Review
Phosphatases in SMAD regulation
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ABSTRACT
SMAD transcription factors are key mediators of the transforming growth factor-beta (TGFβ) family of cytokines. Reversible phosphorylation of SMAD proteins plays a key role in regulating their function. Several phosphatases have been proposed to act on SMAD proteins to influence TGFβ/BMP signalling. Here we provide an overview of the SMAD regulation by different protein phosphatases and review the evidence supporting each phosphatase as a candidate SMAD-phosphatase.

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1. Introduction to the TGFβ signalling

The signalling pathways downstream of the transforming growth factor beta (TGFβ) family of cytokines, including bone morphogenetic proteins (BMPs), are critical during development and in adult tissue homeostasis [1–3]. They control plethora of cellular processes including proliferation, differentiation, extra-cellular matrix production, motility, survival and fate [1,2]. When components of the TGFβ pathway are compromised, numerous human diseases, including fibrosis, cancer progression and metastasis, result [4–7]. For this reason, complex biochemical mechanisms have evolved to intricately control the extent, duration and potency of signalling downstream of the TGFβ ligands. TGFβ ligands initiate signalling by binding to a pair of cell-surface receptor serine threonine protein kinases (termed type II and type I). Upon ligand binding the type II receptors form heterotetrameric complexes with specific type I receptors, which in turn phosphorylate and activate SMAD transcription factors (often termed receptor regulated SMADs or R-SMADs) [8–10]. The phosphorylation of R-SMADs triggers their association with SMAD4 and translocation to the nucleus, where they control the transcription of hundreds of TGFβ/BMP-target genes [2]. The TGFβ subfamily of ligands can broadly be divided into two groups based on their ability to preferentially trigger the activation of specific SMAD transcription factors. The TGFβ subfamily (including TGFβ, Activin and Nodal) activates SMADs 2 and 3, while the BMP subfamily (including BMPs, GDFs and AMH) activates SMADs 1, 5 and 8 [2]. The TGFβ subfamily of ligands signals through specific type II (TGFβR-II or ActR-IIB) and type I (ALK4, ALK5 or ALK7) receptors while the BMP subfamily of ligands employs selective type II (BMPR-II or ActR-IIAB or AMHR-II) and type I (ALK1, ALK2, ALK3 or ALK6) receptors [2].

2. R-SMADs: the key mediators of the TGFβ signals

SMAD transcription factors are highly conserved from Drosophila to mammals. Three distinct structural features define R-SMADs: the highly conserved Mad Homology 1 (MH1) domain at the N-terminus and the Mad Homology 2 (MH2) domain at the C-terminus. The MH1 domain exhibits sequence-specific DNA binding activity and negatively regulates the function of MH2 domain [1,2]. The MH2 domain is responsible for receptor interaction, formation of hetero-omer SMAD complexes as well as interaction with DNA binding partners and transcription factors. The phosphorylation of the two C-terminal Ser residues in the Ser-Xaa-Ser motif of the MH2 domain drives the activation of R-SMADs [1,2]. The divergent linker region contains several Ser/Thr residues that are phosphorylated by various kinases in response to different stimuli (Fig. 1). Additionally there is a PPXY motif that mediates interaction with selective WW-domain containing E3 ubiquitin ligases (Fig. 1).

R-SMADs are indispensable for mediating the cellular responses to TGFβ/BMP signals. Consequently, R-SMADs are key targets for...
regulatory inputs from various proteins and downstream of other signalling networks that modulate the outcome of TGFß/BMP signals. Various post-translational modifications of R-SMADs have been reported to affect their activity, stability and localization in cells [11,12]. Reversible phosphorylation of R-SMADs, mediated by protein kinases and phosphatases, is crucial in regulating the precise nature of cellular responses to TGFß/BMP ligands. The mechanisms by which phosphorylation of R-SMADs regulates their activity, localization and stability are generally well established [11,12]. However our understanding of the mechanisms by which different phosphatases regulate R-SMADs is still emerging. Despite numerous R-SMAD phosphatases proposed to impact TGFß/BMP signalling, the field still remains largely sceptical on their roles. This review focuses on all the proposed R-SMAD phosphatases and discusses the further evidence needed for each phosphatase to establish it as a convincing candidate.

### 3. Phosphorylation of R-SMADs is key to their function

R-SMADs are directly phosphorylated at multiple Ser/Thr residues in response to different agonists by distinct protein kinases (Fig. 1A and B). Two R-SMAD phosphorylation events are best characterized: (a) the phosphorylation of C-terminal SXS motif (termed tail-phosphorylation) by the type I receptor kinases and (b) the phosphorylation of multiple Ser/Thr residues in the linker-region (termed linker-phosphorylation) by various proline-directed kinases. Most proposed R-SMAD phosphatases to date have only addressed the dephosphorylation of these events. However, it is noteworthy that there are additional Ser/Thr residues within R-SMADs that are also phosphorylated by different kinases in response to different agonists (Fig. 1A and B). Any R-SMAD-associating phosphatase could potentially also act as a R-SMAD phosphatase against any number of these phosphorylation sites. Below we discuss the phospho-regulation of R-SMADs.

