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Review

Regulation of BMP/Dpp signaling during embryonic development

T. Nakayama†, Y. Cui† and J. L. Christian*

Department of Cell and Developmental Biology, L215, School of Medicine, Oregon Health Sciences University, 3181 SW Sam Jackson Road, Portland (Oregon 97201-3098, USA), Fax + 1 503 494 4253, e-mail: christia@ohsu.edu

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Abstract. Bone morphogenetic protein-4 (BMP-4) and its *Drosophila* ortholog, decapentaplegic (Dpp), are multifunctional developmental regulators. Both gain-of-function and loss-of-function studies demonstrate that the biological activity and signaling range of these morphogens must be strictly regulated to ensure normal embryonic patterning. BMP-4 and Dpp are produced from inactive precursors that are proteolytically cleaved, following which the active ligand is secreted into the extracellular space. Binding of BMP-4 or Dpp to its cognate receptor leads to phosphorylation of intracellular signal-transducing Smad proteins that then form hetero-oligomers, translocate to the nucleus and modulate transcription of target genes. Recent studies have shown that the BMP signal transduction cascade can be modulated at every step of this process.

Key words. Bone morphogenetic protein (BMP); Smad, decapentaplegic (Dpp); embryo; signal transduction.

Introduction

Bone morphogenetic proteins (BMPs) were initially identified as components of bone extracts that can induce ectopic cartilage and bone formation [1]. These early studies identified four proteins, three of which belong to the transforming growth factor-β (TGF-β) superfamily of secreted signaling molecules and one of which (BMP-1) is a metalloprotease. During the past decade, over a dozen additional members of the BMP subclass of the TGF-β family have been identified and have been shown to regulate many fundamental biological processes such as cell proliferation, differentiation, apoptosis, migration and adhesion. In addition, these molecules are involved in the development of almost all tissues and organs [2].

* Corresponding author.
† Co-first authors.

BMP-4, and the closely related molecule BMP-2, are among the best-studied members of the BMP family. Targeted mutation of either gene in mice results in embryonic lethality, thereby demonstrating that BMP-2 and BMP-4 play nonredundant roles during embryogenesis [3, 4]. Mice mutant for *BMP-2* die during the 2nd week of development and show defects in the formation of extraembryonic tissues and the heart [3]. In contrast, most mouse embryos homozygous for a null mutation in the *BMP-4* gene form very little mesoderm and die in the early stages of gastrulation [4]. Analysis of *BMP-4* heterozygous mutants, and of the minority of *BMP-4* homozygous mutant mice that survive past gastrulation, has revealed that BMP-4 functions repeatedly throughout development and is required for development of the lens [5], germ cells [6], limbs [7] and many other tissues and organ systems. Studies in lower vertebrate organisms suggest that BMP-2 or -4 is also required for establishment of dorsoventral pattern in the mesoderm

and ectoderm. In *Xenopus*, for example, overexpression of BMP-2 or -4 causes prospective dorsal mesodermal (notochord and muscle) and ectodermal (brain) cells to adopt more ventral (e.g. blood and skin) fates [8–12]. Conversely, when endogenous BMP signaling is blocked in ventral cells, by introduction of dominant interfering forms of either the BMP ligand or receptor, a secondary dorsal axis consisting of muscle and neural tissue is formed (fig. 1) [13–18]. Thus, BMP-2/-4 appears to be required for ventral mesoderm formation and for repression of neural fate [19]. This interpretation is supported by analysis of zebrafish *swirl* mutants in that ventral cells take on a more dorsal fate as a result of mutations in the *zbmp-2* locus [20]. Furthermore, genetic analysis of flies mutant for *decapentaplegic* (*dpp*), the *Drosophila* ortholog of *BMP-2/-4* [21], demonstrates that the role of BMP/Dpp in establishment of the dorsoventral axis is conserved between vertebrate and invertebrate organisms [22].

Given that BMP-2/-4 and Dpp play many critical roles throughout embryogenesis, it is perhaps not surprising that multiple mechanisms exist to regulate their biological activities. This review will focus on recent studies of how BMP/Dpp activity is regulated. We will not discuss further the developmental roles of BMPs, or their properties as morphogens, for which several excellent reviews are available [2, 19, 22–26].

Signal transduction downstream of BMP/Dpp

Like other members of the TGF-β superfamily, BMP-2 and -4 bind to a receptor complex that is believed to be a tetramer composed of dimers of type I and type II transmembrane serine/threonine kinases (reviewed by [27, 28]). Following ligand binding, type II receptors transphosphorylate type I receptors which then propagate the signal. Biochemical studies have shown that BMP-2 and -4 can each bind and signal through multiple different type I and II receptors, some of which are also shared by more distantly related members of the TGF-β family. *Drosophila* Dpp can also bind to at least two type I receptors, Thick veins (Tkv) and Saxophone (Sax), in vitro, but recent studies suggest that Dpp signals primarily or solely through Tkv, whereas Sax serves as the receptor for the related ligands, Screw (Scw) and Glass bottom boat [29–31]. It is possible that BMP-2/-4 signaling is modulated, in part, by using different combinations of type I and type II receptors. It is not yet clear, however, which receptor combinations exist in vivo or whether different combinations trigger different downstream pathways.

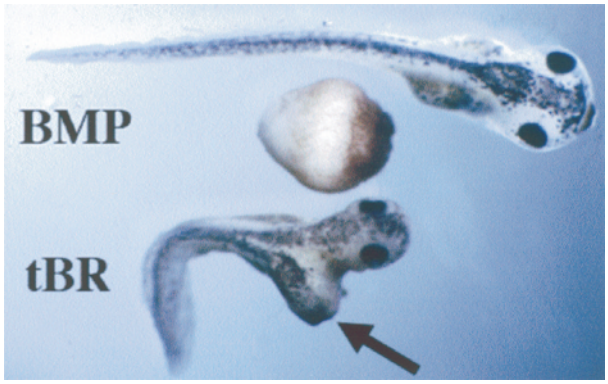

Figure 1. Upregulation of BMP activity in the dorsal side of *Xenopus* embryo leads to a loss of dorsal and anterior structures, whereas blockade of BMP activity in the ventral side, by injection of a dominant-negative BMP receptor (tBR), leads to duplication of dorsal structures such that a siamese twin embryo forms.

