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REVIEW

Intracellular BMP Signaling Regulation in Vertebrates: Pathway or Network?

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Bone morphogenetic proteins (BMPs), members of the TGF-β superfamily of secreted signaling molecules, have important functions in many biological contexts. They bind to specific serine/threonine kinase receptors, which transduce the signal to the nucleus through Smad proteins. The question of how BMPs can have such diverse effects while using the same canonical Smad pathway has recently come closer to an answer at the molecular level. Nuclear cofactors have been identified that cooperate with the Smads in regulating specific target genes depending on the cellular context. In addition, the pivotal role BMP signaling plays is underscored by the identification of factors that regulate members of this pathway at the cell surface, in the cytoplasm, and in the nucleus. Many of these factors are BMP-inducible and inhibit the BMP pathway, thus establishing negative feedback loops. Members of the BMP–Smad pathway can also physically interact with components of other signaling pathways to establish crosstalk. Finally, there is accumulating evidence that an alternative pathway involving MAP kinases can transduce BMP signals. The evidence and implications of these findings are discussed with an emphasis on early embryonic development of *Xenopus* and vertebrates.

Key Words: **TGF-β; Smad; signal transduction; MAP kinase; Ca^{2+}/calmodulin; JAK–STAT; Wnt; Xenopus.**

INTRODUCTION

Bone morphogenetic proteins (BMPs) were originally identified as molecules that can induce ectopic bone and cartilage formation in rodents (Wozney *et al.*, 1988; reviewed in Hogan, 1996). With the exception of BMP1, a metalloprotease, they are all members of the transforming growth factor-β (TGF-β) superfamily of secreted signaling molecules. BMPs are conserved broadly across the animal kingdom, including vertebrates, arthropods, and nematodes. In *Drosophila*, the BMP ligands Decapentaplegic (Dpp), screw, and 60A (also known as gbb) have been shown to participate in developmental events as diverse as oogenesis, the development of the imaginal discs, and the regulation of dorsal–ventral patterning in the early embryo (Raftery and Sutherland, 1999). In vertebrates, BMPs also play roles in dorsal–ventral patterning of the early embryonic mesoderm and specification of epidermis. In *Xenopus*, for example, BMP2, -4, and -7 ventralize early mesoderm and act as negative regulators of neuralization (Harland, 1994; Hemmati-Brivanlou and Melton, 1997). In addition, vertebrate BMPs play roles in limb development, generation of primordial germ cells, tooth development, and the regulation of apoptosis, to name a few (Hogan, 1996). How can BMPs elicit such wide biological responses in different biological contexts? This diversity appears to be partly due to intracellular cofactors that participate in BMP signal transduction, as well as crosstalk between BMPs and other signaling pathways. In this review, we will focus on how the intracellular BMP signal transduction pathway is regulated to better understand the nature of complex biological responses. Particular emphasis will be placed on the roles of recently identified intracellular cofactors involved in BMP signaling regulation and crosstalk between the BMP and other signaling pathways.

THE TGF-β SIGNALING PATHWAY

The TGF-β superfamily is a large group of secreted polypeptide growth factors, roughly grouped into three

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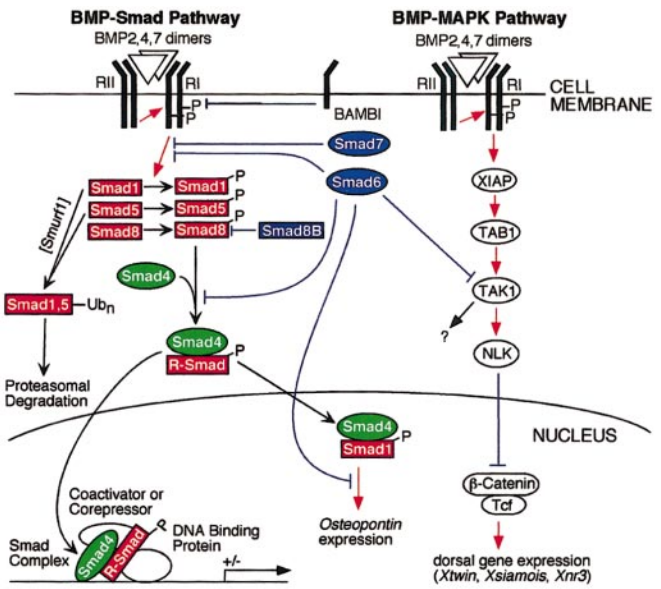

FIG. 1. The "canonical" BMP–Smad pathway and the BMP–MAPK pathway. In the BMP–Smad pathway, BMP2, -4, or -7 dimers bind to the receptor complex, leading to phosphorylation of the type I receptor (RI) by the type II receptor (RII), which in turn phosphorylates an appropriate R-Smad (Smad1, -5, or -8). This phosphorylation enables the R-Smad to complex with the Co-Smad, Smad4, and the R-Smad/Smad4 complex enters the nucleus to activate or repress target genes depending on which nuclear cofactors are present (compare Fig. 2). Although shown as heterodimers, the stoichiometry between R-Smads and Co-Smad is unclear. Smad6, Smad7, Smad8B, BAMBI, and Smurf1 all inhibit the BMP–Smad pathway at different levels. Smad6, Smad7, and BAMBI are induced by BMP signaling, establishing negative feedback loops. In the BMP–MAPK pathway, activated BMP receptors may interact with XIAP, which in turn activates the MAPKKK TAK1 by interacting with TAB1. It is unclear whether and where the BMP–MAPK pathway intersects with the BMP–Smad pathway (question mark). TAK1 can activate NLK and inhibits the DNA binding activity of the β-catenin/TCF complex, which normally activates dorsally expressed target genes of the Wnt/β-catenin pathway. Smad6 inhibits the BMP–MAPK pathway as well. Red indicates activation and blue indicates inhibition. Smads that are part of or regulate the BMP-Smad pathway are colored: Smad1, -5, and -8 are red, the Co-Smad Smad4 is green, and the I-Smads and Smad8B are blue.

