosome-localized protein that shares sequence similarity with SARA (Seet and Hong 2001) can enhance TGF-β signaling by facilitating Smad4 recruitment to the activated Smad2 and Smad3 (Chen et al. 2007). Endofin also acts as an anchor for BMP-specific R-Smad in the context of BMP receptor-dependent activation, analogous to the function of SARA in TGF-β receptor signaling (Shi et al. 2007; Goh et al. 2015). The molecular mechanism underlying endofin's multiple activities and the regulation of each activity need to be resolved in the future.

It was originally proposed that Axin, a negative regulator of Wnt signaling, interacts with Smad3 and facilitates its activation by TGF-β under conditions, in which Smad3 is expressed at higher than normal levels (Furuhashi et al. 2001), but it was later found that Axin reduces the level of Smad3 protein by promoting ubiquitylation and degradation (Guo et al. 2008). Furthermore, Axin acts as a scaffold protein to bring together Smad7 and the E3 ubiquitin ligase RNF111 (RING finger protein 111, also known as Arkadia) to promote Smad7 degradation (Liu et al. 2006). Therefore, the regulatory role of Axin in TGF-β signaling is pleiotropic and likely context-dependent. Dok-1 (Docking protein 1, also known as p62), a Ras-GAP (Ras GTPase-activating protein)-binding protein, has been reported to interact with Smad3 and with both the type I and type II activin receptors, therefore acting as an adaptor to bridge the activin receptors with Smad proteins to promote B-cell apoptosis (Yamakawa et al. 2002). DRAK2, which is a death-associated protein kinase (DAPK) family member and was identified by mass spectrometry-based proteomic screen of TβRI-associated proteins, is induced by TGF-β and antagonizes TGF-β/Smad signaling by interfering with the recruitment of Smad2 and 3 to TβRI (Yang et al. 2012). Ventricular zone expressed PH domain-containing 1 (VEPH1), the human ortholog of *Drosophila* Melted, however, blocks TGF-β by impeding Smad2 release from TβRI (Shathasivam et al. 2015). Similarly, c-Ski, which disrupts the functional R-Smad–Smad4 complex or represses Smad transcriptional activity (Deheuninck and Luo 2009), can also impair the activation and subsequent nuclear translocation of Smad2 by inducing its stable interaction with TβRI (Ferrand et al. 2010).

Several proteins have been indicated to balance TGF-β-induced Smad signaling against

non-Smad signaling. In addition to its regulatory function in modulating the trafficking of TβRII from the early endosome to the recycling endosome (Penheiter et al. 2010), the adaptor protein Disabled-2 (Dab2) can associate with and facilitate the activation of Smad2 and Smad3 by TGF-β (Hocevar et al. 2001). Dab2 also interacts with TβRI, enhances its clathrin-mediated endocytosis, and inhibits TGF-β-induced JNK (c-Jun amino-terminal kinase) activation (Shapira et al. 2014). In contrast, the adaptor protein ShcA (also known as Shc1) can sequester TβRI in caveolin-1-positive compartments and promote Erk and Akt signaling while attenuating Smad3 signaling (Muthusamy et al. 2015). These results are consistent with the requirement of lipid raft localization of TGF-β receptors for TGF-β-mediated MAPK activation (Zuo and Chen 2009). The membrane compartmentalization of TβRI is also regulated by PICK1 (protein interacting with carboxykinase 1) that enhances the TβRI interaction with caveolin-1 and promotes the caveolae-mediated internalization of TβRI, enhancing its degradation (Zhao et al. 2012). Similarly, the localization of BMP receptors in distinct membrane domains also modulates BMP signaling. BMP receptors mediate BMP-induced Smad1 and Smad5 phosphorylation in non-raft membrane regions, whereas their localization in lipid rafts is required for BMP-stimulated expression of alkaline phosphatase (Hartung et al. 2006). Several other receptor-interacting proteins link TGF-β receptors to non-Smad signaling, such as TRAF6 in TGF-β-mediated activation of MAPKs and Par6 in TGF-β-induced epithelial-mesenchymal transition (Table 2).