#### 3.1. R-SMAD tail phosphorylation

Upon formation of the ligand-receptor complexes, the constitutively active type II receptor kinases phosphorylate the ‘GS’ domain of the type I receptor kinases, which activates them [10,13] (Figs. 2 and 3). The activated type I receptors in turn bind to the R-SMADs through their MH2 domains and rapidly phosphorylate the dual Ser residues in the C-terminal Ser-Xaa-Ser motif of R-SMADs [14,15]. In the BMP pathway, activated ALKs 1, 2, 3 or 6 can phosphorylate SMAD1 at Ser463 and Ser465 and corresponding residues on SMADs 5 and 8. In the TGFß pathway, activated ALKs 4, 5 or 7 phosphorylate SMAD2 and SMAD3 at Ser465/Ser467 and Ser423/Ser425, respectively [1,2]. Phosphorylation of R-SMADs at the tail triggers association with SMAD4 and nuclear translocation [16,17]. SMAD4 lacks the critical SXS motif but is essential and non-redundant in mediating TGFß/BMP signals. The phosphorylation of SXS motif in SMADs is indispensable for cellular responses to TGFß/BMP ligands [1,2]. Indeed mouse embryos in which endogenous SMAD1 gene is replaced with SMAD1(SVS-AVA) mutant display many of the SMAD1-null phenotypes [18].

#### 3.2. R-SMAD linker phosphorylation

The linker region of R-SMADs is divergent in that the sequence similarity in this region between the TGFß SMADs (2 & 3) and the BMP SMADs (1, 5 & 8) is low. However within each subfamily there is a high degree of sequence similarity. The linker region of all R-SMADs has two key features: the presence of a PPXY motif and a high degree of sequence similarity. The linker region of all R-SMADs is divergent in that the sequence similarity in this region between the TGFß SMADs (2 & 3) and the BMP SMADs (1, 5 & 8) is low. However within each subfamily there is a high degree of sequence similarity. The linker region of all R-SMADs has two key features: the presence of a PPXY motif and the abundance of proline-directed Ser/Thr residues. The PPXY motif is known to interact with selective WW-domain containing...
proteins [19–23], while the proline-directed Ser/Thr residues can be phosphorylated by any number of proline-directed protein kinases (Fig. 1). Several MAPKs downstream of mitogens, growth factors and stress induce the phosphorylation of several proline-directed Ser/Thr residues in R-SMADs, including Ser187, Ser195, Ser206, Ser214 and Thr222 in SMAD1 and Thr179, Ser204, Ser208 and Ser213 in SMAD3 [20,21,24–28] (Fig. 1A and B). Additionally, GSK-3 can phosphorylate Ser210, Thr202, Ser198 and Ser191 in SMAD1 following priming phosphorylation at Ser214, Ser206 and Ser195 [21]. Interestingly, TGFβ and BMP ligands themselves induce the phosphorylation of the same proline-directed Ser/Thr residues in the linker region of the respective R-SMADs (Fig. 1A and B) [21,25]. The ligand-induced phosphorylation of the linker region follows tail-phosphorylation and is mediated by...
nuclear kinases, CDK8 and CDK9 [24]. The phosphorylation of the linker region of SMADs modulates the R-SMAD function by affecting their localisation, transcriptional ability and turnover [20,21,24–28]. Phosphorylation of SMAD1 at the linker region by proline-directed kinases and GSK-3 primes SMAD1 for recognition and polyubiquitylation by SMURF1 [21,25]. Similarly SMAD3 linker phosphorylation triggers its recognition and polyubiquitylation by NEDD4L [20]. Conversely the ligand-induced linker phosphorylation mediated by CDK8/9 can promote SMAD transcriptional action prior to turnover [24]. An effector of the Hippo signalling pathway, YAP, which is required for the BMP-dependent suppression of neural differentiation of mouse embryonic stem cells, is recruited by linker phosphorylated SMAD1 [24]. Additionally CDK2/4 have been reported to mediate the phosphorylation of Thr179 and Ser213 of SMAD3 leading to inhibition of TGFβ-transcriptional activity [28].

3.3. Other phosphorylation sites in R-SMADs

In addition to the tail- and linker-phosphorylation sites discussed above, several other phospho-sites in R-SMADs have been reported (Fig. 1A and B). A Ste20 kinase Misshapen (a MINK1 ortholog) was shown to inactivate Mad by phosphorylating it at Thr312 (equivalent to Thr322 in SMAD1) [29]. Furthermore, we have identified a novel phospho-Ser132 peptide by mass-spectrometry from SMAD1-immunoprecipitates, although kinase(s) mediating this phosphorylation and its role are unclear (data not shown) (Fig. 1A). CamKII has been reported to phosphorylate SMAD2 at Ser110, Ser240 and Ser260 leading to inhibition of the TGFβ pathway [30]. WNK1/4 have been reported to modulate SMAD2 levels and localisation by phosphorylating SMAD2 at Ser110, Ser260 and Ser423 [31]. PKC was shown to inhibit TGFβ signalling by phosphorylating SMAD3 at Ser37 and Ser70 [32]. Similarly, PKG has been reported to inhibit SMAD3 nuclear localisation by phosphorylating SMAD3 at Ser309 and Thr368 in the MH2 domain [33], SMAD3 stability is reportedly controlled in part by phosphorylation of at Thr66 by GSK-3 [34] and at Ser418 by CSNK1G2 [35]. Furthermore, we have identified a novel phospho-Thr132 peptide from SMAD3-immunoprecipitates (data not shown). The above studies clearly demonstrate that R-SMADs are regulated by phosphorylation at multiple residues, on which a phosphatase could potentially act to reverse the phosphorylation.