Following receptor activation, BMP signals are transduced intracellularly by a novel family of proteins, the Smads. The first *Smad* family member, *Drosophila Mothers against dpp* (*Mad*), was identified in a genetic screen for a modifier of Dpp activity (reviewed by [32–38]). Based on structural and functional features, Smads have been divided into three subclasses [32–38]: pathway-restricted or receptor-regulated Smads (R-Smads), common Smads (Co-Smads) and inhibitory Smads (Anti-Smads). R-Smads and Co-Smads contain two highly conserved regions, termed MH1 (for Mad homology 1) and MH2 domains that are separated by a less conserved linker region. In the case of R-Smads, multiple functions have been assigned for each of these domains as illustrated in figure 2 and discussed below. Sequence identity between Anti-Smads and other classes of Smads is limited and is restricted almost entirely to the MH2 domain.

The R-Smads include vertebrate Smad1, 2, 3, 5 and 8, and *Drosophila* Mad and dSmad2 (also known as Smox) [32–38]. These Smads are direct substrates for activated type I receptors and are phosphorylated at two serines located in a carboxy(C)-terminal Ser-Ser-X-Ser (SSXS) motif. Smad1, 5, 8 and Mad are phosphorylated and activated by BMP/Dpp receptors, whereas Smad2, 3 and dSmad2 transduce TGF-β/Activin-like signaling. The interaction of R-Smads and type I receptors is transient: phosphorylation induces a conformational change that leads to its dissociation from the type I receptor, relieves an inhibitory interaction between the MH1 and MH2 domains and allows it to form heteromers with the vertebrate Co-Smad, Smad4, or its *Drosophila* ortholog, Medea (Med).

The observation that at least three vertebrate R-Smads (Smad1, 5, 8) can be activated by a common combination of type I and type II receptors to mediate BMP-2/-4 signaling raises the question of whether these are

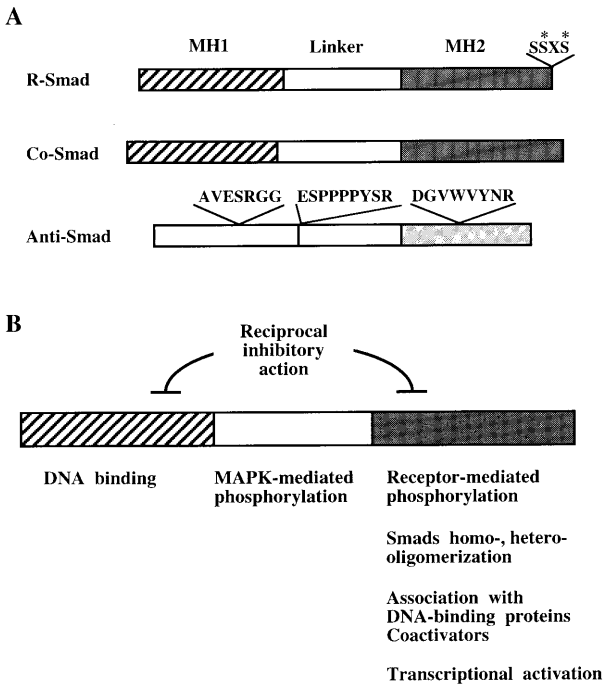

Figure 2. Schematic representation of Smad family members. (*A*) Three classes of Smad proteins. R-Smads contain an SSXS motif at the C-terminus, where the last two Ser residues (*) are phosphorylated by the type I receptor. Both R-Smads and Co-Smads have highly conserved MH1 domains (hatched) and MH2 domains (darkly shaded). Anti-Smads shows limited homology with R-Smads and Co-Smads, and this homology is restricted to the MH2 domain (lightly shaded). Anti-Smads share several short highly conserved amino acid motifs (shown above Anti-Smad). (*B*) Summary of functional properties found in each domain of R- and/or Co-Smad proteins.

functionally redundant, or whether BMP-2/-4 signaling might be varied by using specific combination of R-Smad and receptors. At present, it is not known whether specific combinations of receptor complexes can couple to distinct R-Smads in vivo. It is unlikely that Smad1, 5 and 8 are completely redundant since mice homozygous for a null mutation in the *Smad5* gene die at mid-gestation [39, 40], thereby demonstrating that Smad1 and/or 8 cannot fully substitute for Smad5 activity. Overexpression of Smad1, but not Smad5, in zebrafish leads to a ventralized phenotype [41] and can rescue the zebrafish *zbmp-2* mutant, *swirl* [42], providing further evidence that these two proteins are functionally distinct. Conversely, a zebrafish *Smad5* mutation (*somitabun*, *sbn*) has been identified which encodes an antimorphic form of Smad5 and leads to a strongly dorsalized phenotype [43]. *Sbn* mutant Smad5 protein can dominantly inhibit the function of Smad1 as well as Smad5, however, and thus it is not clear whether the dorsalized phenotype reflects a unique requirement for Smad5 in dorsoventral patterning.