Specificity of Smad Activation by the Receptors

Two major Smad pathways are activated in response to TGF-β family proteins. In response to TGF-β and TGF-β/activin-like proteins, Smad2 and Smad3 are specifically phosphorylated at their carboxy-terminal tails by the type I receptors ACVR1B/ActRIB/ALK-4, TβRI/ALK-5, and ACVR1C/ALK-7, whereas in response to BMPs and related proteins Smad1, Smad5, and Smad8 are activated by the ACVRL1/ALK-1, ACVR1/ALK-2, BMPRIA/ALK-3, and BMPRIB/ALK-6 receptors (Massagué and Chen 2000; Feng and Derynck 2005). Considering that the receptors and R-Smads are highly conserved, great attention has been given to how the specific activation of R-Smads is achieved. Detailed functional mapping of the regions in the TβRI/ALK-5 receptor identified a critical role of the L45 loop between its kinase subdomains IV and V in specifying TGF-β responses (Feng and Derynck 1997). Swapping the L45 loop sequence between the TβRI/ALK-5 and BMPRIB/ALK-6 receptors can switch the signaling specificity in Smad activation and transcriptional responses (Chen et al. 1998; Persson et al. 1998). The L45 loop is not required for the kinase activity of the type I receptors, but determines the specificity of the Smad interaction (Chen et al. 1998; Yu et al. 2002; Itoh et al. 2003). Similarly, the search for the regions in Smad2 that is important for the interaction with TβRI/ALK-5 identified the L3 loop in the MH2 domain (Lo et al. 1998). The exchange of two amino acid residues in the L3 loop sequence of human Smad1 (His425 and Asp428) and human Smad2 (Arg427, Thr430) can switch the specific receptor-Smad interaction and Smad activation. The L45 loop of the type I receptors functionally interacts with the L3 loop of R-Smads (Chen et al. 1998; Wu et al. 2000). Interestingly, although the L45 loop of TβRI/ALK-5 is essential for Smad signaling, it is not important for TGF-β-induced activation of the MAP kinases p38 and JNK (Yu et al. 2002; Itoh et al. 2003).

NUCLEOCYTOPLASMIC SHUTTLING AND INTRACELLULAR MOVEMENT OF Smads

Regardless of the presence or absence of ligands, Smads constantly shuttle between the cytoplasm and the nucleus. Receptor-mediated phosphorylation and association with Smad4 retain R-Smads in the nucleus, where they function as transcription factors and miRNA regulators.

Therefore, nuclear transport offers another level of regulation in the control of Smad activity (Reguly and Wrana 2003; Xu and Massagué 2004; Hill 2009). Smad proteins can be transported into the nucleus via importin-mediated or nuclear pore protein-mediated mechanisms. Although Smad1, Smad2, Smad3, and Smad4 contain Lys-rich nuclear localization signal (NLS)-like motifs in the MH1 domains (Fig. 1), their modes of nuclear import are distinct. Through their NLS-like motifs, Smad3 and Smad4 interact with and are transported by importin-β and importin-α, respectively (Xiao et al. 2000, 2003b; Kurisaki et al. 2001). Smad2, Smad3, and Smad4 can also be imported into the nucleus by directly interacting with the nuclear pore proteins Nucleoporin 153 (Nup153) and Nup214 (Xu et al. 2002, 2003). In addition, importin 7 and 8 and their *Drosophila* ortholog Msk may also mediate nuclear import of Smad1, Smad2, and Smad3 (Xu et al. 2007).

The nuclear export of Smad4 is mediated by exportin 1 (also known as CRM1), as apparent by the nuclear accumulation of Smad4 in the presence of the exportin 1 inhibitor leptomycin B, even in the absence of ligand (Pierreux et al. 2000; Watanabe et al. 2000), and requires a nuclear export signal (NES) in the linker region of Smad4 (Fig. 2) (Watanabe et al. 2000). Smad1 uses a similar mechanism for nuclear export and two NES sequences have been identified (Xiao et al. 2001, 2003a). Although these two NES sequences are conserved in Smad2 and Smad3, they do not mediate the nuclear export of Smad2 and Smad3. The nuclear export of Smad3 is instead mediated by exportin 4 and a Ran GTPase (Kurisaki et al. 2006). RanBP3, which is known as a cofactor of exportin 1 (Lindsay et al. 2001), preferentially recognizes dephosphorylated Smad2 and Smad3 and exports them from the nucleus (Dai et al. 2009). Exportin 4 and RanBP3 do not display extensive sequence similarity, and it is unknown whether they share the same export machinery. In a similar manner, RanBP3L recognizes dephosphorylated Smad1, Smad5, and Smad8 and mediates their nuclear export in a Ran-dependent fashion (Chen et al. 2015).

Several models have been proposed for ligand-induced Smad nuclear accumulation. One of them is the cytoplasmic retention model, which is supported by several lines of evidence. Smad proteins are retained in the cytoplasm in the absence of ligands, whereas receptor-mediated phosphorylation favors their stay in the nucleus by enhancing their interaction with nuclear factors. SARA, for example, can sequester inactive Smad2 and Smad3 in the cytoplasm (Xu et al. 2000). Akt/PKB (protein kinase B) can also directly interact with Smad3 to block its phosphorylation and nuclear accumulation, and this effect is independent of the kinase activity of Akt/PKB (Conery et al. 2004; Remy et al. 2004). After Smads enter the nucleus, they can be retained in the nucleus through their association with DNA and nuclear proteins, such as Fast1/FoxH1 (Xu et al. 2002) and TAZ (Varelas et al. 2008). In the case of Smad3, receptor-mediated phosphorylation can increase its interaction with importin β1 and promote nuclear import (Kurisaki et al. 2001). The nuclear accumulation of Smad4 may depend on its interaction with nuclear R-Smads. For instance, phospho-Smad3 can block Smad4 interaction with exportin 1, therefore promoting nuclear accumulation of Smad4 (Chen et al. 2005). Dephosphorylation of R-Smads by phosphatases, such as PPM1A, may promote their nuclear export (Lin et al. 2006).