families: the TGF-βs, the activins, and the BMPs. The basic mechanism of the TGF-β superfamily signal transduction pathway has been well characterized in recent years (reviewed in Heldin *et al.*, 1997; Whitman, 1998; Massagúe and Chen, 2000; Miyazono *et al.*, 2000). Transduction of TGF-β signals involves two distinct kinds of transmembrane serine/threonine kinase receptors, type I and type II. The prevailing view is that TGF-β ligands bind to the type II receptor, which then recruits a type I receptor. Following formation of a ligand/type II/type I ternary complex, the type II receptor phosphorylates serine and threonine residues within the intracellular GS (glycine–serine-rich) domain of the type I receptor subunit (Wrana *et al.*, 1994). The activated type I receptor kinase, in turn, phosphorylates particular members of the Smad family of proteins, called receptor-regulated Smads (R-Smads), at serines in a conserved C-terminal SSXS motif, to elicit cellular responses. The R-Smads can be subdivided into two classes: Smad2 and -3 transduce activin/TGF-β signals (see Fig. 3), while Smad1, -5, and -8 preferentially transduce BMP signals (Fig.

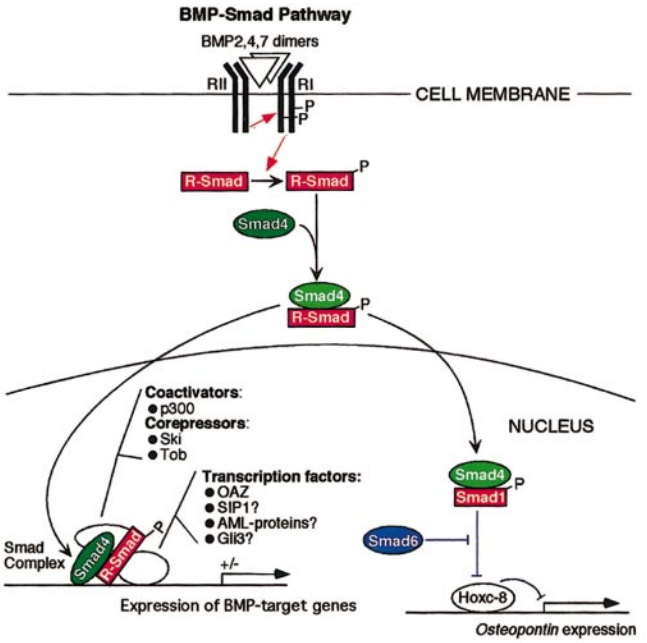

FIG. 2. Nuclear factors involved in the regulation of BMP-target genes by the BMP–Smad pathway. After entering the nucleus, the complex of Smad4 and the activated R-Smad is thought to bind DNA via the MH1 domain and to activate or repress target genes depending on which nuclear cofactors are present. These cofactors include the general coactivator p300, which binds to the MH2 domain of Smad1 and -4 and activates transcription through its histone acetylase activity, and corepressors such as Ski and Tob. Ski binds to the MH2 domain of Smad1, -4, and -5 and represses transcription by recruiting histone deacetylases. Tob also binds to BMP-regulated Smads and can inhibit transcriptional activation of a BMP target gene. Tob is induced by BMP signaling, establishing a negative feedback loop. DNA binding transcription factors such as OAZ are thought to cooperate with the Smad complex in regulating specific target genes. The transcription factors SIP1, Gli3 (C-terminally truncated), and members of the AML family of transcription factors have been shown to bind to BMP-regulated Smads, but their involvement in the regulation of BMP target genes is unclear. Smad1/4-mediated BMP signaling can activate transcription of the *Osteopontin* gene by dislodging the transcriptional repressor Hoxc-8 from its promoter. Smad6 can inhibit this activation by binding to Hoxc-8 while keeping it bound to the promoter. Red indicates activation while blue indicates inhibition. RI and RII represents type I and type II receptors, respectively.