Analyses of the expression patterns of mammalian Smad1, 5 and 8 have revealed that each of these genes is broadly expressed and transcripts can be detected in most, if not all, tissues [39, 40, 44–48]. Some differences in embryonic expression patterns have been detected, however. For example, murine Smad1 transcripts are enriched within the developing nervous system and at sites of mesenchymal-epithelial interactions during organogenesis [47], whereas Smad5 is uniformly expressed in all three embryonic germ layers early in development [40, 44] and is somewhat enriched in somites and mesenchyme at later stages [39]. Zebrafish *Smad1* is first expressed shortly before the onset of gastrulation, and this expression requires an intact BMP signaling pathway [42]. Smad1 transcripts are initially ubiquitously expressed but become restricted to the ventral side by the midgastrula stage and are later enriched in the eyes, somites, hemangiogenic region and in selected regions of the central nervous system [41–43]. By contrast, *Smad5* is expressed maternally and zygotically, independent of BMP signaling, and transcripts are ubiquitously distributed at least until the gastrula stage [41–43]. During later stages of embryogenesis, Smad5 transcripts are enriched in the forming gut and in the dorsal neural tube [41].

Only a single Co-Smad (Smad4/Med) has been identified in mammals and *Drosophila*. Smad4/Med lacks the C-terminal SSXS motif and is not a substrate for type I receptors, but functions as an essential co-factor for all R-Smads. In *Xenopus*, a second Smad4-like protein (Smad4β/Smad10) has been identified that shares $\sim$90% identity with Smad4 in its MH1 and MH2 domains but less than 40% identity in the linker region [49–51]. Like Smad4, Smad4β/Smad10 can associate and functionally cooperate with both Smad1 and Smad2 [50, 51]. Unlike Smad4, however, Smad4β/Smad10 is constitutively phosphorylated and shows a predominantly nuclear localization in both stimulated and unstimulated cells [50]. Also unlike Smad4, Smad4β/Smad10 has potent ventralizing activity when overexpressed on the dorsal side of *Xenopus* embryos, suggesting that it may be sufficient to transduce BMP-like signals [50]. Inconsistent with this finding, studies in another lab have shown that Smad4β/Smad10 can dorsalize ventral mesoderm and neuralize ectoderm when misexpressed in *Xenopus* embryos [49]. Further studies will be required to reconcile these discrepancies and to determine whether Smad4β/Smad10 functions as a Co-Smad or as an independent signaling agent.

Once the R-Smad/Co-Smad complex has formed, it translocates into the nucleus and functions to regulate transcription of target genes [32–38]. Co-Smads and some R-Smads have been shown to bind directly in a

sequence-specific manner to the promoter regions of target genes through their MH1 domains. In addition, the MH2 domains of some R- and Co-Smads can function as direct transcriptional activators. Other R-Smads, however, function as part of a transcription factor complex in association with unrelated DNA-binding factors. Depending upon which co-factor is recruited, the Smad-containing transcription factor complex can either activate [32–38] or repress [52–55] expression of target genes. Although most studies of Smad function have focused on the role of Smad2/Smad4 or Smad3/Smad4 complexes in regulating expression of TGF-β/Activin-response genes [32–38, 52–55], recent analysis of Mad/Med-mediated regulation of Dpp-target genes suggests that BMP-target genes are regulated in a similar manner [56–58]. For example, it has been shown that Mad directly binds Dpp-responsive elements of the *vestigial* and *Ultrabithorax* genes, and that Mad and Med both bind the *tinman* gene [56–58]. More recently, a BMP-responsive element has been identified in the promoter of the *Xvent-2* gene that is activated by direct binding of the zinc finger protein OAZ in association with BMP-activated Smad1 [59].

The third class of Smads, Anti-Smads, includes vertebrate Smad6 and Smad7 and *Drosophila* daughters against dpp (Dad) (reviewed in refs 32-38). These Smads, which inhibit rather than transduce TGF-β family signals, will be discussed in more detail in a later section.

In summary, upon BMP/Dpp binding to its receptor complex, a specific R-Smad is activated, forms a complex with a Co-Smad and translocates to the nucleus to participate as a component of a transcription factor complex that activates or represses target gene expression (fig. 3). In the following sections, we will focus on the multiple mechanisms that exist to fine-tune this signaling cascade.