4. Protein phosphatases reverse the action of protein kinases

Given that intracellular signalling by R-SMADs is reliant on their phosphorylation by different protein kinases under different biological contexts, removal of phosphates from phospho-residues catalysed by protein phosphatases is the most effective way of reversing the phospho-dependent R-SMAD signalling and restoring the R-SMAD to baseline. Dephosphorylation of proteins by protein phosphatases is a fundamental regulatory mechanism in controlling the activity of many proteins in signal transduction. While the protein kinase family is one of the largest enzyme groups encoded by the human genome (~518 genes), there are relatively fewer protein phosphatases encoded (~147 phosphatases) [36,37]. Protein phosphatases are therefore far more promiscuous with regards to their substrate specificity compared to protein kinases, which display a high degree of substrate specificity. Phosphatases are classified according to their substrate preference: 38 are protein tyrosine phosphatases (PTPs), 40 are serine-threonine phosphatases (S/TPs) and the remainder (DUSPs) display dual (Ser/Thr-Tyr) substrate-specificity. All reported phosphorylation sites in R-SMADs are on Ser/Thr residues. Therefore S/TPs have been the major focus for research on SMAD phosphatases. S/TPs are further classified into three groups based on the amino acid sequence composition and structure of the catalytic domain: the PPP (Phospho-Protein Phosphatase) family, of which PP1 is the prototypic member; the PPM (protein phosphatase, Mg²⁺/Mn²⁺-dependent) family, of which PPM1A/PP2C is the prototypic member; and FCP (transcription factor IIIF-interacting CTD phosphatase 1) family, of which FCP is the founding member. The PPP and PPM family display little amino acid sequence similarity surrounding the active sites but the three-dimensional structures of the catalytic subunits are similar [38]. The PPP subfamily often requires one or more regulatory subunits for catalysis and is robustly inhibited by okadaic acid [38]. The PPM family of phosphatases are monomeric and generally consist of a catalytic domain that is flanked by short N- and C-terminal sequences. The FCP family, which includes Small C-terminal domain Ser/Thr phosphatases (SCP5s), possesses a distinct amino acid consensus sequence and catalytic mechanism [38].

5. Role for R-SMAD phosphatases in TGFβ/BMP signalling

Evidence for the role and nature of potential R-SMAD phosphatases has been highlighted by many early observations, primarily on the dynamics and kinetics of tail-phosphorylated R-SMADs in cells. Early observations established that R-SMADs constantly shuttle between the cytoplasm and the nucleus [39–42]. Treatment of cells with Si-431542, a relatively selective inhibitor of the type I TGFβ receptors [43], resulted in rapid dephosphorylation of SMAD2 and redistribution from the nucleus to the cytoplasm [40]. These observations pointed to the existence of a R-SMAD phosphatase in the nucleus. Indeed in vitro nuclear export assays of SMAD2 using isolated nuclei from TGFβ-treated HeLa cells demonstrated that only dephosphorylated SMAD2 was exported from the nucleus [42]. More recently, a mathematical model, built using the observations of real-time nuclear-cytoplasmic shuttling of SMAD2 upon ligand treatment, permits for a nuclear SMAD2-tail phosphatase [39]. Furthermore, proteasomal inhibitors such as MG-132 only partially rescue the levels of tail-phosphorylated SMADs upon removal of ligands or inhibition of the type I receptors [44]. This indicates that ubiquitylation and degradation of R-SMADs play a significant but partial role in terminating R-SMAD activity. Collectively these observations have formed the basis for the search for R-SMAD phosphatases, focussing primarily on the activating tail-phosphorylation (SXS) sites.

