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REVIEW

Phospho-control of TGF-β superfamily signaling

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Members of the transforming growth factor-β (TGF-β) family control a broad range of cellular responses in metazoan organisms via autocrine, paracrine, and endocrine modes. Thus, aberrant TGF-β signaling can play a key role in the pathogenesis of several diseases, including cancer. TGF-β signaling pathways are activated by a short phospho-cascade, from receptor phosphorylation to the subsequent phosphorylation and activation of downstream signal transducers called R-Smads. R-Smad phosphorylation state determines Smad complex assembly/disassembly, nuclear import/export, transcriptional activity and stability, and is thus the most critical event in TGF-β signaling. Dephosphorylation of R-Smads by specific phosphatases prevents or terminates TGF-β signaling, highlighting the need to consider Smad (de)phosphorylation as a tightly controlled and dynamic event. This article illustrates the essential roles of reversible phosphorylation in controlling the strength and duration of TGF-β signaling and the ensuing physiological responses.

***Keywords*:** Smad, phosphorylation, phosphatase, TGF-β signaling

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TGF-β superfamily signaling

TGF-β superfamily signaling controls a diverse set of cellular responses, including cell proliferation, differentiation, extracellular matrix remodeling, and embryonic development. Consequently, when not strictly controlled, TGF-β signaling can play a key role in the pathogenesis of cancer and fibrotic, cardiovascular and autoimmune diseases. The phospho-relay from TGF-β superfamily receptor kinases to downstream Smad transcription factors has long been established as the route to active canonical signaling. More recent work in the field reveals how dephosphorylation of several pathway components can serve as a means to either prevent or terminate active signaling. This review will examine our current knowledge of how dynamic phosphorylation controls TGF-β superfamily signaling at each step in this physiologically important pathway.

Phospho-relay in the TGF-β signaling pathway

TGF-β superfamily ligands, including TGF-βs, activins and bone morphogenic proteins (BMPs), signal through heteromeric complexes of type II and type I transmembrane serine/threonine kinases (Figure 1). The receptor complex usually comprises two type II receptors and two type I receptors, and the receptors are classified based on their structural and functional properties. Within this receptor complex the cytoplasmic domain of the type II receptor is constitutively active, and it phosphorylates the type I receptor on serine and threonine residues in the GS domain in response to ligand binding. Activated type I receptors then traditionally phosphorylate downstream Smads in their distal C-terminal SXS motif, activating them to transduce the signal to the nucleus.

There are five mammalian type II receptors: TβRII, ActR-II, ActR-IIB, BMPR-II, AMHR-II, and seven type I receptors which are referred to as activin receptor-like kinases 1-7 (ALK1-7). The Smad family comprises 8 Smad proteins which are classified into three subgroups: receptor-activated Smads (R-Smads; Smad1/2/3/5/8), the common Smad (Smad4), and the inhibitory Smads (I-Smads; Smad6 and Smad7). The specificity for Smad phosphorylation lies in the distinctive structural features in paired type I receptors and R-Smads [1-3]. Of the type I receptors ALK5 is thought to be specific for TGF-β ligands, and ALK4 and ALK7 are thought to mediate signaling via activins and nodal. In canonical signaling, activated

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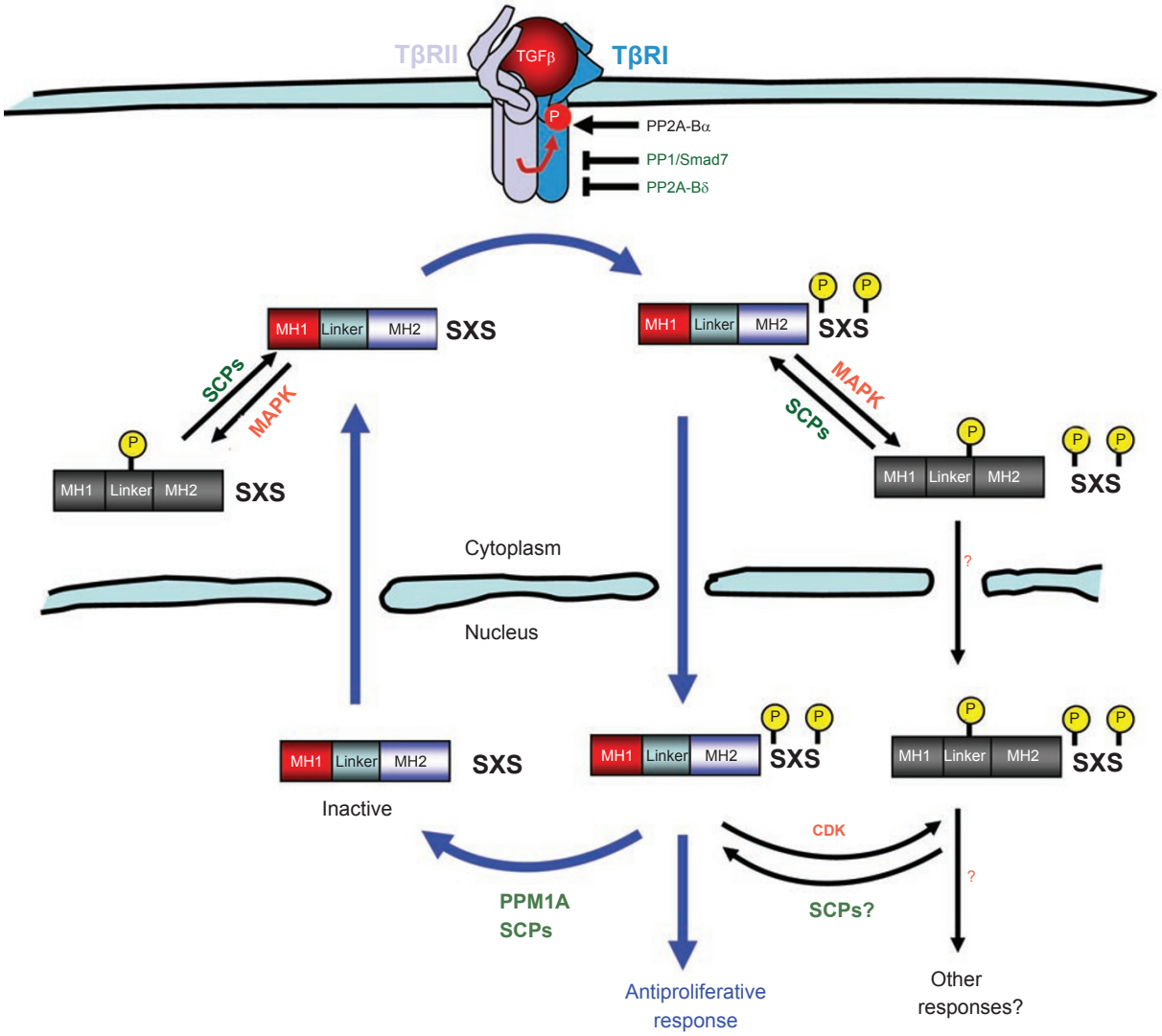