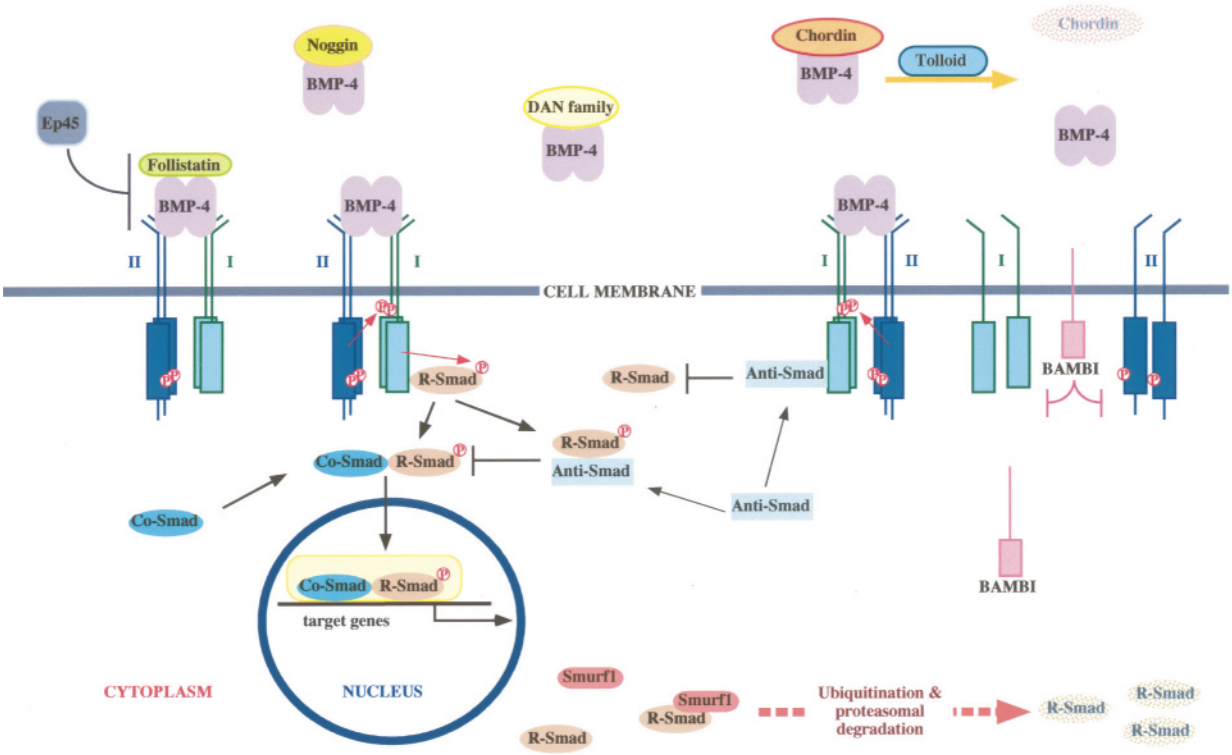

Figure 3. Partial summary of modulation of BMP-4 signaling. Upon BMP-4 binding to its receptor complex, the type II receptor phosphorylates the type I receptor which then phosphorylates an appropriate R-Smad, enabling it to complex with Co-Smad. This complex translocates to the nucleus to form a transcription factor complex (yellow box) that activates (or represses) the expression of target genes. Several BMP antagonists, such as Anti-Smads and BAMBI, are expressed in response to BMP signaling and form a negative feedback loop. In the extracellular region, BMP-4 activity is regulated by many binding proteins (Noggin, Chordin, Tolloid, Follistatin, Ep45 and DAN family members). At the membrane, the pseudoreceptor BAMBI can block formation of a functional receptor complex. Inside the cell, Anti-Smads can block signal transduction by R-Smads, and Smurf1 leads to rapid degradation of R-Smads (see text for detail).

Table 1. Comparison of Selected BMP precursor cleavage sites.

BMPs	Second cleavage site↓	Linker	First cleavage site↓	
BMP-2	-H-V -R-I-S-R	$-X_{30}$	-R-E-K-R	-mature ligand
BMP-4	-H-V -R-I-S-R	$-X_{31 \text{ or } 32}$	-R-X-K-R	-mature ligand
Dpp	-H-V -R-L-R-R	$-X_{27}$	-R-S-I-R	-mature ligand
BMP-7	-H-L None	None	-R-S-X-R	-mature ligand

Sequences of BMPs are represented as consensus of human, chick and *Xenopus* (X, Ala or Ser in BMP-4, Val or Ile in BMP-7). Second cleavage sites are not confirmed by protein sequencing.

Regulation of BMP/Dpp activity upstream of receptor activation

Regulation of intracellular processing

Like other members of the TGF-β superfamily, BMP-4 is initially synthesized as an inactive precursor. ProBMP-4 forms homodimers or heterodimers (with other members of the BMP family) within the secretory pathway. It is then cleaved by members of the pro-protein convertase family of serine endoproteases [60, 61] to release the biologically active C-terminal dimer from the inactive amino (N)-terminal proregion. Recent studies have shown that BMP-4/BMP-7 heterodimers have more potent activities than do BMP-4 homodimers [62–64]. It is not known whether BMP-4/BMP-7 heterodimers normally form in vivo, but the possibility exists that BMP-4 activity is spatially or temporally regulated at the level of ligand dimerization.

ProBMP-4 is cleaved at two sites: initially at a site adjacent to the mature ligand domain and then at an upstream site within the inactive prodomain (ref 60 and Y. Cui, F. Jean, G. Thomas and J. Christian, unpublished data). Interestingly, the existence of two cleavage sites is a common feature of proDpp and proBMP-2/-4 from all species whereas other BMP family members, such as BMP-7, have only a single cleavage site (Table 1). The conservation of these cleavage sites raises the possibility that they function in some way to regulate the bioactivity of mature BMP-2, -4, or Dpp.

Ordered proteolytic cleavage of proBMP-4 may regulate the signaling range of mature BMP-4 in a tissue-specific fashion (Y. Cui, F. Jean, G. Thomas and J. Christian, unpublished data). In *Xenopus* embryonic ectoderm, where BMP-4 can signal only to adjacent cells [65], mutations of the second cleavage site which disrupt this ordered cleavage greatly increase the signaling range of the cleaved ligand. By contrast, in *Xenopus* embryonic mesoderm, mature BMP-4 signals over a distance of many cells [66] regardless of whether it is generated from native or cleavage mutant forms of the precursor. BMP-4 and Dpp are known to signal at either short or long range in a tissue specific fashion [65–67] and differential use of cleavage sites within the prodomain could provide a mechanism for tissue-specific regulation of the range of BMP-4/Dpp action. Alternatively, or in addition, ordered cleavage may enable the mature ligand to bind to tissue-restricted factors that impede its diffusion or compromise its stability. Consistent with the latter possibility, the prodomain of Nodal, a BMP family member, can regulate the stability of the mature protein [61].

Regulation of BMP-4 by extracellular binding proteins

Recent studies have shown that BMP-4 activity is regulated in the extracellular space by a number of proteins that bind BMPs with different affinities and binding specificities. Several examples of BMP-binding regulatory proteins are described below and illustrated in figure 3.