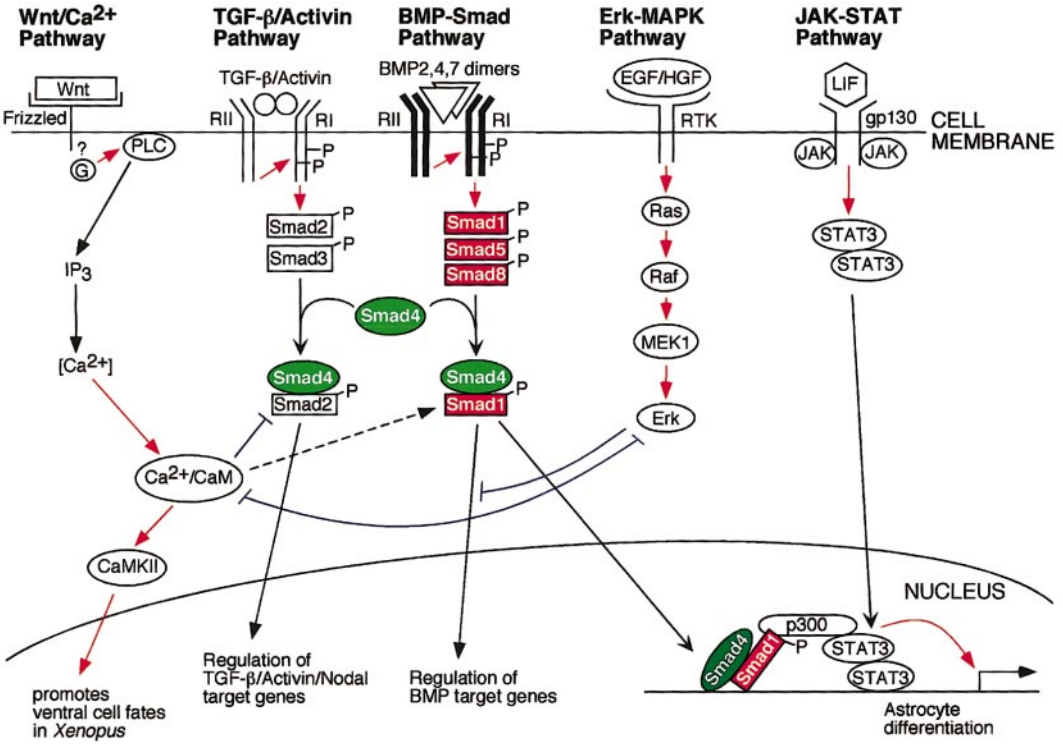

FIG. 3. Crosstalk of the BMP–Smad pathway with other signal transduction pathways: the Wnt/Ca^{2+}, the TGF-β/activin, the Erk–MAPK, and the JAK–STAT pathway. Note that the depicted interactions do not necessarily occur in the same biological context, cell type, or organism, but rather represent a schematic summary of what is known. While there are experimental data suggesting that G-proteins are required for the Wnt/Ca^{2+} pathway, no direct interaction of G-proteins with frizzled receptors has been shown (question mark). Red indicates activation and blue indicates inhibition. RI and RII represents type I and type II receptors, respectively. A dashed arrow denotes conflicting results as to whether activation or inhibition is involved (see text).

1). However, Smad1 and -5 have also been reported to be activated by TGF-β in certain cell types (Bruno *et al.*, 1998; Liu *et al.*, 1998b; Yue *et al.*, 1999; Oh *et al.*, 2000). Following their activation, different type I receptor kinases specifically recognize and phosphorylate distinct R-Smads to activate specific pathways (Hoodless *et al.*, 1996; Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996; Kretzschmar *et al.*, 1997b). Thus, R-Smads are thought to play an important role in transducing specific TGF-β signaling pathways. Upon phosphorylation, R-Smads are released from the receptor and interact with Smad4 (also known as the Co-Smad), which is "shared" between several TGF-β superfamily signal transduction pathways (see Fig. 3). Phosphorylation also results in nuclear translocation of these otherwise cytoplasmically localized factors to permit the assembly of Smad/transcription factor complexes on the promoters of target genes (Baker and Harland, 1996; Lagna *et al.*, 1996; Liu *et al.*, 1996; Zhang *et al.*, 1996, 1997).

Structurally, R-Smads and the Co-Smad are similar in that they share two highly conserved regions, an N-terminal MH1 domain (Mad Homology 1) and a C-terminal MH2 domain (also called N- and C-domain) separated by a less conserved linker region. However, the Co-Smad does not have the C-terminal SSXS phosphorylation motif present in R-Smads, and is thus not phosphorylated by the receptor (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996). The MH1 domain of the Co-Smad and of all R-Smads except for Smad2 can bind to specific DNA sequences, whereas the MH2 domain mediates protein–protein interactions with Smads, transcriptional coactivators, or corepressors (Kim *et al.*, 1997; Dennler *et al.*, 1998; Shi *et al.*, 1998; Zawel *et al.*, 1998). Both R-Smads and the Co-Smad activate transcription primarily through their MH2 domain (Liu *et al.*, 1996). This activity results, at least in part, from the ability of the MH2 domain to recruit the general transcriptional coactivators p300 and CBP (Fig. 2; Feng *et al.*, 1998; Janknecht *et al.*, 1998; Pouponnot *et al.*, 1998; Shen *et al.*, 1998; Topper *et al.*, 1998). Both p300 and CBP have histone acetylase activity, enabling them to increase transcription of target genes by loosening of the chromatin structure. Nuclear factors such as the adenoviral oncoprotein E1A and the transcriptional repressor SNIP1 are thought to inhibit TGF-β signaling by inhibiting this interaction between Smads and CBP/p300 (Nishihara *et al.*, 1999; Kim *et al.*,

2000). While Smads alone can bind to specific DNA sequences, their binding affinity is considered to be too weak to serve as effective and highly specific DNA binding proteins *in vivo* (Shi *et al*., 1998). Thus, additional DNA binding partners are thought to be required for efficient DNA binding. The first such DNA binding partner, *Xenopus* FAST-1, was shown to function in the activin/TGF-β pathway (Chen *et al*., 1996, 1997). FAST-1 is a member of the winged-helix family of DNA binding proteins and has been shown to associate with Smad2 (or Smad3) and Smad4 upon activin stimulation. It binds to an activin response element in the promoter of the *Xenopus Mix.2* gene to affect its transcription. Subsequently, mammalian FAST-1 homologs were shown to be involved in the transcriptional activation of the *gsc* and *Mix.2* genes by TGF-β/activin and the activation of the *lefty-2* and *nodal* genes by nodal (Labbé *et al*., 1998; Zhou *et al*., 1998; Liu *et al*., 1999; Saijoh *et al*., 2000). In addition, transriptional activation of the *gsc* gene by TGF-β/activin appears to involve the *Xenopus* homeodomain proteins mixer and milk (Germain *et al*., 2000). For BMP signaling, the zinc finger protein OAZ has been identified as a DNA binding cofactor involved in regulating the direct BMP target gene, *Xvent-2* (Hata *et al*., 2000).