Figure 1 Regulation of phospho-relay in canonical TGF-β-signaling. The type II receptor kinase is constitutively active, and phosphorylates the type I receptor kinase in response to ligand. The type I receptor subsequently phosphorylates the C-terminal distal SXS motif of R-Smads, which is the key step in initiating signal transduction. Several proteins regulate receptor kinase function and activity, as indicated. Phosphorylated R-Smads form a complex with Smad4 and move into the nucleus to regulate transcription. PPM1A can dephosphorylate the phospho-SXS in Smad1/2/3, and SCPs 1, 2 and 3 can dephosphorylate the phospho-SXS in Smad1 as well as the Smad1/2/3 linker region. The exact localization of R-Smad linker phosphorylation by MAPK, and linker dephosphorylation by SCPs, is not known. Proteins named in red positively contribute to phospho-relay; Proteins named in green inhibit or reverse phospho-relay. However, it is important to note that not all phospho-relay positively contributes to TGF-β signaling. For example, phosphorylation of the Smad linker by MAPK is often inhibitory. Some of the proteins indicated in this diagram may be specific for certain cell types or specific branches of TGF-β superfamily signaling.

ALK4/5/7 phosphorylate downstream Smad2 and 3. The remaining ALKs, ALK1/2/3/6, phosphorylate R-Smad1, 5 and 8 following specific activation by ligands such as BMPs [4]. It is remarkable how R-Smad-SXS phosphorylation controls a cascade of ligand-specific downstream events. Phosphorylated R-Smads undergo hetero-oligomeric complex formation with Smad4, named the common Smad due to its role in all branches of TGF-β superfamily signaling, and this complex accumulates in the nucleus to regulate gene transcription in conjunction with a variety of transcriptional cofactors [4-6]. The interactions of Smads with DNA-binding transcription fac-

tors and transcriptional co-activators and co-repressors, as well as the target genes and the resulting biological responses, have been recently reviewed in detail [4, 5, 7-9]. Thus, phospho-relay from the constitutively active type II receptor to the type I receptor, and subsequently to the relevant R-Smads is essential to activate canonical TGF-β superfamily signaling. This process is positively and negatively regulated at all stages of the pathway by various proteins, as will be discussed in this review.

Regulation of receptor kinase activity

The constitutively active type II receptor requires autophosphorylation of Ser213 and Ser409 for full kinase activity and the ability to interact with and activate the type I receptor [10]. Interestingly, if Ser416 is autophosphorylated instead of 409, receptor function is inhibited [10]. The type II receptor can also autophosphorylate on Tyr259, 336 and 424, which is thought to play a role in regulating type II receptor kinase activity [11], and undergo Src-mediated phosphorylation at Tyr284 (discussed below) [12, 13]. In addition to the long established phosphorylation of serine and threonine residues in the GS domain by the active type II receptor [14], the type I receptor has also recently been shown to be tyrosine phosphorylated in response to TGF-β [15]. Research over the years has identified several key proteins that positively or negatively regulate the kinase activity of TGF-β superfamily receptors, and the downstream phospho-relay as a consequence, including several phosphatases.

The immunophilin FKBP12 was identified as a cytoplasmic interacting protein of TGF-β type I receptors in a yeast two-hybrid system, and found to have an inhibitory role in TGF-β signaling [16]. It binds to the type I receptor in the absence of ligand, and is released upon type II receptor-mediated phosphorylation of the type I receptor [16]. Chen *et al*. concurred that FKBP12 binds the type I receptor (ALK5) to inhibit signaling [17] and found that disruption of this interaction results in receptor activation in the absence of ligand. This suggests FKBP12 might normally inhibit type I receptor phosphorylation by the type II receptor, and thus provide a safeguard against the initiation of signaling in the absence of ligand. Indeed, the crystal structure of a fragment of unphosphorylated ALK5 containing the GS region and the catalytic domain, in complex with FKBP12 [18], revealed that ALK5 adopts an inactive conformation that is maintained by the unphosphorylated GS region. FKBP12 binds to the GS region of the receptor, capping the type II receptor phosphorylation sites and further stabilizing the inactive conformation of ALK5. Huse and colleagues subsequently showed that phosphorylation of the GS region activates ALK5 by creating a binding site for Smad2, whilst concomitantly eliminating the binding site for FKBP12. This enhances the ability of ALK5 to target the C-terminal SXS motif of Smad2 [19].

It has also been suggested that FKBP12 acts as a negative regulator of TGF-β receptor endocytosis, and the type II receptor kinase enhances receptor internalization by promoting the dissociation of FKBP12 from the type I receptor [20]. Activin has also been shown to induce the dissociation of FKBP12 from the type I receptor ALK4 [21]. In this study, FKBP12 was shown to also interact with inhibitory Smad7, in an activin-dependent manner, and form a complex with Smad7 on the type I receptor. Thus, FKBP12 may also serve as an adaptor protein for the Smad7-Smurf1 complex to promote the ubiquitination of the type I receptor by Smurf1 [21]. In short, all of these studies concur that FKBP12 has a negative effect on TGF-β type I receptor kinase activity, and must be dissociated from the receptor for the induction of signaling.