6. SMAD2/3-tail phosphatases in the TGFβ pathway

Protein phosphatase Mg²⁺/Mn²⁺ dependent 1A (PPM1A; also known as PP2Cα) was the first phosphatase proposed to dephosphorylate the SMAD2/3-tail SXS motif (Fig. 2) [45]. In HEK293T cells overexpressing constitutively active rat type I TGFβ receptor, Feng and coworkers observed that overexpression of only PPM1A but not 38 other S/TPs resulted in reduced levels of tail-phosphorylated SMAD2/3 [45]. The ability of DUSPs or PTPs to yield any changes in the levels of phospho-SMAD2/3 was not assayed in this study. The dephosphorylation of type I TGFβ receptors upstream of SMAD2/3 would also be predicted to lead to the reduction in levels of phospho-SMAD2/3. In order to demonstrate that PPM1A acts directly on SMAD2/3, the authors showed that PPM1A bound to SMAD2/3 and dephosphorylated phospho-SMAD2/3 in vitro. It was then demonstrated that overexpression of PPM1A in cells enhanced the TGFβ-induced phosphorylation of SMAD2/3 and transcription of TGFβ-target genes. Conversely, knocking down PPM1A by shRNA resulted in enhanced TGFβ-induced phosphorylation of SMAD2/3 and transcription of TGFβ-target genes. The overexpression of PPM1A in Zebrafish embryos caused fusion of the eyes and thinner posterior notochord, phenotypes consistent with an inhibition of nodal signalling, which signals through SMAD2/3. The authors also
demonstrated that PPM1A was detected exclusively in the nuclear fractions isolated from HaCaT keratinocytes, thus appearing to resolve the long-standing search for a nuclear SMAD2/3 phosphatase in the TGFβ pathway [45]. The nuclear localisation of PPM1A presented by the authors in this study contradicts our observations of PPM1A localisation in at least 10 different cell lines, including HaCaT cells, in which we find that endogenous PPM1A is exclusively cytoplasmic (unpublished findings). Nonetheless, the ideal system to validate the role of PPM1A as a bona fide SMAD2/3 phosphatase would be in cells derived from PPM1A knockout mice or from mice in which the wild type PPM1A is homozygously replaced with a catalytically inactive mutant. Cells or tissues derived from PPM1A-null mice and corresponding wild type mice should also help address definitively the subcellular localisation of PPM1A. Indeed Feng and coworkers recently reported isolation of fibroblasts from PPM1A-null mice [46]. Surprisingly, the effects of PPM1A knockout on TGFβ-induced phosphorylation of SMAD2/3, gene transcription as well as the phenotype of PPM1A-null mice were not discussed in this report [46]. Instead it was reported that PPM1A dephosphorylates RanBP3 and enhances its ability to export SMAD3, but not SMAD2, only under hypoxic conditions [47]. The report claimed that during cutaneous wound healing, the re-epithelialization and keratinocyte migration was delayed in a SMAD2-dependent manner in PPM1A-knockout mice compared to the wild type. Interestingly, the report demonstrated a slight enhancement of phospho-SMAD2 levels in wound tissues derived from PPM1A knockout mice compared to wild type, although the number of representative samples used was very limited [47].

A detailed kinetic analysis of SMAD2/3 dephosphorylation following TGFβ stimulation in cells derived from PPM1A-null mice was lacking [47]. Now that two groups have independently generated PPM1A knockout mouse lines, we can anticipate definitive studies to establish whether PPM1A indeed acts as a SMAD2/3 phosphatase in the TGFβ pathway. It should be noted that PPM1A, like many other phosphatases, most likely dephosphorylates many other substrates. Indeed PPM1A has been reported to dephosphorylate p38 MAPK, RanBP3, CDK2, Axin and IKKβ [46,48–52]. Therefore it is important to understand the precise molecular mechanisms of spatial and temporal action of PPM1A on phospho-SMAD2/3.

In addition to PPM1A, other phosphatases have been proposed to act as SMAD2 and or SMAD3 phosphatases. PP2A was reported to mediate the dephosphorylation of SMAD3, but not SMAD2, only under hypoxic conditions [53]. The study showed that PP2A associates with SMAD3 only in hypoxia. This association affects TGFβ-induced nuclear accumulation of phospho-SMAD3 as well as the transcription of SMAD3-dependent target genes [53]. The report is intriguing in that one of the most promiscuous phosphatases, PP2A, can selectively act on a specific SMAD protein (in this case SMAD3) only under hypoxia. It also highlights an important point that even though many phosphatases may be able to dephosphorylate numerous phospho-proteins under overexpression conditions or in vitro, substrate specificity in cells is most likely defined by how the phosphatase is recruited to its target. Under normoxic conditions however, PP2A did not appear to act as a SMAD3 phosphatase, suggesting specific factors or modifications induced by hypoxia may mediate the interaction between SMAD3 and PP2A [53].

The role of PP2A as SMAD3 phosphatase has not yet been evaluated in mouse models displaying abrogated PP2A activity. Recently, a study reported that myotubularin related protein 4 (MTMR4), a DUSP family member, bound to and dephosphorylated SMAD2/3 to attenuate TGFβ signalling [54]. MTMR4 was chosen for this study as a putative SMAD2/3 phosphatase as it possessed a FYVE domain, similar to the one present in SARA that is known to mediate the interaction with SMAD2/3 in cells [54]. The study shows that overexpression of MTMR4 resulted in the sequestration of TGFβ-induced phospho-SMAD3 in early endosomes thus inhibiting nuclear accumulation of active SMAD3 [54]. On the other hand, knocking down MTMR4 led to sustained SMAD3 activation [54]. However, the evidence to suggest that MTMR4 acts directly on phosphorylated SMAD2/3 was lacking. As with other proposed SMAD2/3 phosphatases, many questions on the mechanisms of action and regulation of MTMR4 remain unanswered.