Noggin. Noggin was originally identified using an expression cloning strategy and was shown to be a dorsalizing factor that can induce a secondary axis in *Xenopus* embryos [68]. Subsequently, it was shown to bind BMP-2 and -4 with high affinity, and BMP-7 weakly, and thereby prevent interaction of these proteins with their cell-surface receptors [69]. In *Xenopus* embryos, *noggin* is expressed in cells of the Spemann organizer, which are characterized by their ability to emit signals that dorsalize mesoderm and neuralize ectoderm. Noggin is believed to participate in Spemann organizer function by inhibiting the ventralizing effects of BMP-4. In zebrafish, three *noggin* homologs have been identified, *noggin1*, *noggin2* and *noggin3*. Of these, *noggin1* is expressed in the fish organizer and is able to dorsalize embryos when ectopically expressed, supporting a role for Noggin in dorsoventral patterning [70]. It is not clear, however, whether this role is evolutionarily conserved, since a *Drosophila* homolog of Noggin has not yet been identified. Furthermore, although Noggin homologs have been characterized in chicken [71] and mouse [72], evidence that these function in establishment of the dorsoventral axis is lacking. Specifically, dorsoventral pattern is not perturbed in *noggin* null mutant mice [72] and ectopically expressed Noggin does

not affect embryonic axis formation in chick embryos [71]. Mice mutant for *noggin* do show a variety of other defects, including abnormalities in neural tube and somite development [72], skeletal defects [73] and retardation of hair-follicle induction [74], all of which may be attributable to misregulation of BMP.

Chordin/Short gastrulation (Sog). *Chordin* was identified in a screen for genes that are differentially expressed on the dorsal side of *Xenopus* embryos and was characterized as a dorsalizing factor [75]. It was subsequently shown to be the ortholog of *Drosophila Short gastrulation (Sog)* [76] and zebrafish *Chordino* [77]. Like Noggin, Chordin binds directly and with high affinity to BMP-2, BMP-4 and BMP-4/BMP-7 heterodimers and blocks BMP function by preventing the ligand from interacting with its receptor [78]. Consistent with the proposal that Chordin dorsalizes embryos via antagonism of BMPs, *Drosophila* Sog acts to antagonize Dpp [76] and/or the closely related BMP family member, Scw [30, 31] and zebrafish *chordino* null mutants display a ventralized phenotype [77].

Interactions between BMP and Chordin are modulated by selected members of a family of astacin-like metalloproteases that includes vertebrate BMP-1 and *Drosophila* Tolloid (Tld). BMP-1 was purified as an osteogenic component of bone extracts [1]. Subsequently, *Drosophila tld* was shown to encode a BMP-1-related molecule and to be a positive regulator of Dpp activity [79, 80]. Since this time, a number of Tld/BMP-1-related genes have been identified [81–86]. A subset of these have been shown to be capable of cleaving vertebrate Chordin/Chordino or *Drosophila* Sog thereby liberating active BMP/Dpp from the inactive complex [84–87]. Thus, these Tld/BMP-1-related gene products may regulate the amount of bioactive BMP/Dpp that is available for receptor binding by regulating the levels of the inhibitory binding protein, Chordin/Sog.

Chordin/Sog may function to attenuate BMP/Dpp signaling in some contexts but enhance it in others. Recent data in *Drosophila* indicate that whereas Dpp is inhibited by high levels of Sog that are present nearby Sog-producing cells, Dpp signaling is enhanced by low levels of Sog at distal sites and this process requires Tld [88]. These results suggest the possibility that Chordin/Sog might function to facilitate diffusion of BMP/Dpp, allowing the inactive complex to accumulate and then be activated by Tld-mediated cleavage at sites distant from the Chordin/Sog source. Alternative explanations for the ability of Sog to enhance Dpp signaling, such as the generation of bioactive Chordin/Sog cleavage products that enhance binding of BMP/Dpp to its receptor, are also possible.

Follistatin. Follistatin, which was originally characterized as a protein that binds and antagonizes the activity of Activin [89], has recently been shown to bind to BMPs as well [90, 91]. Unlike Chordin and Noggin, Follistatin does not block BMP from interacting with its receptor but instead forms a trimeric complex with BMP and its receptor and inhibits receptor activation [91]. *Follistatin* is expressed in Spemann organizer cells in *Xenopus* embryos and may function, together with Chordin and Noggin, to specify dorsal fate by inhibiting the ventralizing activities of BMPs. If Follistatin does function in this capacity, this role is not evolutionarily conserved since *follistatin* is not expressed in organizer-equivalent regions of zebrafish or mouse embryos [92–94] and axial patterning is not perturbed in *follistatin*-null mice [95]. Follistatin may function as a BMP antagonist in other contexts later in embryogenesis. For example, the regular arrangement of feather buds in chick embryos is established by a process of lateral inhibition in which one developing bud secretes BMPs and thereby prevents the formation of another bud in the immediate vicinity. *Follistatin* is specifically expressed in the feather placode and may enable the feather bud itself to escape this inhibition [96].

DAN family. The first member of the DAN family, DAN (initially called N03), was identified as a candidate tumor suppressor whose expression is downregulated in v-*src*-transformed rat fibroblast cells [97]. DAN was initially proposed to be a DNA-binding protein based on the presence of a cysteine knot domain similar to that found in zinc-finger containing transcription factors [97]. A novel gene containing this same domain, *drm*, was subsequently identified in a screen for genes that were downregulated in v-*mos*-transformed rat cells [98]. It has since been shown that a large family of proteins containing this cysteine knot motif exist [99–104] and all family members that have been tested are secreted proteins that bind to, and inhibit, one or more members of the TGF-β family [100, 101, 103–106]. In *Xenopus*, for example, two members of this family have been identified: Cerberus, which can bind and inhibit BMPs, Nodal and possibly Activin [100, 106], and Gremlin (the *Xenopus* ortholog of DRM), which appears to be a BMP-specific antagonist [100]. *Xenopus* Cerberus binds and inhibit Wnts in addition to TGF-β family members, and can function as a head inducer [106]. Characterization of the expression pattern and activity of the murine Cerberus homologue, Cer1 (also known as Cer-l, mCer-1 or Cerr1), suggested that this function may be conserved [101, 107–109]. Recent genetic data has shown, however, that *cer1* is not required for anterior patterning or even normal mouse morphogenesis [110]. It is possible that there is redundancy of *cer1* function, or that *cer1* is not the murine ortholog of *Xenopus cerberus*. Functional analysis of the BMP antagonist Gremlin/DRM has shown it to be involved in limb patterning and outgrowth [111–113]. A chicken DAN family member, *caronte* is specifically expressed

on the left side of the node and functions in the establishment of left-right asymmetry by antagonizing BMPs to induce asymmetric expression of *nodal* [102–104]. A murine DAN family member, *dante*, is also expressed asymmetrically around the node during gastrulation, and might also contribute to patterning the left-right axis [101]. To date, DAN homologs have not been identified in *Drosophila*, although a *Caenorhabditis elegans* homolog exists [101], suggesting that this family is of ancient origin.