Several proteins in addition to FKBP12 bind to TGF-β receptors. A TGF-β receptor regulator called TGF-β receptor interacting protein 1 (TRIP-1) was identified as a WD40 repeat-containing protein that can associate with, and be phosphorylated by, the TGF-β type II receptor [22]. Later studies show that TRIP-1 expression represses the ability of TGF-β to induce transcription [23]. STRAP, also a WD-containing protein, interacts with both TGF-β receptor types, as well as Smads 2, 3 and 7 [24, 25]. STRAP is thought to stabilize the association of inhibitory Smad7 with the receptor, which inhibits TGF-β-dependent transcription, probably by interfering with Smad2/3 binding to the receptors or by recruiting a receptor phosphatase (see below). Another protein called TRAP-1 binds only to the activated TGF-β receptors and inhibits TGF-β-induced signaling [26]. Interestingly, TLP (a TRAP-1-like protein) associates with both active and kinase-deficient TGF-β/activin type II receptors, as well as with Smad4 only in the presence of TGF-β/activin signaling [27], and differentially regulates Smad2 and Smad3 signaling.

On the flip side of TGF-β receptor regulation, the FYVE domain-containing protein SARA (Smad anchor for receptor activation) aids phospho-relay by bringing the Smad2/3 substrate to the receptor complex [28]. SARA was shown to recruit Smad2 to the type I TGF-β receptor both by controlling the subcellular localization of Smad2 and by interacting with the receptor complex. Phosphorylation of Smad2 induces its dissociation from SARA, with concomitant formation of Smad2/Smad4 complexes and their nuclear translocation [28]. The chaperone protein Hsp90 also positively regulates TGF-β

receptor activity. Both the type I and type II receptors were found to interact with, and be stabilized by, Hsp90 [29]. Inhibition of Hsp90 using small molecule inhibitors leads to receptor degradation mediated by the Smurf2 E3 ubiquitin-ligase, and a subsequent block in Smad-mediated cellular responses [29]. However, it is not clear whether Hsp90 binding to the receptors depends on and/or regulates the receptor kinase activity.

With regards to TGF-β receptor phosphatases, studies in *Drosophila melanogaster* first implicated protein phosphatase 1c (PP1c) in receptor dephosphorylation. Bennett and Alphey identified a binding motif for PP1c in SARA [30]. Further investigation revealed that expression of a modified SARA protein that could not bind PP1c resulted in hyperphosphorylation of the type I receptor and active TGF-β signaling. In addition, reducing PP1c activity enhanced Dpp (Decapentaplegic) signaling stimulated by ectopic expression of the type II receptor [30]. This result suggests that SARA may target PP1c to Dpp receptor complexes where PP1c can negatively regulate Dpp signaling by affecting the phosphorylation state of the type I receptor.

The role of PP1 proteins as TGF-β type I receptor phosphatases has been extended to mammalian systems by several groups. Shi *et al.* found that inhibitory Smad7 can interact with growth arrest and DNA damage protein 34 (GADD34), a regulatory subunit of the PP1 holoenzyme, which subsequently recruits the catalytic subunit of PP1c to dephosphorylate the type I TGF-β receptor in Mv1Lu mink lung epithelial cells [31]. Similar to the findings in *Drosophila*, SARA enhanced the recruitment of PP1c to the Smad7-GADD34 complex. Significantly, Smad7-recruited GADD34-PP1c inhibits TGF-β-mediated cellular responses. Thus, dephosphorylation of the type I receptor mediated by Smad7 is an effective mechanism governing the negative feedback in TGF-β signaling. In endothelial cells, a role for PP1α in the regulation of type I receptor activity was identified [32]. Smad7 and PP1α mRNA expression levels were specifically upregulated by the non-canonical TGF-β/ALK1 branch of signaling, but not the canonical TGF-β/ALK5 branch of signaling. Data from Smad7 or PP1α gene over-expression and knock-out experiments suggest that Smad7 induced by the TGF-β/ALK1 pathway may recruit PP1α to ALK1 and thereby negatively regulate TGF-β/ALK1-induced Smad1/5 phosphorylation (described below), but not the traditional TGF-β/ALK5 signaling [32].

Finally, several studies have shown that regulatory subunits of the protein phosphatase PP2A associate with TGF-β receptors [33-35]. Interestingly, two highly related regulatory subunits of PP2A, Bα and Bδ, modulate TGF-β/Activin/Nodal signaling in opposite ways [33]. The Bα subunit of PP2A enhances or mediates the anti-mitogenic signaling of TGF-β [35], and its knockdown in *Xenopus* embryos or mammalian cells suppressed TGF-β/Activin/Nodal-dependent responses [33]. In contrast, knockdown of the Bδ subunit enhanced these responses. Bα is thought to enhance TGF-β/Activin/Nodal signaling by stabilizing basal levels of the type I receptor, whereas Bδ is thought to negatively modulate these pathways by restricting receptor activity. Thus, these highly related members of the B family regulatory subunits of PP2A have opposing effects on the ability of the type I receptor to initiate downstream phospho-relay.

Although these studies, and others, have furthered our knowledge of how receptor phosphorylation is both initiated and reversed, more work is still required in this area. For example, most of the work on TGF-β superfamily receptors focuses on TβRII and ALK5/TβRI. It has not yet been clearly defined whether the identified receptor interacting proteins and phosphatases will also function in a similar manner on other type II and type I receptors for the TGF-β superfamily.

Dephosphorylation of R-Smads at the C-terminal SXS motif

As TGF-β superfamily signaling activates a broad range of cellular responses, it stands to reason that signaling via the activated Smad complex must be stringently controlled to allow for normal cellular responses and the maintenance of tissue homeostasis. As R-Smad C-terminal SXS phosphorylation is the key step in activating Smad-mediated transcription, two possible mechanisms to terminate Smad functions in the nucleus can be envisioned: phosphatase-mediated dephosphorylation or ubiquitination-dependent degradation of phosphorylated R-Smads. The nuclear export of R-Smads has been shown to depend on their dephosphorylation and the subsequent dissociation of Smad complexes [3, 36-38]. Indeed, a recent mathematical model explains how Smad phosphorylation, Smad complex formation, Smad nucleocytoplasmic shuttling and Smad dephosphorylation work together to transduce TGF-β signals, and this model indicates that R-Smad dephosphorylation occurs in the nucleus [39]. There is also experimental support that activated R-Smads undergo faster degradation in the nucleus [40-42], although phospho-Smad degradation alone as a signal termination mechanism does not account for the constant level of total R-Smads, or the nuclear export of inactive Smad molecules. As a result of several recent and significant studies it is now possible to describe how dephosphorylation, by specific phosphatases, can act as a

critical regulatory mechanism in the termination of Smad signaling.