The nucleocytoplasmic shuttling of Smads is not only regulated by TGF-β family ligands, but also by other signaling events. The phosphorylation of the linker region of R-Smads by Erk MAPK (Kretzschmar et al. 1997), CDKs (Matsuura et al. 2004; Wang et al. 2009), and GSK3β (Fuentealba et al. 2007; Millet et al. 2009) has been shown to inhibit Smad nuclear accumulation. Thus, intracellular signals are integrated to control the subcellular localization of Smad proteins and finely tune TGF-β signaling (Schmierer et al. 2008).

Smad intracellular trafficking between the cell membrane and the nucleus is also under the control of microtubules and associated motor proteins, such as kinesin (Batut et al. 2007). Dynein light chain protein km23-1 (DYNLRB1) plays a role in Smad movement toward the nu-

cleus after activation by the receptor kinases (Jin et al. 2009). These studies imply that Smad movement within the cell is an active and directed process but not passive diffusion.

POSTTRANSLATIONAL MODIFICATIONS OF SMADS AND MODULATION OF THEIR ACTIVITY

In addition to the two critical regulatory steps that lead to Smad activity (i.e., phosphorylation of the SSXS motif and nucleocytoplasmic trafficking), Smad proteins are subject to various posttranslational modifications that occur upon specific stress or growth factor stimulation. In the section below, we summarize well-described posttranslational modifications found in Smad proteins.

Ubiquitylation and Sumoylation

E3 ubiquitin ligases are recruited to Smad proteins to promote ubiquitin-mediated degradation of Smads and Smad partners. Smurfs and other HECT domain E3 ligases contain WW domains that interact with conserved PPXY (Pro–Pro–X–Tyr) motifs in the linker region of R-Smads and I-Smads (Fig. 2). Both R-Smads and I-Smads recruit HECT domain E3 ligases to target either their own degradation or that of binding partners. I-Smads recruit Smurfs to the receptors and trigger their ubiquitin-mediated degradation (Murakami et al. 2003; Ogunjimi et al. 2005). Upon TGF-β stimulation, R-Smads recruit Smurfs and mediate degradation of the transcriptional corepressor SnoN (also known as Ski-like), which in turn promotes transcriptional regulation by the Smad complex (Deheuninck and Luo 2009). Smurf1 causes degradation of BMP-regulated R-Smads and inhibits BMP signaling (Zhu et al. 1999). Smurf1 also modulates the inhibitory activity and the stability of Smad7 and, hence, the TGF-β signaling output (Zhu et al. 1999; Suzuki et al. 2002). Smurf2 is also known to mediate target degradation of the TGF-β-regulated R-Smads (Lin et al. 2000; Zhang et al. 2001). The HECT domain E3 ligase NEDD4-2 causes degradation of Smad2 (Kuratomi et al. 2005) and Smad4 (Morén et al. 2005).

Members of the RING-finger class of E3 ligases also mediate degradation of Smads or Smad partners. RNF111/Arkadia induces Smad7 ubiquitylation and degradation to promote nodal signaling. Upon TGF-β treatment, RNF111 interacts with R-Smads, mediates degradation of the corepressors Ski and SnoN, and facilitates transcriptional regulation by the Smad complex (Levy et al. 2007; Nagano et al. 2007; Le Scolan et al. 2008). The RING finger E3 ligase complex SCF (Skp1, Cullin1, and Fbw1a)/ROC mediates TGF-β-dependent degradation of Smad3 and Smad4 and termination of TGF-β signaling (Fukuchi et al. 2001; Wan et al. 2004), whereas the U-Box-dependent E3 ligase STUB1 (STIP1 homology and U-Box containing protein 1, also known as CHIP) negatively regulates the BMP-regulated R-Smads and Smad-mediated signaling (Li et al. 2004). Another RING finger E3 ligase, anaphase-promoting complex (APC), interacts with Smad3, mediates ubiquitin-dependent degradation of SnoN, and promotes TGF-β signaling (Stroschein et al. 2001).

In addition to promoting the proteosomal degradation of Smad proteins or their partners, monoubiquitylation can modulate Smad activities. In the MH2 domain of Smad3, ablation of lysine residues that are monoubiquitylated by Smurf2 has no impact on protein stability but inhibits Smad3 signaling (Tang et al. 2011). Conversely, the deubiquitylating enzyme USP15 reverses this modification and restores responsiveness to TGF-β (Inui et al. 2011). The RING ubiquitin ligase TRIM33 (tripartite motif containing 33, also known as TIF1γ or ectodermin) monoubiquitylates Smad4 in the MH2 domain and efficiently inhibits both TGF-β and BMP signaling, presumably by disrupting the R-Smad/Smad4 complex and promoting Smad4 translocation to the cytoplasm (Dupont et al. 2005). The deubiquitylating enzyme USP9X (also known as FAM) reverts the effects of TRIM33 on Smad4 and restores TGF-β signaling (Dupont et al. 2009). Ubiquitylation of Smad4 by TRIM33 is regulated by association of the PHD finger-bromo domain of TRIM33