Ep45. The serine protease inhibitor Ep45 was recently identified as a BMP-4 binding protein [114]. Since Ep45 binds BMP-4 with higher affinity than Follistatin, but with lower affinity than Chordin and Noggin, it may function as a specific inhibitor of BMP-Follistatin interactions. If so, Follistatin-mediated inhibition of BMP-4 is most likely not required for Spemann organizer function since overexpression of Ep45 in *Xenopus* embryos has no effect on dorsoventral patterning.

Instead of, or in addition to, inhibiting BMP-Follistatin interactions, Ep45 may function to protect BMP-4 from serine protease-mediated cleavage [114]. Several serine proteases are capable of cleaving off the N-terminus of BMP-4. This cleavage removes potential heparin binding sites yet maintains an active, and potentially more diffusible, ligand. If this type of cleavage occurs in vivo, Ep45 may regulate when and where it takes place and thus modulate interaction of BMP-4 with the extracellular matrix.

Regulation of Dpp by proteoglycans

Proteoglycans modulate the function of intracellular signaling molecules in many ways (reviewed in ref [115]) and genetic studies have shown that the *Drosophila* glypican homologue, *division abnormally delayed* (*dally*), is required for signaling by both Dpp and the unrelated patterning molecule, Wingless (Wg) [116–118]. Similar to its vertebrate relatives, Dally is a glycophosphatidyinositol (GPI)-linked proteoglycan that specifically bears heparan sulfate side chains [118]. The mechanisms by which Dally regulates Dpp and/or Wg signaling are not understood, but Dally has been suggested to limit diffusion of Wg, thereby increasing the local concentration available to interact with its receptor, to generate a higher affinity site for Wg binding to its receptor, and/or to function as an obligate co-receptor. Whether these same mechanisms apply to regulation of Dpp signaling is unknown. Analysis of Dally function is further complicated by the finding that it does not serve the same role in all tissues [116–118]. For example, Dally is required for Wg, but not Dpp signaling in the embryonic epidermis and the opposite is true in the genitalia [118]. Furthermore, Dally can either enhance or inhibit cellular responses to Dpp, depending on the tissue being examined [116]. At this point, no equivalent regulation of BMP signaling has found in vertebrates.

Regulation of BMP/Dpp activity at the receptor level

Regulation by pseudoreceptors

An expression cloning approach was used to identify a novel transmembrane protein, BAMBI [119]. BAMBI shares sequence similarity with TGF-β-family type I receptors but lacks an intracellular kinase domain and stably associates with TGF-β-family receptors to prevent the formation of functional receptor complexes (fig. 3). Thus, BAMBI functions as a naturally occurring dominant mutant to block signal transduction downstream of TGF-β, Activin and BMPs. In *Xenopus* embryos, expression of *BAMBI* is induced by BMP-4, suggesting that it functions as part of a negative feedback loop to downregulate endogenous BMP signals. Interestingly, the human ortholog of *BAMBI*, *nma*, is downregulated in metastatic melanoma cell lines raising the possibility that it functions as a tumor suppressor by inhibiting TGF-β, Activin or BMP signals.

Regulation of Dpp activity by its receptor

Recent studies have shown that the signaling range and activity of Dpp are regulated by the level of expression of the Dpp receptor. In the developing wing disc, for example, Dpp negatively regulates the expression of its own type I receptor, *tkv* [120]. An additional level of regulation is conferred by Hedgehog, which further represses expression of *tkv* in cells that synthesize Dpp [121]. Thus, Tkv levels are lowest in Dpp-expressing cells and highest in cells furthest from the source of Dpp [29, 120, 121]. Proper regulation of Tkv levels is essential for Dpp-dependent patterning of the wing [121]. Low levels of Tkv attenuate responsiveness of cells to Dpp and facilitate the spread of Dpp over long distances [29, 120, 121]. By contrast, high levels of Tkv sensitize cells to low levels of Dpp [120], limit the spread of Dpp and most likely generate a border beyond which Dpp cannot move [29, 120]. As a result, a Dpp activity gradient is established that is highest adjacent to Dpp-expressing cells but is low at a distance and is very low within Dpp expressing cells themselves [121].

Regulation of BMP/Dpp activity downstream of receptor activation

Signal inhibition by competition between R-Smads

Smad4/Med appears to be an obligate component of the signal transduction machinery downstream of all TGF-β family members and limited availability of

Smad4/Med within cells may lead to competition between different signaling pathways. For example, in overexpression experiments using *Xenopus* embryos, Smad1 and Smad2 compete for binding to a limited pool of Smad4 [122]. This competition leads to transduction of either BMP (Smad1) or TGF-β/Activin (Smad2) signals, but not both. Thus, the final output downstream of activation of a given TGF-β family receptor may depend not only on the absolute level of activation of the corresponding R-Smad but also on whether other distinct R-Smads have been activated in the same cell. Whether this same type of competition between endogenous R-Smads occurs in vivo is not known.