In addition to this "canonical" TGF-β signaling pathway, recent studies suggest that a mitogen-activated protein kinase (MAPK) pathway may mediate TGF-β signal transduction (Fig. 1). These studies have shown that BMP signals can be transduced by TGF-β activated kinase 1 (TAK1), a MAP kinase kinase kinase (MAPKKK), and TAK1 binding protein 1 (TAB1; Yamaguchi *et al*., 1995; Shibuya *et al*., 1998). In *Xenopus*, both TAK1 and TAB1 are expressed maternally and throughout embryonic development. When TAK1 alone is overexpressed in *Xenopus*, it induces apoptosis. However, coinjection of TAK1 together with an apoptosis inhibitor induces ventral mesoderm in animal caps and ventralizes embryos, thus mimicking BMP overexpression phenotypes. TAB1 activates TAK1 by directly binding to its catalytic domain, and overexpression of TAB1 enhances the ventralizing activity of TAK1 when coinjected. Importantly, overexpression of a kinase-deficient form of TAK1 partially blocks BMP-mediated ventralization and ectodermal differentiation in *Xenopus* embryos (Shibuya *et al*., 1998). These results suggest that TAK1 mimics BMP-like activity and is required for robust BMP signaling in early *Xenopus* development. While the mechanism of the transduction of BMP signals by this BMP-TAK1 pathway is unclear, it is possible that activation of the BMP-TAK1 pathway leads to phosphorylation of transcription factors that then synergize with BMP-regulated Smads in the activation of BMP-target genes. Interestingly, IAP (inhibitor of apoptosis protein) was identified as a possible adaptor protein linking the receptors and TAB1–TAK1 in both *Drosophila* and *Xenopus* (Oeda *et al*., 1998; Yamaguchi *et al*., 1999). While it is unclear whether TAK1 has a role in *Drosophila* dpp signaling (Takatsu *et al*., 2000), these findings suggest that the BMP–TAK1 pathway is evolutionarily conserved and can transduce BMP signals during early dorsal–ventral patterning of *Xenopus*. It should be noted that, in addition to BMPs, other ligands such as TGF-β, interleukin-1, and tumor necrosis factor (TNF)-α have been reported to activate TAK1 in certain contexts (Yamaguchi *et al*., 1995; Sakurai *et al*., 1998, 1999; Ninomiya-Tsuji *et al*., 1999; Yao *et al*., 1999), and that TAK1 has been implicated in the activation of the JNK and p38 MAPK signaling pathways (Moriguchi *et al*., 1996; Shirakabe *et al*., 1997; Wang *et al*., 1997; Yao *et al*., 1999; Takatsu *et al*., 2000). In the remainder of this review, we will refer to the classical BMP pathway as BMP–Smad pathway and to the novel BMP–TAK1 pathway as BMP–MAPK pathway.

REGULATION OF BMP SIGNALING VIA INTRACELLULAR FACTORS

Research in recent years has shown that the BMP–Smad signaling pathway is often subjected to negative autofeedback loop regulation both at extracellular and intracellular levels, suggesting that this mechanism may modulate the duration and/or intensity of BMP signaling. Extracellularly, the activity of BMPs can be regulated by secreted proteins such as chordin, noggin, Gremlin, Cerberus, Tolloid/BMP1-related metalloproteases, and twisted gastrulation. This mode of regulation of BMP signaling will not be discussed here, since it has recently been reviewed elsewhere (Cho and Blitz, 1998; De Robertis *et al*., 2000; Ray and Wharton, 2001). In this review, we will focus on intracellular modes of BMP signaling regulation. This section will describe intracellular antagonisms mediated by recently identified intracellular factors (Figs. 1 and 2), while the next section will discuss potential crosstalk mechanisms between the BMP and other signaling pathways (Fig. 3).

BAMBI. BMP and activin membrane bound inhibitor (BAMBI) has been identified as an inhibitor of BMP signaling during *Xenopus* embryonic development (Fig. 1; Onichtchouk *et al*., 1999). BAMBI shows sequence similarity to TGF-β receptors, but lacks the intracellular kinase domain. Thus, BAMBI can function as a naturally occurring dominant-negative receptor as association of BAMBI with receptors of the TGF-β family prevents the formation of functional receptor complexes and blocks TGF-β, activin, and BMP signaling. BAMBI homologues have been isolated in mouse (Grotewold *et al*., 2001), humans, and zebrafish (Degen *et al*., 1996; Tsang *et al*., 2000). In all species examined, embryonic expression of BAMBI overlaps that of BMPs, and appears to be regulated by BMP ligands. For instance, in both *Xenopus* and mouse, expression of BAMBI is induced by overexpressing BMP4 (Onichtchouk *et al*., 1999; Grotewold *et al*., 2001), and in zebrafish, expression of BAMBI is lost in bmp2b mutants (Tsang *et al*., 2000), suggesting that BAMBI expression requires BMP signaling. Thus, it appears that expression of BAMBI is induced by BMPs and negatively regulates the BMP–Smad signaling pathway in vertebrates. In addition, BAMBI may serve as a

key regulatory molecule to cross-regulate other members of the TGF-β superfamily as its expression interferes with TGF-β and activin signaling as well.