Using a functional genomic approach, Lin *et al*. identified PPM1A/PP2Cα as a Smad2/3 SXS-motif specific phosphatase. PPM1A over-expression abolished Smad2/3 phosphorylation induced by a constitutively active type I receptor, whereas shRNA-mediated depletion of PPM1A increased the durability of Smad2/3 SXS-phosphorylation. Importantly, this was reflected by the TGF-β-mediated biological response with PPM1A over-expression abolishing, and PPM1A depletion enhancing, TGF-β-induced anti-proliferative and transcriptional responses. Furthermore, PPM1A was shown to bind phospho-Smad2/3, and to facilitate nuclear export of dephosphorylated Smad2/3 to the cytosol [43]. Interestingly, a recent study presents data implying that PTEN may have a negative role in TGF-β signaling by serving as a functional co-factor of PPM1A [44]. PTEN was found to mediate a decrease in Smad2/3 phosphorylation, in a lipid phosphatase-independent manner, by forming a complex with PPM1A to protect it from TGF-β-induced degradation. Thus, PTEN helps to abrogate Smad2/3 phosphorylation by stabilizing PPM1A [44]. PTEN localization to the nucleus was required for this phenomenon to take place, and this was dependent on a product of sphingosine kinase called dihydrosphingosine 1 phosphate. This functional relationship between PTEN and PPM1A is in line with the data showing that Smad2 phosphorylation is significantly negatively correlated with PTEN and PPM1A, and that PTEN and PPM1A are positively correlated with carcinogenesis, in hepatocellular carcinoma [45]. These studies illustrate how our recent knowledge of Smad dephosphorylation is already aiding our understanding of TGF-β-signal regulation. Although PPM1A remains the only Smad2/3-SXS-motif-directed phosphatase identified thus far, it is possible that others are yet to be discovered.

Indeed, although PPM1A has also been shown to dephosphorylate the C-terminal SXS motif of phospho-Smad1 [46], it not appears to be unique in its ability to do so. In *Xenopus* embryos Small C-terminal Domain Phosphatases (SCPs) 1, 2 and 3 were found to cause selective SXS dephosphorylation of phospho-Smad1, as compared with phospho-Smad2, thus inhibiting Smad1-dependent transcription and altering normal embryo development [47]. Additionally an RNAi screen for the *Drosophila* MAD phosphatase in *D. melanogaster* S2 cells identified Protein Pyruvate Phosphatase (PDP) as such, and PDP was shown to inhibit DPP signal-transduction [48]. In human cells RNAi-mediated depletion of SCP1 and SCP2 [47], and PDP [48], increased the level (and duration in the case of SCPs) of Smad1 phosphorylation. However, over-expression of mammalian SCPs and PDP did not reduce Smad1 SXS phosphorylation when compared with PPM1A [46], thus these phosphatases may require additional co-factors in over-expression studies.

Regardless of how many R-Smad C-terminal SXS phosphatases are identified in the future, the exciting discovery of the phosphatases thus far opens up an entirely new and critical level of research on TGF-β superfamily signaling in both normal cells and diseases.

Reversible phosphorylation of R-Smads in the linker

Although C-terminal SXS phosphorylation by the type I receptor is the key event in Smad activation, additional phosphorylation by intracellular protein kinases can also positively and negatively regulate Smads. R-Smads contain two conserved polypeptide segments, the MH1 and MH2 domains, joined by a less conserved linker region. R-Smad linker regions are serine/threonine-rich and contain multiple phosphorylation sites for proline-directed kinases. They are phosphorylated by mitogen-activated protein kinases (MAPKs) and cyclin-dependent kinases (CDKs), which exhibit some preference for specific serine residues in the linker [2, 5, 49] (Figure 2).

In the nucleus CDK2/4-mediated phosphorylation of Smad3 occurs mostly at Thr8, Thr179, and Ser213 [50]. CDK-dependent phosphorylation of Smad3 inhibits its transcriptional activity, negating the anti-proliferative action of TGF-β and serving as a novel means by which CDKs promote aberrant cell cycle progression and confer cancer cell resistance to the growth-inhibitory effects of TGF-β. Interestingly, MAPK-mediated phosphorylation appears to have a dual role in Smad2/3 regulation. Mitogens and hyperactive Ras result in ERK-mediated phosphorylation of Smad3 at Ser204, Ser208, and Thr179, and of Smad2 at Ser245/250/255 and Thr220. Mutations of these sites increase the ability of Smad3 to activate target genes, suggesting that MAPK phosphorylation of Smad3 is inhibitory [51]. However, in contrast, ERK-dependent phosphorylation of Smad2 at the N-terminal Thr8 enhances its transcriptional activity [52]. Phosphorylation of Smad3 by p38 MAPK and ROCK (Ser204, Ser208, and Ser213) and c-Jun N-terminal kinase (JNK) (Ser208 and Ser213; analogous to Ser250 and Ser255 in Smad2) may also enhance Smad2/3 transcriptional activity, suggesting that Smads and the p38/ROCK/JNK signaling pathways might cooperate in generating a more robust TGF-β response [53-55]. In accordance, a significant increase in Ser208/Ser213 phosphorylation of Smad3 is associated with late stage colorectal tumors, suggesting that the linker-phosphorylated Smad3 may