7. SMAD1/5/8-tail phosphatases in the BMP pathway

The mode of activation and the nature of the SXS phosphorylation motif of BMP-SMADs are almost identical to the TGFβ-SMADs. However, distinct phosphatases have been proposed to catalyse the removal of phosphates from the SXS motif of BMP-SMADs. A mitochondrial enzyme, pyruvate dehydrogenase phosphatase (PDP) was the first phosphatase to be proposed as a SMAD1-tail phosphatase (Fig. 3) [55]. PDP is a member of the PPM serine/threonine phosphatase family. By employing RNA interference based screening to deplete 44 serine/threonine phosphatases from Drosophila S2 cells, PDP was identified to be essential for dephosphorylation of MAD, the Drosophila homologue of SMAD1. Several biochemical assays on both Drosophila and human PDP demonstrated that PDP could act as a SMAD1-tail phosphatase in vitro and in cells overexpressing PDP. Furthermore, depletion of PDP enhanced BMP/DPP responses in cells [55]. The molecular mechanisms by which PDP recognizes and dephosphorylates BMP-SMADs in the cells remain to be elucidated.

The discovery of PDP as a mitochondrial BMP-SMAD-phosphatase left the nuclear phosphatase(s) still elusive. Knockaert et al. proposed Small C-terminal domain phosphatases (SCPs) as nuclear BMP-SMAD tail-phosphatases (Fig. 3) [44]. In a phenotype-based screen, Xenopus SCP2 mRNA microinjected into the ventral marginal zone of Xenopus embryos caused partial duplication of axis, phenotype consistent with inhibition of the BMP pathway [44]. The human SCPs, (SCP1–3) were also able to induce partial duplication of axis in Xenopus embryos. The study further established that SMAD1-tail was dephosphorylated directly by SCPs, both in vitro and in cells. However SCPs did not dephosphorylate SMAD2/3-tail. SCPs associated with SMAD1 with a higher affinity compared to SMAD2/3 [44]. Depletion of SCP2 from multiple human cell lines was sufficient to enhance and sustain high levels of tail-phosphorylated SMAD1 following BMP-treatment. Consequently, depletion of SCP2 also enhanced BMP transcriptional responses in cells. Furthermore, a siRNA-resistant silent mutant of SCP2 was able to rescue the enhanced BMP-induced phospho-SMAD1 levels seen with RNAi-mediated depletion of SCP2. It was also reported that SCP2 and tail-phosphorylated SMAD1 co-localise in the nucleus upon BMP treatment [44]. It is still unclear precisely how the activity of SCP2 as well as its recognition of BMP-SMADs is regulated. SCP2-null or activity deficient mouse models would be the only definitive way to address the potential role of SCP2 in the BMP pathway. In a proteomic approach we undertook to identify potential regulators of SCP2, we isolated over 600 proteins that associated with SCP2 (unpublished data). This indicates that SCPs may be very promiscuous with regards to the number of proteins they might dephosphorylate. Recently, a large proline-rich protein BAT3 was identified as a co-factor of the phospho-tail SMAD1-SCP2 complex [56]. BAT3 has been implicated in various signalling networks and appears to potentiate the interaction between SMAD1 and SCP2 after receptor phosphorylation, with the knockdown of BAT3 increasing the expression of BMP-responsive genes [56]. More recently, a study demonstrated that SCP1 overexpression inhibited BMP-induced osteoblastic differentiation [57]. Rather surprisingly the osteoblastic differentiation induced by
overexpression of tail-phospho-mimetic SMAD1 and constitutively active BMPR1 was also inhibited by SCP1 in a phosphatase activity-dependent manner [57]. These results imply that dephosphorylation of non-SMAD targets may also contribute to the inhibition of the BMP pathway by SCPs [57].

In addition to dephosphorylating SMAD2/3 and attenuating TGFβ signalling, PPM1A was also proposed to act as SMAD1-tail phosphatase leading to the inhibition of BMP signalling (Fig. 3) [58]. PPM1A overexpression resulted in the inhibition of BMP-induced phospho-SMAD1 as well as the expression of BMP-target genes, while RNAi-knockdown enhanced BMP responses [58]. While PPM1A overexpression in Zebrafish embryos resulted in phenotypes consistent with the inhibition of nodal signalling, no effects resulting from its inhibition of the BMP signalling were reported [58]. Furthermore, the role of PPM1A on BMP-SMADs has not yet been reported in cells derived from PPM1A-null mice [46,47]. A recent study suggested that PPM1A inhibits BMP signalling by promoting the proteasomal degradation of SMAD1 [59]. PPM1A was shown to have a negative impact on BMP signalling even when a constitutively active form of SMAD1 was overexpressed. Similarly, PPM1A overexpression led to a reduction of SMAD1 protein levels, which was blocked by Lactacystin, a proteosomal sens. Similarly, PPM1A overexpression led to a reduction of SMAD1 even when a constitutively active form of SMAD1 was overexpres-