I-Smads. Inhibitory Smads (I-Smads) consist of vertebrate Smad6 and Smad7 and *Drosophila* daughters against dpp (Dad). Unlike R-Smads, which augment the signaling of the members of the TGF-β superfamily, I-Smads inhibit TGF-β superfamily signaling (Fig. 1; Hayashi *et al*., 1997; Imamura *et al*., 1997; Nakao *et al*., 1997; Tsuneizumi *et al*., 1997). I-Smads can bind stably to the intracellular domain of activated BMP/Dpp type I receptors, thereby inhibiting BMP signaling by preventing phosphorylation of R-Smads by the receptor (Imamura *et al*., 1997; Inoue *et al*., 1998; Souchelnytskyi *et al*., 1998). In addition, Smad6 has been suggested to inhibit BMP signaling by competing with Smad4 for binding to receptor-activated Smad1, yielding apparently inactive Smad1–Smad6 complexes (Hata *et al*., 1998).

In *Xenopus*, overexpression of either Smad6 or -7 can phenocopy the effect of blocking BMP signaling, in that it leads to the formation of a secondary axis when injected ventrally into whole embryos or to direct neural induction in ectodermal explants (Bhushan *et al*., 1998; Casellas and Brivanlou, 1998; Nakayama *et al*., 1998a,b). However, Smad7, and possibly Smad6, can also target other TGF-β family pathways. In biochemical studies, Smad6 and -7 can inhibit phosphorylation of Smad2 and/or -3 by binding TGF-β/activin type I receptors (Hayashi *et al*., 1997; Imamura *et al*., 1997; Nakao *et al*., 1997). In *Xenopus* embryos, Smad7 overexpression can phenocopy the effects of blocking activin like signaling pathways (Nakao *et al*., 1997; Bhushan *et al*., 1998; Casellas and Brivanlou, 1998; Nakayama *et al*., 1998b), and in mouse B cells, Smad7 can inhibit activin-induced growth arrest and apoptosis (Ishisaki *et al*., 1998, 1999). While Smad6 can partially block activin signaling in *Xenopus* embryos as well (Nakayama *et al*., 1998a), there is evidence suggesting that endogenous Smad6 may preferentially or selectively inhibit the BMP–Smad pathway (Hata *et al*., 1998; Ishisaki *et al*., 1999).

Interestingly, expression of I-Smads appears to be part of a negative feedback loop. The expression patterns of Smad6, Smad7, and Dad are similar to the expression of BMP2/4 in *Xenopus* and Dpp in *Drosophila* embryos (Tsuneizumi *et al*., 1997; Casellas and Brivanlou, 1998; Nakayama *et al*., 1998a,b), and the expression of Smad6 and -7 can be induced rapidly and in some cases directly by BMP, activin, and/or TGF-β in cultured cells (Nakao *et al*., 1997; Afrakhte *et al*., 1998). Recently, the promoter region of the mouse Smad6 gene has been shown to contain a BMP responsive element, which directly binds a Smad4/5 complex (Ishida *et al*., 2000). In addition, Smad3 and -4 can directly bind to the Smad7 promoter to mediate activation of this promoter by activin or TGF-β (Nagarajan *et al*., 1999; von Gersdorff *et al*., 2000). Finally, in *Xenopus* embryonic explants and in the developing *Drosophila* wing, BMP/Dpp signaling is necessary and sufficient for the expression of Smad7 or Dad (Tsuneizumi *et al*., 1997; Nakayama *et al*., 1998b). Taken together, these findings suggest that the negative feedback mechanism established by I-Smads is evolutionarily conserved and involves direct regulation of I-Smads by TGF-β signaling.

Interestingly, Smad6 has recently been shown to directly bind to and inhibit TAK1 (Kimura *et al*., 2000), suggesting that I-Smads not only inhibit the canonical BMP–Smad pathway, but the TAK1-mediated BMP–MAPK pathway as well. Thus, Smad6 can inhibit BMP–Smad signaling at several levels, and it also inhibits the BMP–MAPK pathway by interacting with TAK1 (Fig. 1). Recently, mice lacking Smad6 were generated and shown to display relatively minor defects. This mild phenotype (Galvin *et al*., 2000) may be due to the fact that these authors only deleted the MH2 domain of Smad6, since the MH1 domain of Smad6 alone can still inhibit BMP signaling, at least in *Xenopus* embryos (Nakayama *et al*., 2001). It will be interesting to see the effect of a complete knockout of Smad6 in mice.

Smurfs. Smurf1 (Smad ubiquitination regulatory factor-1) is a new member of the HECT (homologous to E6-associated protein C terminus) class of E3 ubiquitin ligases. Smurf1 interacts with Smad1 and -5, but not with Smad2 and -4. The interaction with Smad1 has been shown to occur through a PPXY motif (also called PY motif) located in the linker region of Smad1. Smad5 also contains this motif, suggesting that it interacts with Smurf1 through this motif as well. Smurf1 specifically targets Smad1 and -5 for ubiquitination, leading to proteasomal degradation (Fig. 1; Zhu *et al*., 1999). In *Xenopus*, overexpression of Smurf1 mRNA blocks Smad1-dependent induction of ventral mesodermal marker genes (Zhu *et al*., 1999). Interestingly, the degradation of Smad1 and -5 by Smurf1 occurs independent of BMP receptor activation, indicating that Smurf1 does not function downstream of activated Smads to turn off BMP signals, but may rather adjust the basal level of Smads available for BMP signaling (Zhu *et al*., 1999).