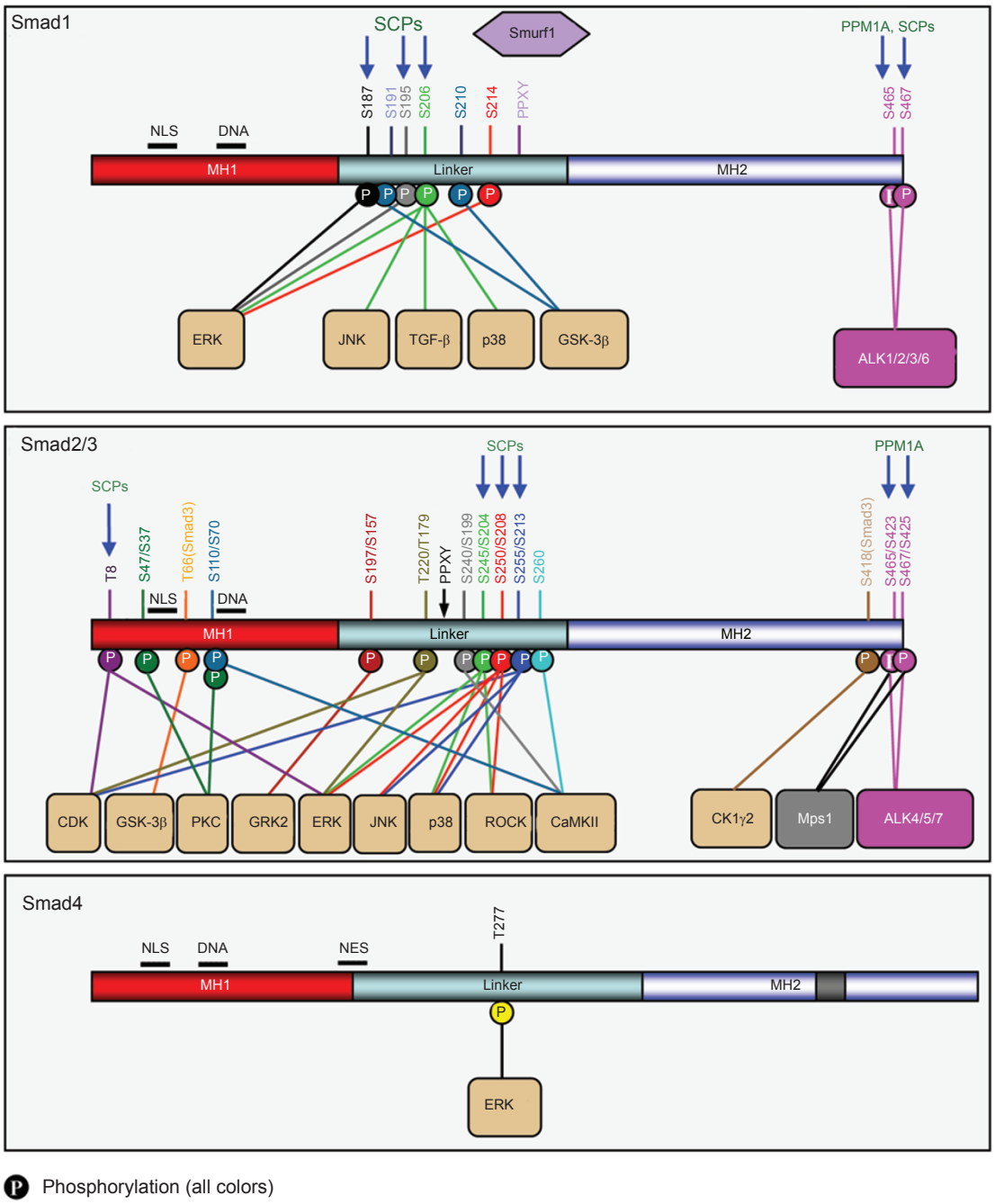

Figure 2 Smad Phosphorylation. All amino acid numbers are based on human sequences for Smad1 (NM_005900), Smad2 (NP_005892), Smad3 (NP_005893) and Smad4 (NP_005350). In the Smad2/3 box, amino acid residues are numbered in the Smad2/Smad3 order. Note that the Smad3 sequence is incorrectly numbered in many publications. Smad2 exon 3, inserted between the NLS and the DNA-binding domain is not shown. Several other kinases such as CK1ε and MEKK also phosphorylate R-Smads at unidentified sites. The linker region of Smad1 contains two other potential MAPK sites at Ser209 and Ser210. MAPK-mediated phosphorylation of Smad1 primes the linker for phosphorylation at Ser191 and Ser210 by GSK-3β. Smurf1 selectively binds linker-phosphorylated Smad1, targeting it for ubiquitin-mediated degradation and also blocking Smad1 nuclear translocation. Smad5 and 8 may be similarly regulated by (de)phosphorylation at analogous sites.

mediate the tumor-promoting role of TGF-β in late tumorigenesis [56]. In BMP signaling, the linker region

of Smad1 consists of four MAPK phosphorylation sites (Ser187, Ser195, Ser206, and Ser214) which can be phosphorylated by ERK [57], and two other potential MAPK sites at Ser209 and Ser210 [58]. Additionally, MAPK-mediated phosphorylation of Smad1 primes the linker region for phosphorylation at Ser191 and Ser210 by the signaling kinase glycogen synthase kinase 3β (GSK-3β) [59], which enhances Smad1 ubiquitination and degradation. Fuentealba *et al*. extend this idea by showing that the BMP receptor first causes Smad1 C-terminal phosphorylation and nuclear translocation; Smad1 is then phosphorylated by MAPK enzymes (Erk, p38 and JNK) in the nucleus, and finally GSK-3 recognizes the pre-phosphorylated linker region and phosphorylates Smad1 at an unknown cellular location [60]. Here phosphorylation at the GSK-3 sites was found to repress the transcriptional activity of Smad1 by enhancing its ubiquitin-mediated degradation [60]. Activated MAPK-directed phosphorylation of the Smad1 linker has been shown to cause the nuclear exclusion of Smad1 even in the presence of BMP, and consequently desensitize cells to BMP and promote neural induction in *Xenopus* embryos [61]. Notably, analysis of mutant mice carrying mutations in the six potential MAPK sites of Smad1 supports the inhibitory role of linker phosphorylation, and also points to additional important functions of MAPK-directed linker phosphorylation [58]. Additional kinases, *e.g.* CK1γ2, CK1ε, CaMKII, PKC, GRK2, and MEKK-1, target R-Smads and regulate Smad-dependent transcriptional responses, and TGF-β may also induce phosphorylation in the linker region of Smad1/2/3 as well as at the SXS motif of Smad2/3 [62]. Thus, the Smad linker region is emerging as an important and critical regulatory platform in the fine-tuning of TGF-β signaling.