8. R-SMAD linker phosphatases

R-SMAD phosphorylation at the linker region is a very prominent event mediated by multiple Ser/Thr protein kinases downstream of a variety of agonists and environmental factors (Fig. 1). Therefore, the linker region provides an important avenue for integration of regulatory inputs from multiple signalling networks to influence the outcome of TGFβ/BMP signalling. When R-SMADs are overexpressed in cells, they are in fact rapidly and spontaneously phosphorylated at the linker region, mainly on the proline-directed Ser/Thr residues, even in the absence of ligands [60]. Two groups independently reported SCPs as prominent R-SMAD linker phosphatases (Figs. 2 and 3) [60,61]. In overexpression assays, SCP1-3 were able to efficiently dephosphorylate the SMAD2-linker sites at Ser245, Ser250 and Ser255 and analogous SMAD3 sites [60,61]. Interestingly Ser1P1s were unable to dephosphorylate Thr179 of SMAD3, although most proline-directed kinases that phosphorylate the SMAD2/3-linker also induce the phosphorylation of Thr179 [61]. RNAi-mediated depletion of SCP1-3 resulted in enhanced phosphorylation of SMAD2/3-linker but did not affect the levels of SMAD2/3-tail phosphorylation [60,61]. Overexpression of SCPs enhanced TGFβ-induced phosphorylation of SMAD2 as well as TGFβ-induced gene transcription [60,61]. Conversely, RNAi-mediated depletion of SCP1/2 resulted in the inhibition of TGFβ-induced transcription of several target genes [60,61]. This is consistent with the notion that enhanced SMAD2/3-linker phosphorylation inhibits TGFβ-responses. In addition to the SMAD2/3-linker, the linker sites in SMAD1 were also targeted by SCP1-3 in both mammalian cells and in Xenopus embryos [60]. SCP1-3 dephosphorylated SMAD1-linker sites at Ser187, Ser195, Ser206 and Ser214 as detected by a PXS’P (where S’ is a phospho-Ser) antibody [21,60]. As discussed earlier, SCPs also dephosphorylated SMAD1-tail sites [21]. RNAi-depletion of SCP1/2 resulted in the enhancement of the levels of BMP-induced proline-phospho-linker as well as phospho-tail SMAD1 resulting in enhanced transcription of BMP target genes [21,60]. The distinction in substrate specificity displayed by SCPs towards BMP vs TGFβ-SMADs is quite intriguing. This could perhaps be explained by the relatively higher affinity SCPs have for BMP-SMADs over TGFβ-SMADs [44]. It is likely that by dephosphorylating SMAD2/3-linker but not tail-sites, the SCPs enhance TGFβ-signalling but by dephosphorylating both SMAD1-linker and tail sites, the SCPs reset SMAD1 to the basal unphosphorylated state [44,60]. In this review and in many reports, linker phosphorylation (and dephosphorylation) of R-SMADs is taken as a single entity. However it is clear that multiple phospho-residues in the linker region are regulated in different ways and follow distinct kinetics of both phosphorylation and dephosphorylation (Fig. 1) [11,20,21,24,28]. More detailed studies on how each of the linker sites is phosphorylated/dephosphorylated may shed light into how SCPs act on individual sites. The mechanisms by which the activity of and substrate recognition by SCPs are regulated remain to be elucidated. Furthermore, in addition to the proline-directed phospho-Ser/Thr residues, there are other non-proline-directed phospho-residues within the R-SMAD-linker that are phosphorylated by different kinases (Fig. 1). It is not known whether SCPs also dephosphorylate these residues.

9. R-SMAD phosphatases that target non-linker and tail phospho-sites

As demonstrated in Fig. 1, the MH1 and the MH2 domains of the R-SMADs are targeted for phosphorylation by several kinases in response to several agonists. Currently we do not know whether any of the proposed SMAD-associating phosphatases also target any of the sites other than the SXS motif and the linker sites already discussed. In most cases, appropriate tools do not exist to probe the phosphorylation or dephosphorylation of many of these sites. The only site outside the linker region that SCPs are reported to dephosphorylate is Thr8 of SMAD3, which is phosphorylated in cells by CDKs and ERK [61].