Signal inhibition by Anti-Smads

Three Anti-Smads, vertebrate Smad6, Smad7 and *Drosophila* Dad, have been identified using a variety of functional or homology-based screens [36]. Anti-Smads lack the C-terminal SSXS phosphorylation motif that is present in R-Smads and share several unique conserved motifs of unknown function (fig. 2). In addition, all Anti-Smads antagonize signal transduction downstream of BMP/Dpp, and possibly other TGF-β family ligands [36]. Recent loss-of-function studies have demonstrated the physiological importance of this regulatory pathway in that targeted mutation of the *Smad6* gene led to developmental abnormalities of the cardiovascular system [123].

Anti-Smads have been shown to bind stably to the intracellular domain of activated BMP/Dpp type I receptors, thereby preventing receptor-mediated phosphorylation of R-Smads, complex formation with Co-Smads and nuclear translocation [124–126] (fig. 3). A distinct mechanism of action for Smad6 has been proposed in which Smad6 forms hetero-oligomers with Smad1 in response to BMP signaling [127]. The Smad6-Smad1 complex is inactive and precludes formation of active Smad1-Smad4 complexes (fig. 3).

In addition to inhibiting signal transduction downstream of BMPs, some Anti-Smads target other TGF-β family pathways. Smad6 and Smad7 have both been shown to bind TGF-β/Activin type I receptors, thereby inhibiting phophorylation of Smad2 and/or 3 [124, 128, 129]. Furthermore, whereas overexpression of Smad7 in *Xenopus* embryos phenocopies the effect of specifically blocking the BMP signaling pathway, it also causes distinct patterning defects, including spina bifida and inhibition of gastrulation, which cannot be attributed to loss of endogenous Activin or BMP signaling [130]. Thus, Smad7, and possibly other Anti-Smads, may target discrete, as of yet unidentified, signaling pathways.

Studies in *Drosophila* and in *Xenopus* suggest that Anti-Smads may function in autoregulatory negative feedback loops (fig. 3). Expression of *Smad7*/*dad* closely mirrors that of *BMP-2*, *-4*/*dpp* throughout development and BMP/Dpp function is both necessary and sufficient for expression of these genes in vivo [130, 131]. *Xenopus Smad6* is also expressed in a spatially restricted pattern that shows extensive overlap with that of *BMPs* at the tailbud stage [132], and recent studies have shown that expression of *Smad6* in the developing chick heart is dependent on BMP signaling [133]. Together these results suggest that Smad6, Smad7 and Dad participate in a conserved negative feedback loop in which BMP/Dpp signaling induces the expression of its own antagonist, which then functions to negatively modulate the amplitude or duration of signaling. Support for this type of autoregulatory negative feedback loop is also provided by the observation that expression of mammalian *Smad6* and *Smad7* is rapidly and directly induced by TGF-β1, Activin and/or BMP-7 in a variety of cultured cell lines [128, 134]. Furthermore, transcription of *Smad7* is regulated by TGF-β/Activin through direct binding of Smad3 and Smad 4 to the *Smad7* promoter [135]. Induction of expression of Anti-Smads is not always dependent of TGF-β family signaling, however, since expression of *Smad7* is also upregulated in response to interferon-γ signaling through the JAK/STAT pathway [136].

Smad8-specific inhibition by Smad8B

A splice variant of Smad8 (Smad8B) has been identified that can act as a dominant inhibitor of BMP signaling [48]. Smad8B encodes a C-terminally truncated form of the protein and thus lacks the SSXS motif that is the target for receptor-mediated phosphorylation. Biochemical studies have shown that Smad8B binds to Smad4 and Smad8, but not to Smad1, 2, 3 or 5, and that it specifically antagonizes BMP signaling mediated by Smad8 but not Smad1. These results suggest that Smad8B is a naturally occurring dominant mutant protein that specifically inhibits Smad8, possibly by preventing association with Smad4 as has been proposed for inhibition of Smad1 by Smad6 [127]. The relative levels of Smad8 and Smad8B transcripts varies from tissue to tissue, raising the possibility that Smad8B functions as a tissue- or cell type-specific inhibitor of Smad8.

R-Smad degradation triggered by Smurf1

Smurf1, a new member of the Hect class of E3 ubiquitin ligases, was identified in a yeast two-hybrid screen for Smad1-interacting proteins [137]. Data from in vitro and in vivo studies has shown that Smurf1 specifically

targets Smad1 and Smad5 for ubiquitination and proteasomal degradation (fig. 3). This occurs independent of BMP receptor activation. Thus, Smurf1 does not function downstream of activated Smads to turn off BMP signals, but rather controls cellular competence to respond to BMPs. Overexpression of Smurf1 in *Xenopus* embryos inhibits Smad1/Smad5-mediated transduction of BMP signals and simultaneously enhances cellular responsiveness to the Smad2 (TGF-β/Activin) pathway. The latter effect is most likely due to enhanced degradation of endogenous Smad1 or Smad5 which frees up more Smad4 to form complexes with Smad2.

Regulation of Dpp-target genes by repression of a transcriptional repressor

Brinker (Brk) is an intracellular antagonist of Dpp signaling that may function as a direct transcriptional repressor of Dpp-target genes [138–140]. Expression of *brk* is negatively regulated by Dpp such that complementary gradients of Dpp signaling activity and Brk inhibitory activity are established. Curiously, in the absence of *brk* Dpp-target genes are expressed independent of Dpp signaling. Specifically, Dpp-target genes are activated in clones of cells that are mutant for *brk* even if these cells are also mutant for *dpp*, *tkv* or *Mad*. These results suggest that Dpp signal transduction through Mad and Med does not play a direct role in transcriptional activation of Dpp-target genes but instead functions solely to repress expression of *brk*. This probably is not strictly true, however, since repression of *brk* alone is not always sufficient for maximal activation of Dpp-target genes and since Mad/Med binding sites have been found in the promoter regions of some Dpp-target genes (e.g. [56–58]). A vertebrate homolog of Brk has not yet been identified, but Brk can antagonize BMP-4 function in *Xenopus* embryos suggesting that its function is evolutionarily conserved.