Despite growing evidence showing phosphorylation in the linker region, the exact mechanism(s) by which this phosphorylation regulates Smad signaling remains to be clearly elucidated. Phosphorylation in the linker may allosterically regulate intramolecular interactions between the MH1 and MH2 domains, and/or intermolecular interactions between Smads and other molecules (*e.g.* cytoplasmic anchors or cofactors). Which Ser/Thr residues, and to what extent they are phosphorylated, would largely be defined by accessible kinases to Smads in a specific location and at a specific time. The variety of linker kinases, regulated by many signaling pathways and even by TGF-β family members themselves, further contribute to the complex pattern of phosphorylation in the linker. The combination of sites phosphorylated may have different or even opposite consequences, and may well explain why Smad phosphorylation by the same kinase, for example ERK, can lead to opposite effects on Smad signaling in different experimental settings. Use of phospho-specific antibodies against each individual pSer/pThr residue is critical in determining both the responsible kinases, and how each single phosphorylation event contributes to Smad regulation [50]. In addition, phosphorylation in the cytoplasmic or nuclear compartment of the cells may have conceptually different effects even though the phosphorylation can take place on the same Ser/Thr residues. It is also conceivable that some phosphorylation may require priming kinases, and conversely, one phosphorylation event may prevent others. Therefore, the physiological outcome of the regulatory phosphorylation by intracellular kinases is largely context-dependent. Caution must be taken to interpret how different kinases work independently, or in concert, to regulate Smad activity in normal cells and diseases.

An important level of complexity comes from the recent discovery of R-Smad linker phosphatases, highlighting the need to consider Smad-linker (de)phosphorylation as a reversible and tightly controlled event. The SCP1, 2 and 3 were found to specifically dephosphorylate certain sites in the Smad2 (Ser245/250/255 and Thr8) and Smad3 (Ser204/208/213 and Thr8) linker/N-terminus, but to have no effect on Smad2/3 C-terminal SXS-phosphorylation [63]. It is intriguing that SCPs were unable to dephosphorylate Smad2/3 at Thr220/179, given that these sites can be phosphorylated by the same kinases as other linker/N-terminal sites; perhaps phosphorylation at Thr220/179 may have a unique role which excludes them from dephosphorylation by SCPs. In fact, of 40 phosphatases screened, none had phosphatase activity towards Smad2/3 at Thr220/179 [63]. Aside from their role in Smad1 SXS dephosphorylation, SCPs also dephosphorylate Smad1 in the linker at Ser206, and presumably Ser187 and Ser195, in both mammalian cells and *Xenopus* embryos [64]. Importantly Sapkota *et al*. showed that SCPs can mediate dephosphorylation of the Smad1 and Smad2 linker in mammalian cells regardless of whether the phosphorylation has occurred in response to BMP, TGF-β or EGF-activated kinases [64]. Smad2/3 linker dephosphorylation clearly results in enhanced TGF-β signaling [63, 64], consistent with earlier findings that mutations of CDK2/4 and ERK phosphorylation sites in the Smad2/3 linker increase the TGF-β response [50, 51, 65]. However, by dephosphorylating Smad1 at both the C-terminus and in the linker, SCPs terminate BMP signaling [47, 64]. The opposing effect of SCPs on TGF-β and BMP signaling outcomes fits with the role of *Xenopus* SCP2 in promoting secondary axis development in embryos [66], by simultaneously promoting Activin signaling (via dephosphorylation of the Smad2/3 linker) and inhibiting BMP signaling (via dephosphorylation of

the Smad1 SXS and linker). Thus, this biological need to differentially regulate Activin and BMP signaling in parallel seems to explain the interesting specificity of SCPs for the Smad1, but not Smad2/3 SXS motif, as well as the more generalized role of SCPs in Smad1/2/3 linker dephosphorylation.

It is possible that additional understanding of the regulatory role of R-Smad linkers in TGF-β superfamily signaling may come from firmly connecting specific linker kinases and phosphatases *in vivo*, and from discovering the physiological conditions under which these (de)phosphorylation events occur.

Smad phosphorylation coupled to ubiquitin-mediated degradation

Whilst it is accurate to say that SXS phosphorylation of R-Smads is essential for Smad transcriptional activity, additional post-translational modifications work in concert to control their final ability to transduce TGF-β signals [62]. One of these modifications is ubiquitination which targets various components of the TGF-β pathway for degradation, including both type II and type I receptors and Smads 1-7 [62]. Ubiquitination occurs through a three-step process involving ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes, and results in covalent attachment of ubiquitin to a substrate and its degradation via the 26S proteasome. In some cases, whether or not proteins in the TGF-β superfamily signaling pathway are targeted for ubiquitin-mediated degradation depends on their phosphorylation status. However, it should be noted that many additional receptor/Smad ubiquitination events are also known to take place, but at this time no data specifically link them to the phosphorylation state of the target.

A link between active signaling termination and ubiquitination was proposed in a study demonstrating that TGF-β induces proteasomal degradation of phosphorylated Smad2 in the nucleus [40]. Indeed, Smurf2 appears to bind to only the activated form of Smad2 to promote its proteasomal degradation [67]. Another study found that the nuclear RING-domain E3 ligase Arkadia directly ubiquitinates phospho-Smad2/3 in embryonic cells, leading to their proteasomal degradation, and that this is coupled with their high activity [41]. As Arkadia was found never to repress, and in some cells even to enhance, signaling, the authors of this elegant study propose that Arkadia provides a mechanism for signaling termination after gene transcription. In short, they suggest that the link between activity and degradation ensures that only phospho-Smad2/3 that have already initiated gene transcription are degraded. A recent interesting finding reveals that CK1γ2-mediated S418 phosphorylation targets activated Smad3 for ubiquitination-dependent degradation [68]. Given the recent progress showing R-Smad phosphatases as a critical mechanism for terminating TGF-β signaling, it will be interesting to see how the ubiquitin-mediated degradation and dephosphorylation events of "used" phospho-R-Smads work together or independently, in any given context, to control the overall duration of Smad signaling.