with unmodified histone H3 tails (Agricola et al. 2011). TRIM33 also competes with Smad4 for binding to phosphorylated Smad2 and Smad3 and, thus, mediates TGF-β-induced and Smad4-independent responses, such as erythroid differentiation in hematopoiesis (He et al. 2006). TRIM33 forms a complex with activated R-Smads, binds to the promoter region of nodal target genes characterized by H3K9 (histone H3 Lys9) trimethylation and H3K18 (histone H3 Lys18) acetylation, and displaces the chromatin-compacting factor chromobox homolog 3 (CBX3, also known as HP1γ) (Xi et al. 2011). This process is a prerequisite for the transcriptional activation of nodal target genes by the Smad complex during differentiation of embryonic stem cells (Xi et al. 2011). Future studies must assess how general the requirement of TRIM33 is for Smad-dependent gene regulation, and what determines whether TRIM33 activates or represses target genes.

Similar to ubiquitylation, sumoylation is a multistep posttranslational modification in which SUMO (a small ubiquitin like modifier protein) is attached to the target protein. Sumoylation of Smad4 by the E2 SUMO ligase Ubc9 and the PIAS (protein inhibitor of activated STAT-1) family members of the E3 SUMO ligases has been implicated in the increased level of nuclear Smad4 and the activation of both TGF-β and BMP signaling (Lee et al. 2003; Lin et al. 2003; Shimada et al. 2008). However, sumoylation of Smad4 can also suppress TGF-β and BMP signaling by repressing the transcriptional activity of Smad4 (Long et al. 2004; Yukita et al. 2012).

Acetylation and ADP-Ribosylation

Both R-Smads and Smad7 are substrates of acetyltransferases. Acetylation of Smad2 and Smad3 in the MH1 or MH2 domains appears to promote TGF-β signaling by enhancing the transactivation activity of Smad proteins (Inoue et al. 2007). Acetylation of Smad7 by the histone acetyltransferase p300 protects Smad7 from proteasomal degradation, as the acetylation occurs at the same lysine residues that the E3 ubiquitin ligase Smurf1 would otherwise ubiquitylate, and also increases Smad7 stability and inhibits TGF-β signaling (Grönroos et al. 2002). Smad3 and Smad4 are also subject to poly-ADP ribosylation by poly(ADP-ribose) polymerase-1 (PARP-1), a modification that interferes with Smad DNA binding and, thus, attenuates transcription (Lönn et al. 2010).

Linker Phosphorylation

The linker region between the MH1 and MH2 domains of Smads is rich in serine and proline residues, which provide prime target sites for modulation of Smad signaling in response to growth factors and other signaling cascades. Signaling pathways cross talk exerts great impact during embryogenesis and in homeostatic processes to generate complex context-dependent biological responses (Moustakas and Heldin 2009; Massagué 2012; Weiss and Attisano 2013).

Smad proteins that are actively engaged in transcription can be phosphorylated in the linker region by cyclin C-CDK8 or cyclin T-CDK9 (Alarcon et al. 2009; Gao et al. 2009). This phosphorylation step primes the secondary phosphorylation of the linker region by GSK3, generates binding sites for E3 ubiquitin ligases, such as Smurf1 and NEDD4-2, and targets Smad proteins for degradation (Alarcon et al. 2009; Gao et al. 2009). Linker phosphorylation by CDK8 or CDK9 also triggers the recruitment of Yes-associated protein (YAP), a signal transducer of the Hippo pathway (Alarcon et al. 2009), or Pin1 (peptidylprolyl *cis*/*trans* isomerase) (Nakano et al. 2009; Matsuura et al. 2010; Aragon et al. 2011; Shen et al. 2012; Ueberham et al. 2014), which modulate the nuclear activity of Smads.

Dephosphorylation of Smads

Following the activating phosphorylation of R-Smad proteins at their carboxyl terminus and inhibitory phosphorylation in the linker region, different phosphatases can reverse the phosphorylation to control the duration and intensity of the Smad signal. Both linker phosphorylation and receptor-mediated carboxy-terminal phosphorylation of R-Smad proteins

can be reversed by small carboxy-terminal domain phosphatases (SCP) 1, 2, and 3 (Sapkota et al. 2006; Bruce et al. 2012). The effects of SCP1, 2, and 3 on TGF-β-regulated and BMP-regulated Smads are different, as SCP1, 2, and 3 undo the phosphorylation of the linker but not of the carboxy terminal of Smad2 and Smad3, and thus enhance TGF-β signaling (Sapkota et al. 2006). On the contrary, SCP1, 2, and 3 dephosphorylate both the linker and carboxyl terminus of Smad1, resulting in an overall inhibitory effect on BMP signaling (Sapkota et al. 2006). SCP1, 2, and 3 are, thus, regulatory molecules that exert opposing controls on the TGF-β and BMP pathways (Sapkota et al. 2006). It is still unclear, however, how the activities of SCP1, 2, or 3 are regulated and coordinated with the phosphorylation step.