An additional Smurf, human Smurf2, which shares 83% sequence identity with Smurf1, has recently been isolated by three different groups (Kavsak *et al*., 2000; Lin *et al*., 2000; Zhang *et al*., 2001). However, the exact role of Smurf2 in the regulation of TGF-β signaling is unclear, as there are conflicting results. While Lin *et al*. (2000) find Smurf2 to be implicated in proteasomal degradation of TGF-β-activated Smad2, Zhang *et al*. (2001) report that Smurf2 functions similar to Smurf1 in that it preferentially targets Smad1 for proteasomal degradation. Finally, Kavsak *et al*. (2000) report that Smurf2 mediates proteasomal degradation of the activated TGF-β receptor via binding to Smad7 as well as downregulation of BMP receptor complexes via binding to Smad6. While these discrepancies need to be clarified, it appears that Smurfs control the competence for the response to and the duration of BMP signaling by possibly acting at two different levels of the BMP–Smad signaling cascade, at the level of R-Smads, and at the receptor level.

Ski. Ski was recently found to act as a transcriptional corepressor of BMP–Smad signaling in the nucleus (Fig. 2).

Ski was originally identified as the product of a retroviral oncogene (*v-ski*) that causes transformation in chick embryo fibroblasts (Li *et al*., 1986). Its cellular counterpart, the product of the proto-oncogene *c-ski*, and the related SnoN protein are transcriptional corepressors that recruit histone deacetylases (HDAC) via the transcriptional corepressor N-CoR (nuclear hormone receptor co-repressor; Luo *et al*., 1999; Nomura *et al*., 1999). Both c-ski and SnoN physically interact with the MH2 domain of Smad2, -3, and -4 and directly repress their ability to activate TGF-β target genes (Akiyoshi *et al*., 1999; Luo *et al*., 1999; Stroschein *et al*., 1999; Sun *et al*., 1999). Recently, Ski was also shown to interact with the MH2 domains of the BMP-specific Smad1 and -5 in a BMP signaling-dependent manner (Wang *et al*., 2000). This interaction of Ski with the Smad1/4 complex antagonizes BMP signaling, causes direct neural induction in *Xenopus* embryonic ectoderm explants, and represses Smad1/4-dependent transcriptional activation of BMP-responsive reporter genes (Amaravadi *et al*., 1997; Wang *et al*., 2000). Similarly, a zebrafish *c-ski* homologue dorsalizes embryos in the mesoderm when overexpressed, an effect that can be rescued by BMP4 (Kaufman *et al*., 2000). In *Xenopus* and zebrafish, Ski mRNA and protein are expressed maternally and throughout early embryogenesis (Sleeman and Laskey, 1993; Amaravadi *et al*., 1997; Kaufman *et al*., 2000), and mice lacking *c-ski* show defects in myogenesis and neural tube formation, resulting in lethality at birth (Berk *et al*., 1997). These findings are consistent with an essential function of Ski in early embryonic development of vertebrates and suggest that Ski is a general nuclear corepressor of TGF-β signaling including the BMP–Smad signaling pathway.

Tob. Another more recently identified cofactor regulating BMP–Smad signaling is Tob (Fig. 2; Yoshida *et al*., 2000). Tob is a member of a novel antiproliferative protein family, known to suppress cell growth when overexpressed in NIH3T3 cells. Mice carrying a targeted deletion of the *Tob* gene have a greater bone mass resulting from an increased number of osteoblasts. This increased number of osteoblasts has been suggested to be the result of enhanced BMP2-induced osteoblast proliferation and differentiation in *Tob* knockout mice. This suggests that Tob is a negative regulator of the BMP signaling cascade in mouse osteoblasts. Consistent with this notion, Tob has been shown to interact with Smad1, -4, -5, and -8 in cell culture and to block Smad1/5/8-dependent transcriptional activation of a BMP2-responsive reporter gene containing multiple Smad1 binding sites (Yoshida *et al*., 2000). Interestingly, *Tob* transcription is rapidly and directly induced in response to BMP2 in osteoblast precursor cells (Yoshida *et al*., 2000). These results suggest that Tob establishes a negative feedback loop controlling BMP–Smad signaling in the nucleus. At present, it is not clear whether Tob is required during early embryonic development since homozygous null (*Tob*−/−) mice display no apparent early phenotypic abnormalities (Yoshida *et al*., 2000). However, the lack of embryonic phenotype in *Tob*-deficient mice may be due to functional redundancy as a related mouse gene, *Tob2*, has been recently identified and shown to be expressed in mouse embryos (Ajima *et al*., 2000).