10. Phosphatases that act on TGFβ/BMP receptors also modulate R-SMAD activity

The type I TGFβ/BMP receptor kinases are themselves activated upon phosphorylation by constitutively active type II receptor kinases prior to phosphorylating and activating R-SMADs. As with the R-SMADs, the type I receptors are regulated by dephosphorylation and degradation, both of which are critical regulatory steps for the termination of the signalling cascade. While the degradation of type I receptors involves various E3 ubiquitin ligases and inhibitory SMADs 6 &7, several phosphatases have been reported to act on type I receptors (Figs. 2 and 3). The majority of phosphatases that have been reported to act on the cell membrane receptors belong to the PPP and FCP families of serine/threonine phosphatases. PP2A, a member of the PPP family, has been reported to act on type I type I TGFβ receptors ALK5 to modulate the TGFβ pathway [62]. Two distinct regulatory subunits of PP2A, Bα and Bδ, appear to regulate the TGFβ pathway in opposite ways. While the knockdown of Bα subunit suppresses nodal signalling in Xenopus embryos, the depletion of Bδ enhanced the nodal signalling responses [62]. Neither subunit was reported to direct PP2A to dephosphorylate ALK4, 5 or 7 suggesting that these subunits may regulate the receptor activity or stability by binding to the receptors [62]. Another PPP family member, PP1, has been reported to function at the type I receptors to impact on TGFβ signalling [63]. A two-hybrid screen identified PPIc to interact with SARA through the PPIc binding RXVF motif [63]. In Drosophila, the interaction with SARA allowed PPI to be recruited to the Decapentaplegic (Dpp) receptor, the homolog of the BMP type I receptor. The expression of SARA mutant that was unable to interact with PPII displayed hyperphosphorylation of the type I receptor [63]. Furthermore, flies with a catalytically inactive PPIc background displayed increased expression of Dpp-target genes [63]. Further studies on mammalian models have also established the role for PP1 in type I receptor
dephosphorylation [64,65]. In mammalian cells, PP1 has been reported to engage SARA as well as SMAD7 and GADD34 (growth arrest and DNA damage protein 34) to enhance its interaction with the type I TGFß receptors [64]. Indeed, phosphorylation of the type I TGFß receptor was reduced in vitro when PP1 was in complex with GADD34 and Smad7. However, this does not appear to be an immediate feedback loop as the association of PP1 with the receptor was only observed after stimulation with TGFß for 16 h [64]. The dependence of PP1 on SMAD7 for it to be targeted to the type I receptors was further highlighted by another study [65]. In endothelial cells, PP1 interacted with ALK1, in a SMAD7-dependent manner, leading to the inhibition TGFß/BMP signalling [65]. Finally, the FCP family member Dullard was shown to be an important player in the induction of neuralisation in Xenopus embryos. Dullard was demonstrated to interact with the BMP type II receptor and promote its degradation as well as the repression of the phosphorylation of the BMP type I receptor. Both processes were reported to require the active phosphatase domain of Dullard [66].

11. Concluding remarks

Although the regulation of R-SMADs by phosphorylation has been known for over 15 years, the first phosphatases proposed to dephosphorylate R-SMADs made their debut just over five years ago [44,45,55,58,60,61]. Since then more R-SMAD phosphatases have been proposed [53,54]. The emerging debate in the field is: which of these phosphatases are in fact true R-SMAD phosphatases and what are the mechanisms that define substrate specificity and activity of these phosphatases? In order to help this debate constructively, we have drawn up a few parameters below that we believe any potential R-SMAD phosphatase has to meet in order to be considered a relevant candidate:

1. The phosphatase has to interact, at least transiently, with phospho-R-SMADs. Defining the mechanisms of this interaction may yield clues to the mechanisms of action for candidate phosphatases.
2. The phosphatase and R-SMADs should co-localise at the site of dephosphorylation.
3. The phosphatase should be able to dephosphorylate the appropriate phospho-residue within R-SMADs in vitro. Often overexpression of phosphatases is used to demonstrate that the phosphatase is able to dephosphorylate the substrate. A negative result under these conditions may simply mean that the phosphatase lacks the equivalent amounts of necessary regulatory subunits to be active in cells. This is particularly true for the PPP family of phosphatases, which are controlled by multiple regulatory subunits. Even for monomeric phosphatases, overexpression may cause non-specific dephosphorylation of multiple targets. By their promiscuous nature, many phosphatases would be expected to pass this test regardless of the substrates used. However it is still important to demonstrate the in vitro activity towards R-SMADs.
4. Abrogation of the phosphatase activity in cells (e.g. by knockdown of the phosphatase or its regulatory subunits) has to impede the rate of R-SMAD dephosphorylation. This would entail an extended half-life of ligand-induced phospho-R-SMAD levels, especially following the removal of appropriate ligands and/or inhibition of the upstream kinases after a pulse of ligand treatment. Consistent with the sustained levels of R-SMAD-dephosphorylation, depletion of candidate phosphatase would be expected to impact on the target gene-expression and downstream cellular responses. Well-established phenotypic screens using Xenopus or Zebrafish embryos may provide further biological insights of the candidate phosphatase.
5. Restoration of wild type phosphatase, but not catalytically inactive or substrate-interaction deficient mutants, in cells where the phosphatase activity is abrogated should be able to rescue the dephosphorylation of R-SMADs. For example, if the phosphatase is silenced by RNA interference, then rescue experiments using RNAi-resistant silent mutants of the wild type phosphatase or catalytically inactive mutant should be performed. This is extremely important in order to demonstrate that the effects on R-SMAD-phosphorylation are due to the predicted abrogation of the candidate phosphatase activity and not due to off-target effects.
6. The ultimate test for a R-SMAD-phosphatase is to confirm findings in a transgenic mouse model in which the candidate phosphatase gene has been knocked out or replaced with a catalytically inactive phosphatase. With global efforts to complete the targeted disruption of every gene in the human genome already underway, we may be able to accomplish this comprehensively within the next decade. If the transgenic mice are embryonic lethal, cells derived from such embryos will be sufficient to verify the role of the candidate phosphatase as a R-SMAD-phosphatase. One problem with the transgenic models may be potential redundancy of the R-SMAD phosphatases. However, if RNAi-knockdown of the candidate phosphatase impacts R-SMAD phosphorylation in cells, knocking out the phosphatase in mice should yield the same outcome.
7. Ideally any candidate R-SMAD phosphatase should be independently verified.