Ubiquitin-mediated degradation has also been shown to have a preference for non-phosphorylated Smad3 which specifically undergoes proteasomal degradation in response to the scaffold protein Axin and its associated kinase GSK-3β [69]. Smad3, but not Smad2, interacts with Axin/GSK-3β only in the absence of TGF-β, resulting in GSK-3β-dependent phosphorylation of non-activated Smad3 at Thr66 and its subsequent ubiquitination and degradation. Accordingly, Smad3 Thr66 mutants show increased protein stability and transcriptional activity [69]. This study suggests that the stability of non-activated Smad3, as well as the level of phospho-Smads, may be important in the potential of cells to respond to TGF-β.

Sapkota *et al*. have elegantly demonstrated the interplay between Smad1 linker phosphorylation and Smad1 ubiquitination mediated by the Smurf1 E3 ligase [59]. Smurf1 was found to selectively bind to linker-phosphorylated Smad1. Not only did this result in the ubiquitination and specific degradation of linker-phosphorylated Smad1, Smurf1 binding also blocked nucleoporin-Smad1 interactions and consequently Smad1 nuclear translocation [59]. These data provide a firm mechanism as to how linker phosphorylation can restrict Smad1 activity. It will be interesting to see if Smad2/3 linker phosphorylation is also involved in the selective binding of any such regulatory factors.

Smad4 is constitutively phosphorylated in cells and although not all of the phosphorylation sites are known, ERK has been shown to phosphorylate Smad4 specifically at Thr277 (equivalent to mouse Thr276) [70]. Substitution of Thr277 to Ala results in reduced nuclear localization of Smad4 through an unknown mechanism [70]. Interestingly, Smad4 phosphorylation also appears to be a prerequisite for the ubiquitination of certain cancer-derived Smad4 mutants by the Skp1-Cul-F-box protein (SCF) E3 ligase - a multi-subunit complex that can initiate ubiquitination of a target protein [71]. Liang *et al*. demonstrated that R100T, G65V, or L43S mutation in the MH1 domain results in massive phosphorylation of Smad4 by JNK/p38 MAPK, resulting in an increased affinity of the mutant Smad4 for βTrCP and Skp2 as compared to wild type Smad4 [71]. Similar mutations

in acute myelogenous leukemia or pancreatic cancer cause rapid $SCF^{\beta TrCP}$-mediated proteasomal degradation of Smad4 [72]. The resistance of wild-type Smad4 to SCF^{Skp2}-mediated degradation could thus be controlled, at least in part, by the fact that JNK/p38-mediated phosphorylation is apparently low or absent in wild-type Smad4. Hyperactive Ras has also been shown to promote degradation of Smad4 in intestinal epithelial cells [73]. As this was dependent on ERK/MAPK activity it further highlights a link between Smad4 phosphorylation and degradation, although no ubiquitin ligase was identified in this study. Further identification of Smad4 phospho-Ser residues and ubiquitination sites should allow us to gain deeper insight into the important relationship between these modifications in Smad4 stability and beyond.

Ligand-induced phosphorylation of non-canonical R-Smads and non-Smad targets

Although the canonical model of TGF-β signaling represents the widely accepted mechanism for downstream phospho-transfer and TGF-β-mediated transcriptional responses, there are exceptions to the rule. Specific ligands have been shown to initiate phosphorylation of an unexpected R-Smad, as well as the phosphorylation of non-Smad protein targets.

Several studies show that TGF-β can induce Smad1/5 phosphorylation as well as the expected TGF-β-induced Smad2/3 phosphorylation. In endothelial cells, the current model proposes that the type I receptor ALK1 is necessary for TGF-β-induced Smad1/5 phosphorylation, in addition to ALK5 activity and the accessory receptor Endoglin [74-76]. In dermal fibroblasts, TGF-β can also induce Smad1 phosphorylation dependent on ALK5 and ALK1 activity, but this also appears to require activation of the ERK1/2 pathway for a sustained response [77]. It has also been shown that TGF-β can stimulate Smad1/5 phosphorylation in HaCaT keratinocytes and various epithelial cell lines dependent on ALK5 activity and a canonical activator of Smad1, most likely ALK2 and/or ALK3 [78, 79]. Thus, these studies reveal specific cross-talk between the TGF-β ligand and the normally BMP-regulated R-Smads. The function of TGF-β-induced Smad1 phosphorylation may be largely cell-type specific as it stimulates proliferation and migration in endothelial cells [74], and it appears to be essential for anchorage-independent growth in epithelial cells [79]. It is interesting that in both of these cell types TGF-β-induced Smad1/5 phosphorylation does not appear to be required for the anti-proliferative effects of TGF-β, and may actually be acting in opposition to the normal role of TGF-β-induced Smad2/3 phosphorylation in these cells. Further study of the role of specific type I receptors in TGF-β superfamily signaling has been made easier by the discovery of type I receptor kinase inhibitors such as SB431542, which specifically inhibits ALK4/5 activity [80], and Dorsomorphin which specifically inhibits ALK2/3/6 kinase activity [81]. Finally, it is worthy to note another layer of complex regulation of R-Smad SXS motif phosphorylation. It is reported that the mitotic kinase Mps1 can phosphorylate the Smad2/3 SXS motif, perhaps independently of type I receptors [82]. This finding suggests a new scheme of TGF-β-independent Smad signaling.

It is clear that so far most of the TGF-β-regulated cellular responses appear to be mediated through the transcriptional functions of Smads, and largely through the canonical receptor/Smad phospho-relay. However, the core Smad complex may not necessarily contain only Smad proteins. Recent work clearly shows that Smad2/3 can form a complex with IKKα or TIF1γ to elicit Smad4-independent signaling events in specific cell types [83, 84]. It will be interesting to investigate whether these functional analogs of Smad4 are subject to phosphorylation by TGF-β receptors and whether they modulate the phosphorylation of R-Smads.