Other Smad-interacting nuclear cofactors (Fig. 2). The zinc finger protein OAZ (Olf-1/EBF associated zinc finger) has been identified as a DNA binding cofactor that directly associates with the MH2 domain of Smad1 *in vitro* (Hata *et al*., 2000). A complex of Smad1, Smad4, and OAZ binds to a BMP-responsive element of the *Xvent-2* gene, a direct BMP target (Candia *et al*., 1997), to increase the transcription of *Xvent-2* upon BMP stimulation in cultured cells (Hata *et al*., 2000). OAZ, a protein with 30 zinc finger domains, can mediate signaling from multiple pathways. While the central zinc fingers are used in BMP signaling, the N- and C-terminal zinc fingers can bind to the SV40 minimal promoter and to the transcription factor Olf1/EBF, respectively, to activate transcription in partnership with Olf1/EBF. In addition, in terminally differentiated olfactory neurons, binding of OAZ's C-terminal zinc fingers to Olf1/EBF can block formation of Olf1/EBF homodimers, which normally activate the transcription of olfactory marker protein (Tsai and Reed, 1997; Hata *et al*., 2000). Thus, OAZ is a multifunctional protein.

In addition to Ski, Tob, and OAZ, several other nuclear proteins have been shown to interact with BMP-regulated R-Smads. For example, Smad1 binds to the transcriptional repressor Hoxc-8 and dislodges it from the *osteopontin* promoter, thus allowing activation of transcription of the *osteopontin* gene by derepression (Shi *et al*., 1999). Interestingly, Smad6 can also bind to Hoxc-8, but is thought to act as a transcriptional corepressor. Smad6 competes with Smad1 for binding to Hoxc-8, and keeps Hoxc-8 bound to the promoter (Bai *et al*., 2000; see Fig. 2). A similar mechanism has been suggested for the transcriptional repressor SIP1 (Smad interacting protein 1). SIP1 is a zinc finger/homeodomain protein, that has been shown to interact with the MH2 domain of Smad1, -2, -3, and -5 in yeast and mammalian cells (Verschueren *et al*., 1999). SIP1 binds to the *Xenopus Xbra* promoter and represses its activity. Smads have been suggested to dislodge SIP1 from the *Xenopus Xbra* promoter, thereby allowing transcription. Other nuclear factors that interact with BMP-regulated R-Smads are the zinc finger transcription factor Gli3 and transcription factors of the acute myelogenous leukemia (AML) family, also known as core-binding factors (CBF), polyoma enhancer binding proteins (PEBPs), or Runt domain transcription factors (Liu *et al*., 1998a; Hanai *et al*., 1999; Pardali *et al*., 2000). While these factors can bind to Smads, the physiological significance of these interactions for BMP signaling remains to be determined.

Many of the intracellular factors that have been shown to interact with BMP signaling appear to be negative regulators. This is surprising and parallels the burgeoning abundance of extracellular BMP inhibitors (Cho and Blitz, 1998; De Robertis *et al*., 2000; Nakayama *et al*., 2000). It raises the possibility that inhibition is a prominent mode of

intracellular BMP signaling regulation, as seen in the extracellular mode of BMP signaling regulation. It is possible, however, that at least some of the observed inhibitory effects are the result of experimental overexpression of proteins leading to fortuitous interactions with Smads or other members of the BMP signaling pathway.

INTERSECTION BETWEEN THE BMP AND OTHER SIGNALING PATHWAYS

Crosstalk between the BMP-Smad and Other TGF-β-Related Signaling Pathways

Signaling by BMPs and members of the activin/TGF-β families has been shown to interact antagonistically in *Xenopus*. In *Xenopus* ectodermal explants, activin/TGF-β can induce dorsal-type mesoderm, whereas BMPs induce ventral mesoderm and can block the dorsalizing effect of activin/TGF-β. Investigation of mechanisms underlying this phenomenon has revealed that this antagonism between BMPs and activin/TGF-β may be explained by intracellular competition for a limited pool of Smad4, in certain physiological situations (Fig. 3; Candia *et al*., 1997). According to this model, the amount of Smad4 may be limited in cells and simultaneous activation of two TGF-β signaling pathways will result in competition for Smad4. The outcome of this competition determines the relative strengths and antagonism of the signals.

Another level of regulation is known to occur by sequestering R-Smads. Smad6 has been shown to interact specifically with Smad1, but not with Smad2, to form an inactive Smad1/6 complex (Hata *et al*., 1998). Thus, in this case, Smad6 competes with Smad4 for binding to Smad1 to block BMP signaling (Fig. 1). This mode of Smad6 action is different from the other receptor-interference model proposed for Smad6 (see above). Similarly, another R-Smad, Smad8, appears to be inhibited by Smad8B, a splice variant of Smad8 that lacks the C-terminal SSXS motif (Nishita *et al*., 1999). Smad8B does not translocate into the nucleus, and associates specifically with Smad8 and -4 in the cytoplasm to inhibit BMP signaling mediated by Smad8. This suggests that Smad8B can act as a naturally occurring dominant inhibitor of Smad8, possibly by preventing its association with Smad4 (Fig. 1; Nishita *et al*., 1999).

Factors that regulate and/or are induced by both BMP signaling and other TGF-β signaling pathways may also provide a basis for crosstalk between these different TGF-β-related signaling pathways. For example, the I-Smads (Smad6 and -7) are induced by BMP, activin, and/or TGF-β, and can inhibit both BMP and TGF-β/activin signaling. Another example is BAMBI, which is induced by BMP signaling, but inhibits not only BMP signaling, but TGF-β and activin signaling as well.