The Bβ subunit of PP2A interacts with the BMP receptors and mediates Smad1 dephosphorylation, mainly in the linker region, leading to amplification of BMP signaling (Bengtsson et al. 2009). The Bα subunit of PP2A, however, interacts with the TGF-β receptor and modulates TGF-β signaling (Griswold-Prenner et al. 1998; Petritsch et al. 2000). PPM1A, the prototype of metal ion-dependent protein phosphatases, also known as PP2C, is the only phosphatase shown to dephosphorylate Smad2 and Smad3 at their carboxy-terminal SXS motif (Lin et al. 2006), whereas several phosphatases can mediate carboxy-terminal dephosphorylation of Smad1. In addition to SCPs (Sapkota et al. 2006), which dephosphorylate the carboxyl terminus of Smad1 to terminate BMP signaling (Duan et al. 2006; Zhao et al. 2014), PPM1H has also been reported to interact with and dephosphorylate activated Smad1 (Shen et al. 2014). An RNA interference screen identified pyruvate dehydrogenase phosphatase (PDP) as a phosphatase for Mad, the homolog of Smad1 and Smad5 in *Drosophila*, that inactivates decapentaplegic (Dpp)/Mad signaling (Chen et al. 2006). Myotubularin-related protein 4 (MTMR4), a FYVE domain-containing dual-specificity protein phosphatase, can dephosphorylate Smad1, Smad2, and Smad3 in the early endosome and block their nuclear accumulation, functioning as a general negative regulator for both TGF-β and BMP signaling (Yu et al. 2010, 2013). Further studies are required to elucidate the mechanism that regulates the activity of phosphatases, which are critical determinants of the duration and intensity of the Smad signal.

REGULATION OF TARGET GENES BY Smads AND THEIR PARTNERS

Once located in the nucleus, Smad complexes can directly bind DNA and modulate transcription. Smad complexes bind with low affinity to a DNA sequence known as either the "Smad-binding element" (SBE) (i.e., GTCT or AGAC) or a GC-rich sequence, and require DNA binding transcription factors as partners to increase specificity and DNA binding affinity. Many DNA binding partners of Smads are tissue-specific transcription factors and, thus, are essential in mediating context-dependent gene regulation. A variety of DNA binding partners of the Smad complex has been identified, including chromatin modifiers, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), or DNA cytosine-5-methyltransferase 3A (DNMT3A), which removes repressive DNA methylation and activates transcription in a TGF-β-inducible manner (Thillainadesan et al. 2012).

DNA binding partners are often prime recipients of cross talk input from other signaling pathways. For example, Wnt cooperates with BMP and TGF-β through co-occupancy of Smad target enhancers by Wnt-activated lymphoid enhancer-binding factor 1 (LEF1, also known as TCF1α) and transcription factor 7-like 2 (TCF7L2) transcription factors (Labbé et al. 2000, 2007; Nakano et al. 2010). Also, the interaction between Smad proteins and FoxO factors provides an integration point of the Akt and TGF-β pathways (Seoane et al. 2004; Naka et al. 2010). Cross talk can also be achieved at the level of Smad target genes. For example, in epithelial cells, the TGF-β-activated Smad complex stimulates the expression of ATF3 (activating transcription factor 3) and Snail1, which then cooperates with Smads to repress *ID1* and *CDH1*, encoding E-cadherin,

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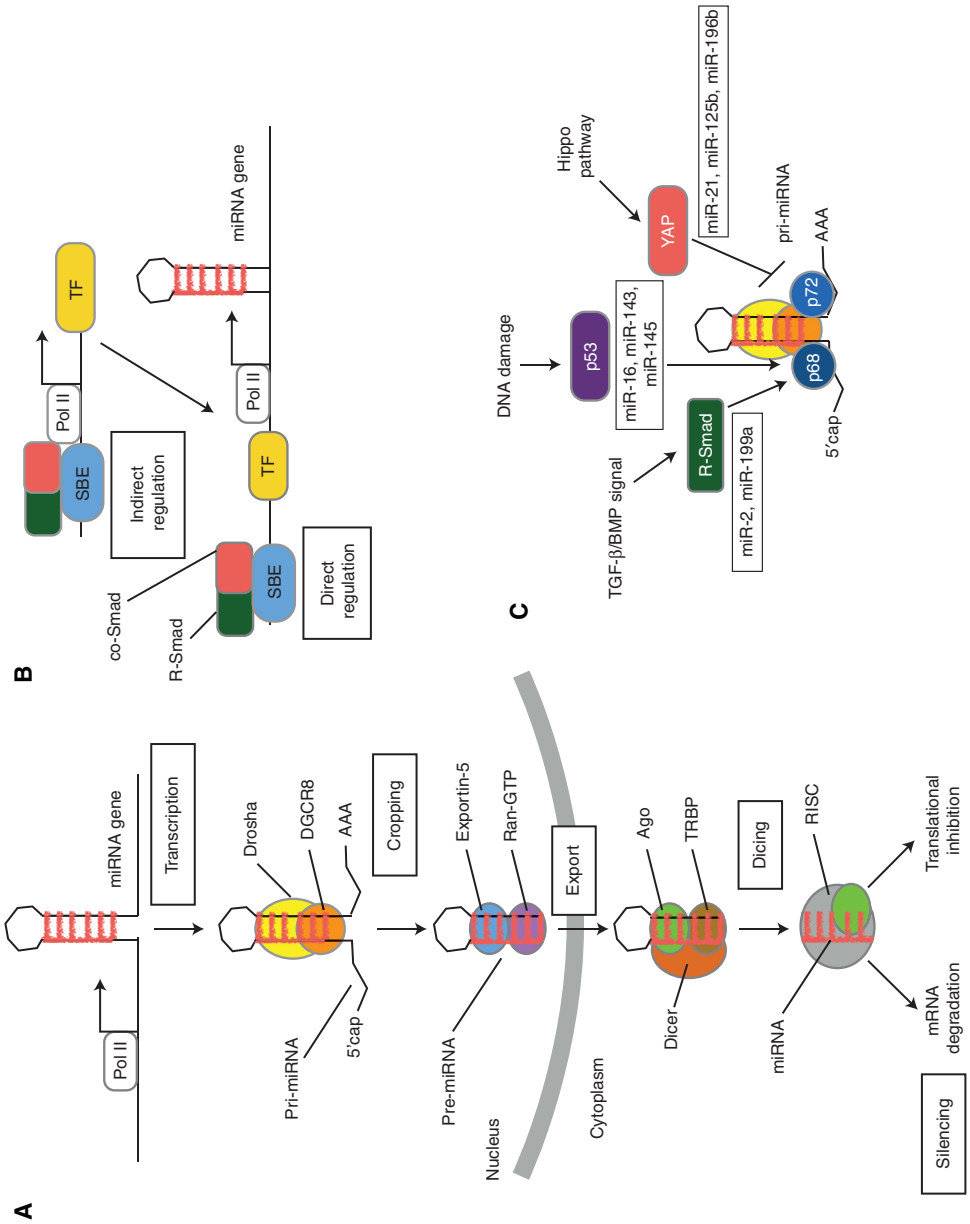