Table 1
Overview of all the reported R-SMAD phosphatases. Using the seven parameters discussed in the text, we have queried all the published SMAD-phosphatases for their ability to act as R-SMAD phosphatases. Abbreviations used: TP-tail phosphorylated; LP-linker phosphorylated. This table is not a critical assessment of the data presented in each study but a mere reflection of the claims made. For most proposed R-SMAD-phosphatases, validation in transgenic mouse models and independent verification are still missing.

| Phosphatase | Target | Interacts with the R-SMAD? | Localises with the R-SMAD? | Dephosphorylates the R-SMAD in vitro? | Affects R-SMAD function upon depletion? | Rescue restores R-SMAD function? | Has been confirmed in mouse models? | Has been independently verified? | Reference(s): |
|-------------|--------|---------------------------|---------------------------|-------------------------------------|--------------------------------------|-------------------------------|---------------------------------|---------------------------------|----------------|
| PDP         | SMAD1-| Yes                       | Yes                       | Yes                                 | Yes                                  | No                            | No                              | No                              | [55]          |
|             | TP     |                           |                           |                                     |                                      |                               |                                 |                                 |                |
| SCPI/2      | SMAD1-| Yes                       | Yes                       | Yes                                 | Yes                                  | Yes                           | No                              | No                              | [44]          |
|             | TP     |                           |                           |                                     |                                      |                               |                                 |                                 |                |
| PP1M1A      | SMAD1/ | Yes                       | Yes                       | Yes                                 | Yes                                  | Yes                           | No                              | No                              | [45,57]       |
|             | 2/3-TP |                           |                           |                                     |                                      |                               |                                 |                                 |                |
| MTMR4       | SMAD2- | Yes                       | Yes                       | Yes                                 | Yes                                  | No                            | No                              | No                              | [54]          |
|             | TP     |                           |                           |                                     |                                      |                               |                                 |                                 |                |
| PP2A        | SMAD3- | No                        | Yes                       | Yes                                 | Yes                                  | Yes                           | No                              | No                              | [53]          |
|             | TP     |                           |                           |                                     |                                      |                               |                                 |                                 |                |
| SCPI/2      | SMAD1/ | Yes                       | Yes                       | Yes                                 | Yes                                  | No                            | No                              | Yes                             | [60,61]       |
|             | 2/3-LP |                           |                           |                                     |                                      |                               |                                 |                                 |                |
We have used these parameters to assess each of the proposed R-SMAD phosphatases to date (Table 1). Once established as a robust candidate R-SMAD phosphatase, it is still very important to elucidate the molecular mechanisms by which the candidate phosphatase recognises and acts on the R-SMAD. This is particularly important as most phosphatases are predicted to act on multiple substrates.

Phosphatases acting directly on R-SMADs are not the only phosphatases that are likely to modulate the levels of R-SMAD-phosphorylation or the outcome of TGFβ/BMP pathways. Clearly the phosphatases that target the TGFβ-receptor complexes have a direct impact on R-SMAD phosphorylation and TGFβ/BMP signalling (Figs. 2 and 3). The same would be expected of any phosphatase that regulates the activity of any upstream R-SMAD kinase. Furthermore dephosphorylation of any number of proteins such as SMURF1, NEDD4L, SMAD4, inhibitory SMADs, and TRIM33 that can modulate R-SMAD activity, stability or subcellular localisation could also impact on the outcome of TGFβ/BMP signalling. Phosphatases that impact on the transcription of R-SMADs or miRNAs that regulate R-SMADs indirectly could also impact R-SMAD activity. These factors have to be considered carefully when setting up genome-wide phosphatase overexpression or knockdown screens to identify novel R-SMAD phosphatases. Additionally some of the proposed R-SMAD phosphatases themselves may modulate the physiological responses to TGFβ ligands by targeting other components of the TGFβ/BMP pathway.[57,59].

The homeostasis of almost every cell in vertebrates is regulated in some way by TGFβ/BMP signalling. As key mediators of TGFβ/BMP signals, SMAD transcription factors are tightly regulated. A balanced cellular response to TGFβ/BMP signals depends partly on fine-tuning the activity of SMAD-transcription factors. SMAD-phosphatases are likely to play a key role in determining the extent and duration of TGFβ/BMP responses in cells. Understanding the regulation of SMAD-phosphatases in the TGFβ/BMP signalling pathways may provide important insights into whether such phosphatases are compromised in diseases that are associated with abnormal TGFβ/BMP signalling.

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