TGF-β receptors can also phosphorylate non-Smad targets to influence gene transcription (See [2, 49] for reviews). Some of the key non-Smad activation events by TGF-β receptors may help to reconcile the difference in TGF-β response between epithelial cells, where TGF-β elicits growth arrest, and fibroblasts where TGF-β elicits proliferation and transformation. Indeed, TGF-β receptor signaling can activate p21-activated kinase 2 (PAK2), a serine/threonine kinase target of the small GTP binding proteins CDC42 and RAC1, in fibroblasts but not epithelial cells [85]. This occurs in a Smad2/3-independent, but Cdc42- and Rac1-dependent, manner. As TGF-β-induced transformation and fibroblast proliferation were inhibited when PAK2 activity was blocked, PAK2 may contribute to the TGF-β signaling outcome in these cells. Further work revealed that TGF-β receptor-mediated activation of phosphatidylinositol 3-kinase (PI3K) is required for PAK2 activity and the corresponding biological response to TGF-β in fibroblasts [86]. Both TGF-β receptors appear to be required for ligand-induced PI3K activation, which is potently stimulated by the active type I receptor [87]. A later study reveals that c-Abl serves as a downstream target of PI3K-activated PAK2 in fibroblasts [88]. Thus, phospho-relay from TGF-β receptors to a PI3K-PAK2-cAbl non-Smad branch of TGF-β signaling may be specific to fibroblasts, and may help explain why these cells respond differently to TGF-β from their epithelial counterparts which don't harbor this line of sig-

naling activation. TGF-β signaling has also been shown to contribute to the metastatic properties and survival of HER2-over-expressing breast cancer cells by activating a Rac1-PAK1 pathway in a PI3K-dependent manner [89, 90]; and BMP-2 may increase migration of human chondrosarcoma cells via the PI3K/Akt pathway [91].

Mitogen activated protein kinases (MAPKs), such as p38 and extracellular signal-regulated kinase (ERK), are another key class of non-Smad targets for TGF-β receptors. Interestingly, several recent studies have shed some light on the underlying mechanisms. One study shows that the type I receptor can recruit and phosphorylate the ShcA adaptor protein on tyrosine and serine residues in response to TGF-β [15]. This results from a novel intrinsic tyrosine kinase activity of the type I receptor first identified here, in addition to its well established serine-threonine kinase function. Phosphorylated ShcA subsequently associates with Grb2 and Sos [15], proteins that are well established to link receptor tyrosine kinases with ERK via Ras and Raf activation [92]. This suggests that TGF-β stimulates ERK phosphorylation by initiating a ShcA-Grb2/Sos-Ras-Raf phospho-cascade. Not only does this study reveal how TGF-β signaling may result in ERK MAPK activation, it also presents the idea that TGF-β may initiate cellular responses that are normally mediated by receptor tyrosine kinases, which should be considered in assessing the functional read-out of TGF-β signaling in the future.

A separate series of studies implicate Grb2 in the transduction of TGF-β signal to p38 MAPK phosphorylation. Galliher and Schiemann discovered that β3 integrin alters TGF-β signaling in mammary epithelial cells, via Src-mediated Tyr284 phosphorylation of the type II receptor [12]. This significantly enhances the ability of TGF-β to induce epithelial-mesenchymal transdifferentiation (EMT) and cell invasion [12], and also appears to be essential for TGF-β-induced p38 MAPK activation in breast cancer cells [13]. Src-mediated phosphorylation of Tyr284 coordinates the docking of Grb2 and Shc to the type II receptor, thereby further associating adaptor proteins with MAPK activation in response to TGF-β, and importantly, it does not affect TGF-β-induced Smad2/3 activation. Interestingly, type II TGF-β receptor mutants that could not be phosphorylated on Tyr284 abrogated breast cancer cell invasion induced by αvβ3 integrin and TGF-β, suggesting a novel αvβ3/Src/TβRII signaling axis that promotes oncogenic signaling by TGF-β in malignant mammary epithelial cells [13]. The ability of TGF-β to stimulate breast cancer growth and pulmonary metastasis in mice was also shown to require type II receptor phosphorylation on Tyr284, which activated p38 MAPK in developing and progressing mammary tumors [93].

Two recent parallel studies reveal an intriguing role for the E3 ubiquitin ligase TRAF6 in mediating TGF-β-induced p38 phosphorylation. Yamashita and colleagues found that TRAF6 interacts with type I and II TGF-β receptors and is required for TGF-β-induced activation of JNK, p38, apoptosis and EMT [94]. TGF-β promoted Lys63 ubiquitination of TRAF6 and its interaction with its downstream target TAK1 (TGF-β-activated kinase-1) which functions upstream of p38 and JNK. A kinase-deficient type I receptor did not support ubiquitination of TRAF6, suggesting a dependence on type I receptor kinase activity for this event [94]. However, data from Sorrentino and colleagues suggest a kinase-independent role for the type I receptor in TRAF6-mediated TAK1 and p38 activation [95]. Here TGF-β was found to stimulate dimerization of TRAF6 molecules bound to the type I receptor ALK5 [95]. Once activated by this association TRAF6 underwent autoubiquitination, and caused Lys63-linked polyubiquitination and activation of TAK1 which resulted in the initiation of p38 phosphorylation via MKK3/6 [95]. The different conclusions in these studies with regards to the requirement of ALK5 kinase activity may result from the different cell lines and experiments used to reach these conclusions. Sorrentino and colleagues treated HEK293T cells with ALK5 kinase inhibitors and found that TGF-β stimulation still resulted in TAK1 and p38 phosphorylation, but not Smad2 phosphorylation [95], whereas Yamashita *et al*. based their conclusion on the fact that kinase-deficient TβRI could not support TRAF6 ubiquitination in RIB17 mink lung epithelial cells [94]. In the future it will be interesting to see how these differences can be reconciled, and also if adaptor proteins such as ShcA, Grb2 and Sos, and ubiquitin ligases such as TRAF6, are involved in TGF-β-induced activation of non-Smad targets in addition to these MAPKs.

Conclusion

Recent advances, such as the discovery of the long sought-after Smad phosphatases, emphasize the importance of considering TGF-β mediated phospho-relay as a dynamic and tightly controlled event. Additionally, our increasing knowledge of TGF-β signaling cross-talk between branches of TGF-β superfamily pathways, and other key signaling pathways in the cell, highlights that cell signaling pathways should not be regarded and studied as discrete events. It is obvious that many phosphatases acting in non-Smad pathways will significantly contribute to the outcome of TGF-β cellular responses. A better understanding of how (de)phosphorylation regu-

lates TGF-β receptor and Smad activity in normal cells, and identifying how its dysregulation contributes to aberrant TGF-β signaling in disease, may lead to novel treatments in the clinic.

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