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and mediates epithelial to mesenchymal transition (Kang et al. 2003; Vincent et al. 2009).

REGULATION OF miRNA EXPRESSION BY SMADS

miRNAs are small (~22 nucleotides) noncoding RNAs that associate with a partially complementary sequence often found in the 3′-untranslated region (UTR) of target mRNAs, and repress their expression either by promoting mRNA degradation or inhibiting translation (Siomi and Siomi 2010; Ha and Kim 2014). The biosynthesis of miRNAs begins with transcription of miRNA genes by RNA polymerase II to generate long primary transcripts known as pri-miRNAs. These contain one or more stable stem-loop structures that will give rise to mature miRNA sequence(s) after two sequential cleavage steps by the RNase III enzymes Drosha and Dicer (Fig. 3A) (Ha and Kim 2014). Gene regulation by miRNAs is an integral component of the control of gene expression exerted by the TGF-β family of ligands (Blahna and Hata 2012, 2013). Because a single miRNA is capable of modulating more than 100 mRNAs simultaneously (Lim et al. 2005; Blahna and Hata 2012), regulation of even a few miRNAs can affect the expression of hundreds of genes and contribute to tissue- and time-controlled biological outcomes mediated by TGF-β family ligands. In general, TGF-β signaling modulates miRNA expression both transcriptionally, via DNA binding activity of Smad complexes, and posttranscriptionally, via the RNA binding activity of R-Smads, as summarized below.

Transcriptional Regulation of miRNA Expression by Smads

Because the promoter structure of genes encoding miRNAs closely resembles that of protein coding genes (Corcoran et al. 2009), Smad complexes control the transcription of miRNA genes by binding to SBEs in their promoter (Figs. 2,3B). In addition, Smads can indirectly modulate miRNA levels through activation of transcription factors that regulate the miRNA promoter activity (Fig. 3B). For instance, during epithelial to mesenchymal transition, the miR-200 family of miRNAs is repressed by TGF-β through induction of the transcriptional repressors ZEB1 (also known as δEF1) and ZEB2 (also known as SIP1) (Gregory et al. 2008). These factors directly bind an E-box