The mechanism by which Brk represses expression of Dpp-target genes is unknown. Epistasis experiments indicate that although *brk* is a Dpp-target gene, Brk does not regulate its own expression even though it negatively regulates other Dpp-target genes. This suggests that *brk* functions in parallel to or downstream of *Mad* to regulate Dpp-target genes.

Crosstalk between BMP and other signal transduction pathways

Intracellular branchpoints, as well as signals initiated by unrelated ligand/receptor combinations, can impinge upon the BMP signaling cascade to modulate its final output. A few examples of this type of signaling crosstalk are described below.

Calmodulin acts as a Smad inhibitor. Calmodulin (CaM), which is the primary intracellular calcium receptor, associates in vitro with Smad1-4 in a calcium-dependent manner [141]. In cultured cells, inhibition of CaM activity enhances Activin-inducible reporter gene expression, whereas overexpression of CaM suppresses expression of the reporter [141]. Similarly, in *Xenopus* embryos, overexpression of CaM suppresses the ventralizing activity (in mesoderm) and anti-neurogenic effect (in ectoderm) of Smad1 [142]. Thus, CaM potentially functions as a general inhibitor of Smad-mediated signal transduction.

TAK1 and BMP signaling. The mitogen-activated protein kinase kinase kinase, TAK1, and its upstream activator, TAB1, have been suggested to function as transducers of BMP signals [143]. Ectopic expression of TAK1 in *Xenopus* embryos induces apoptotic cell death, most likely through activation of the JNK or p38 MAP kinase cascade. When TAK1-mediated cell death is inhibited, however, by coexpression of Bcl-2 or human X-chromosome-linked inhibitor of apoptosis protein (XIAP), embryos instead develop with a ventralized phenotype similar to that observed following overexpression of BMP-4 [143, 144]. Moreover, a kinase-deficient dominant-negative form of TAK1 can partially block phenotypes generated by overexpression of BMP-4, a constitutively active BMP receptor, Smad1 or Smad5. These results suggest that TAB1 and TAK1 may transduce BMP signals either downstream of or in cooperation with R-Smads. Although the mechanism by which the TAK1-mediated MAP kinase pathway transduces BMP signals is unknown, it is possible that it leads to phosphorylation of transcription factors that interact synergistically with Smad1 and/or Smad5.

Interestingly, XIAP has been shown to physically associate with TAB1 and with BMP receptors in mammalian cells. Furthermore, a truncated form of XIAP lacking the TAB1-interaction domain partially blocks phenotypes caused by overexpression of a constitutively active BMP receptor in *Xenopus* embryos. These results suggest that XIAP may be a positive regulator linking BMP receptors and TAB1-TAK1. Activation of BMP receptors by ligand binding may elicit a signal via interaction with XIAP, which in turn might inhibit apoptosis and activate TAK1 by interacting with caspases and TAB1, respectively. Intriguingly, two *Drosophila* IAP homologs have been shown to bind to the Dpp receptor Tkv [145], suggesting that this is an evolutionarily conserved pathway. There is a delicate balance between proteins that promote and inhibit cell death, and IAPs may provide a mechanism that allows BMPs to induce apoptosis in some developmental contexts but not others.

Inhibition of BMP signaling by receptor tyrosine kinases. Whereas TAK1-mediated activation of the JNK

or p38 MAP kinase pathway transduces BMP signals, receptor tyrosine kinase-mediated activation of the Erk MAP kinase pathway inhibits BMP signal transduction [146]. Activation of receptor tyrosine kinases by ligands such as EGF or HGF leads to phosphorylation of Smad1 on serine residues within the linker region. This phosphorylation is directly catalyzed by the Erk family of MAP kinases and is independent of BMP receptor-mediated phosphorylation. Erk-mediated phosphorylation does not inhibit Smad1-Smad4 association, but does inhibit nuclear accumulation, and thus prevents Smad1-Smad4 complexes from activating gene transcription. These findings may provide a mechanistic explanation for the observation that BMPs and agents that activate the Erk MAP kinase pathway show opposing activities in many developmental contexts (e.g. refs [147], [148]).

STAT3-Smad1 complex. Leukemia inhibitory factor (LIF) and BMPs signal through different transcription factors, namely STATs (signal transducers and activators of transcription) and Smads, respectively, but can act synergistically to induce astrocyte differentiation [149]. Recent studies have shown that this synergism is due to the ability of Smad1 and STAT3 to form a complex that is bridged by the coactivator p300 on the promoter of at least one target gene [149]. Since Smads often function in concert with sequence specific DNA binding proteins to activate transcription, it is likely that many more examples of this type of synergism between BMPs and other signaling pathways will be forthcoming.

Conclusions

In this review, we have briefly summarized the many levels at which BMP signaling is regulated. It is interesting to note that several of the regulatory mechanisms used to modulate BMP/Dpp signaling involve negative-feedback loops. Dpp, for example, negatively regulates the level of its own receptor, BMP regulates the level of its antagonist, *BAMBI*, and BMP/Dpp both regulate expression of their respective Anti-Smads. Similar negative feedback loops exist to regulate signaling downstream of Wnts, Hedgehog and other families of signaling molecules that, like BMPs, are used repeatedly throughout embryogenesis to pattern different tissues and cell types [150].

Given the large and diverse range of mechanisms that exist to regulate BMP activity, it is perhaps surprising that these signals are ever successfully transduced to the nucleus. It is probable, however, that these different mechanisms are not simultaneously operative in all cells at all times throughout development. Also, because much of what we have learned about regulation of BMP signaling is inferred from studies involving overexpression of different regulatory components in cultured cells or embryos, the specificity of a given antagonist for different BMP ligands, receptors or Smads remains unknown. Hopefully, this will be sorted out in the near future through specific loss-of-function studies in mouse and *Drosophila* embryos.

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