Figure 3. (*Continued*) MicroRNA (miRNA) biogenesis pathway and its regulation by Smad proteins. (*A*) The miRNA biogenesis pathway. The biogenesis of a miRNA is a stepwise process that includes (1) transcription of a primary transcript (pri-miRNA), (2) nuclear cropping to produce the precursor-miRNA (pre-miRNA), (3) export to the cytoplasm, and (4) cytoplasmic cropping to a double-stranded (ds) miRNA precursor. miRNA genes are generally transcribed by RNA polymerase II (Pol II) as long, 5′-capped and 3′-polyadenylated transcripts (pri-miRNAs) that are processed by the RNase III enzyme, Drosha, in the microprocessor complex to generate hairpin-loop RNAs, known as pre-miRNAs. Pre-miRNAs are recognized by the exportin 5 (Xpo5)/Ran-GTP transporter and exported to the cytoplasm, where another enzyme of the RNase III family, Dicer, catalyzes secondary processing ("dicing") to produce miRNA/miRNA* duplexes. Dicer, TRBP, and Argonaute (Ago) proteins mediate the processing of pre-miRNAs and the assembly of the RNA-induced silencing complex (RISC) in mammalian cells. Ago proteins associate with Dicer in both the cropping and RISC assembly steps (Hata and Lieberman 2015). (From Hata and Lieberman 2015; adapted, with permission, from the authors.) (*B*) Transcription of miRNA genes can be regulated by TGF-β and BMP signaling pathways either by a direct binding of Smad complex (R-Smad/co-Smad) to Smad-binding element (SBE) in the promoter regions of miRNA genes or by transcriptional regulation of other transcription factors, which, in turn, modulate the transcription of miRNA genes. (*C*) Pri-miRNA to pre-miRNA processing of a subset of miRNAs catalyzed by Drosha and its cofactors p68 and p72 is positively regulated by transcription factors, such as R-Smad and p53, and negatively regulated by YAP, a signal transducer of the Hippo pathway. Specific binding of R-Smads to a dsRNA sequence motif located in the pre-miRNA provides specificity of this regulation. TF, Transcription factor. (From Hata and Lieberman 2015; adapted, with permission, from the authors.)

proximal promoter element and repress the transcription of miR-200, and are reciprocally targeted by miR-200 (Burk et al. 2008; Korpal et al. 2008).

Posttranscriptional Regulation of miRNA Biogenesis by Smads

Besides acting as transcriptional regulators, R-Smad proteins promote the processing of a subset of miRNAs and rapidly enhance their expression on ligand stimulation (Figs. 2,3C) (Davis et al. 2008, 2010; Blahna and Hata 2012). In the first processing step, the RNase III enzyme Drosha in complex with cofactors DGCR8 (DeGeorge critical region 8, also known as Pasha), DEAD-box RNA helicases p68 (DDX5), and p72 (DDX17) cleave the pri-miRNAs to generate precursor miRNAs (pre-miRNAs) in the nucleus (Figs. 2,3C) (Blahna and Hata 2012). Both TGF-β- and BMP-regulated R-Smad proteins interact with p68 in the nucleus and facilitate the processing of pri-miRNAs by Drosha (Davis et al. 2008). The carboxyl-terminus phosphorylation by the receptor kinase is required for nuclear translocation of R-Smads but dispensable for the regulation of Drosha activity (Davis et al. 2008). Furthermore, unlike the transcriptional control by R-Smads that require co-Smad, R-Smads are capable of modulating the processing activity of Drosha in the absence of co-Smad (Davis et al. 2008), possibly explaining instances of Smad4-independent gene regulation (Bardeesy et al. 2006). In addition to their interaction with p68, Smads also directly associate with a 5-nucleotide double-stranded RNA (dsRNA) sequence motif that closely resembles the SBE and is enclosed within the mature miRNA sequence. This RNA motif specifies the pri-miRNAs that are regulated by R-Smads (Davis et al. 2010). miRNA-mediated gene regulation by Smad proteins can play a central role under stresses, such as hypoxia, during which transcription is compromised. Simi-

Table 3. List of miRNAs whose expression is regulated by the TGF-β family of ligands

miRNA	Ligand	References
let-7b, let-7c, miR-19b, miR-221, miR-222	Activin A	Tsai et al. 2010
miR-17 ~ 92 cluster	TGF-β	Luo et al. 2014
miR-21	TGF-β	Zhong et al. 2011
	BMP-4	Ahmed et al. 2011
	BMP-6	Du et al. 2009
miR-22	BMP-2	Berenguer et al. 2013
miR-23a cluster	TGF-β	Huang et al. 2008
miR-24-1, miR-31	BMP-2	Sun et al. 2009; Dunworth et al. 2014
miR-30b/c	BMP-2	Balderman et al. 2012
miR-96	BMP-4	Kim et al. 2014
miR-140-5p, miR-455-3p	TGF-β	Swingler et al. 2012
miR-141, miR-200a	BMP-2	Itoh et al. 2009
miR-143/145	TGF-β and BMP-4	Davis-Dusenbery et al. 2011
	Activin A	Blumensatt et al. 2013
miR-181a	BMP-2	Dunworth et al. 2014
miR-181b	Activin, TGF-β	Wang et al. 2010a; Neel and Lebrun 2013
miR-181c/d, miR-341 ~ 3072 cluster	TGF-β	Redshaw et al. 2013
miR-192	BMP-6	Hu et al. 2013
	TGF-β	Sun et al. 2011
miR-200 family	TGF-β	Gregory et al. 2008
	BMP-7	Samavarchi-Tehrani et al. 2010
miR-206	BMP-2	Sato et al. 2009
	Nodal	Liu et al. 2013
miR-302/367	BMP-4	Lipchina et al. 2011

This list contains both direct and indirect transcriptional targets of Smad complexes.

Table 4. List of miRNAs and their targets in the TGF-β family of signaling pathways

miRNA	miRNA targets	References
let-7	ACVR1B/ALK4	Colas et al. 2012
miR-14, miR-200c, miR-203	Noggin, Bmper (Crossveinless-2)	Cao et al. 2013
miR-15, miR-16	ACVR2A	Martello et al. 2007
miR-17 ~ 92 cluster	TβRII, Smad2, Smad4	Li et al. 2012
	BMPR2	Luo et al. 2014
miR-18	Smad2	Colas et al. 2012
miR-20a	BMP2	Tiago et al. 2014
miR-21	Smad3	Kim et al. 2009
miR-22	BMP6, BMP7	Long et al. 2013
miR-23a	Smad2, Smad3, Smad4	Huang et al. 2008
miR-23b cluster	Smad3, Smad4, Smad5	Rogler et al. 2009
miR-24	ACVR1B/ALK4	Wang et al. 2008
miR-26a	Smad4	Liang et al. 2014
miR-27a	Smad2	Bao et al. 2014
miR-30	Smad1	Wu et al. 2012
miR-34a	INHBB (Activin B)	Tu et al. 2014
miR-92	Noggin3	Ning et al. 2013
miR-98	ACVR1B/ALK4	Siragam et al. 2012
miR-106b	TGFβR2	Wang et al. 2010b
miR-130a	ACVR1/ALK2	Zumbrennen-Bullough et al. 2014
miR-134	CHRDL1	Gaughwin et al. 2011
miR-135	Smad5	Li et al. 2008
miR-140-5p	BMP2	Hwang et al. 2014
	TβRI	Yang et al. 2013
miR-141, 192, 194, 215, 200c	ACVR2B	Senanayake et al. 2012
miR-145	Smad2, Smad3	Kim et al. 2011
	ACVR1B/ALK4	Yan et al. 2012
miR-146a	Smad2, Smad3	Cheung et al. 2014
	Smad4	Lv et al. 2014
miR-148a	ACVR1/ALK2	Song et al. 2012
miR-155	Smad2	Xiao et al. 2009
	Smad5	Rai et al. 2010
	Smad1	Yin et al. 2010
miR-181a	ACVR2A	Zhang et al. 2013b
miR-195	ACVR2A	Bai et al. 2012
miR-199a-5p	ACVR1B/ALK4	Lin et al. 2014
miR-199-3p	Smad1	Lin et al. 2009
miR-204-5p	Smad4	Wang et al. 2013
miR-210	ACVR1B/ALK4	Mizuno et al. 2009
miR-224	Smad4	Yao et al. 2010
miR-302	TOB2, DAZAP2, and SLAIN1	Lipchina et al. 2011
miR-370	TβRII	Lo et al. 2012
miR-376c	TβRI, ACVR1C/ALK7	Fu et al. 2013
miR-378	TGF-β1	Nagalingam et al. 2014
	Nodal	Luo et al. 2012
miR-455-3p	ACVR2B, Smad2	Swingler et al. 2012
miR-656	BMPR1A/ALK3	Guo et al. 2014

larly to Smads but in response to different stimuli, other transcription factors, such as p53 (Fukuda et al. 2007; Suzuki et al. 2009) and YAP (Mori et al. 2014), associate with the Drosha microprocessor complex and modulate processing of a set of pri-miRNAs. Thus, R-Smads are not unique in terms of modulating gene expression through two mechanisms: regulation of transcription via DNA binding and miRNA biogenesis via RNA binding.

CONTROL OF TGF-β SIGNALING PATHWAY MEDIATORS BY miRNAS

It has been estimated that the translation of more than 30% of the coding genes is regulated by miRNAs. Molecules of the TGF-β signaling pathway are no exceptions. Protein expression of ligands, receptors, and Smads is under the control of miRNAs: one more regulatory layer for TGF-β signaling (Blahna and Hata 2012). Deregulation of miRNA expression, therefore, can lead to aberrant activity of TGF-β signaling and contribute to the pathogenesis of various disorders, including tumorigenesis (Blahna and Hata 2012). miRNAs often act in a tissue-specific manner, because of either tissue-specific expression of miRNA and targets, or tissue-specific variation of the length of the 3′-UTR of target mRNAs (Blahna and Hata 2012). These constraints contribute to limited expression of many genes, including those controlled by TGF-β. Furthermore, miRNAs whose expression is regulated by TGF-β family pathways (in Table 3) often target mRNAs encoding mediators of the TGF-β signaling pathway (in Table 4), indicating a regulatory feedback loop between miRNAs and targets.

CONCLUDING REMARKS

About two decades have elapsed since the discovery of the receptors and signal transducers of the TGF-β family ligands. Despite a detailed understanding of the signaling principles and the effectors of its regulation, the TGF-β pathway remains rather mysterious as it is still unclear how ligands can transmit context- and concentration-dependent signals through a deceptively simple signaling pathway. The mere number of different regulatory proteins that modulate the TGF-β signaling pathway at different steps underscores the complex mode by which a specific biological outcome is generated. A major challenge at the current stage is to elucidate the logic that integrate these various regulatory inputs to explain the multifunctional nature of the TGF-β pathway during embryogenesis and in the maintenance of homeostasis. Understanding the precise nature of context-dependent signal transduction has tremendous medical relevance to numerous pathological conditions and developmental defects linked to deregulation of the TGF-β signaling pathways.

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