Crosstalk between BMP- and Wnt/β-Catenin Signaling

There has been some speculation as to whether BMP signaling and Wnt signaling can directly crosstalk. In vertebrates, certain members of the Wnt family of secreted glycoproteins are known to be involved in organizer formation, among other functions (reviewed in Moon *et al*., 1997; Sokol, 1999). For example, overexpression of the Wnt1 class of Wnts, which includes Wnt1, Wnt3A, Wnt8, and Wnt8b (Moon *et al*., 1997), induces an ectopic organizer and activates organizer-specific genes when overexpressed ventrally in cleavage-stage *Xenopus* embryos. These Wnts bind to certain members of the Frizzled family of Wnt receptors, thus activating the canonical Wnt/β-catenin pathway. This stabilizes a cytosolic pool of β-catenin, which then enters the nucleus to form complexes with Lef/Tcf transcription factors to activate Wnt target genes. The Wnt/β-catenin pathway is conserved in *Drosophila*. In the patterning of the *Drosophila* leg discs, both Wingless (Wg), the *Drosophila* orthologue of Wnt1, and Dpp inhibit the expression of each other (Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Theisen *et al*., 1996). While this evidence shows that Wg and Dpp do interact, it is not clear whether the Wg- and Dpp-signaling pathways crosstalk intracellularly. It is equally possible that Wg indirectly regulates the expression of Dpp to repress Dpp signaling, and/or *vice versa*. In *Drosophila* endoderm formation, input from both Wg and Dpp signaling is required, and the signaling inputs of both pathways converge at the midgut enhancer of the *Ultrabithorax* (*Ubx*) gene, which possesses adjacent Wg- and Dpp-responsive elements to enhance *Ubx* expression (Riese *et al*., 1997). In *Xenopus* organizer formation, recent evidence suggests synergistic crosstalk between activin/Vg1/nodal-like signaling and the canonical Wnt/β-catenin pathway in that Smad4 and Lef1/Tcf can interact directly in inducing the expression of the direct Wnt/β-catenin target gene *Xtwn* during gastrula stages (Nishita *et al*., 2000).

There is also evidence suggesting that BMP and Wnt/β-catenin signaling interact in early *Xenopus* embryos, although the molecular nature of the interaction (direct vs. indirect) is not clear. First, early Wnt/β-catenin signaling by Wnt1 class protein inhibits BMP4 expression in the *Xenopus* ectoderm (Baker *et al*., 1999). Second, after the midblastula transition (MBT), Xwnt8 is similar to BMP2/4 in that it can induce ventral mesoderm when overexpressed, and it is expressed in the ventral marginal zone of gastrulating *Xenopus* embryos (Dale *et al*., 1992; Jones *et al*., 1992; Christian and Moon, 1993). This zygotic Xwnt8 activity should be distinguished from its Wnt1-like ability to induce a secondary axis when overexpressed before MBT. Third, ectopic BMP signaling can affect zygotic Xwnt8 expression in the ventral marginal zone, suggesting that Xwnt8 cooperates with BMP2/4 in specifying ventrolateral mesoderm in *Xenopus* (Hoppler and Moon, 1998; Marom *et al*., 1999). In addition to these regulatory interactions between BMPs and Wnts, the induction of the BMP-target gene *Xvent-2*

appears to require Wnt/β-catenin signaling, whereas the expression of the Wnt/β-catenin target genes *Xtwin*, *Xsiamois*, and *Xnr3* does not require BMP signaling (Laurent and Cho, 1999; Kazanskaya *et al*., 2000).

There are also results suggesting that the noncanonical BMP–MAPK pathway may interact with Wnt/β-catenin signaling. The MAPKKK TAK1, which can be activated by the BMP–MAPK pathway (see Fig. 1), has been shown to antagonize induction of Wnt/β-catenin target genes (Ishitani *et al*., 1999). In a search for downstream targets of TAK1, Ishitani *et al*. (1999) showed that TAK1 can activate MAPK-related NEMO-like kinase (NLK), which, in turn, down-regulates Lef/Tcf mediated Wnt-dependent transcription (Fig. 1). In *Xenopus*, NLK phosphorylates Lef/Tcf, and this phosphorylation prevents the binding of the β-catenin-Tcf complex to DNA (Ishitani *et al*., 1999). These observations raise the interesting possibility that a TAK1-regulated protein, NLK, participates in blocking Wnt/β-catenin signaling in the ventral region of *Xenopus* embryos where BMP signaling is active, ensuring to maintain the ventral state. However, this scenario is questionable, since BMP signaling does not affect the expression of *Xsiamois*, *Xtwin*, and *Xnr3*, direct dorsal target genes of the Wnt/β-catenin pathway (Laurent and Cho, 1999), and since factors other than BMPs are known to activate TAK1. An alternative possibility is that activation of the BMP–MAPK pathway ventralizes *Xenopus* embryos by phosphorylating transcription factors that then synergize with BMP-regulated Smads in the activation of BMP-target genes. Since interaction of TAK1 with Smad6 has recently been shown (Kimura *et al*., 2000), it will be interesting to see if members of the BMP–MAPK pathway can interact with BMP-regulated R-Smads as well.

There is also evidence for synergistic interaction of BMP–Smad signaling with a novel Wnt/Ca^{2+} signaling pathway on the ventral side of the *Xenopus* embryo, which will be discussed further below. First, we will discuss evidence for crosstalk between BMP–Smad and Ca^{2+}/calmodulin signaling.

Crosstalk between BMP–Smad and Ca^{2+}/Calmodulin Signaling

Calmodulin, the primary intracellular Ca^{2+} receptor, is part of the classical inositol–phospholipid pathway found downstream of certain G-protein-linked receptors (Fig. 3). Ligand binding to these receptors activates G-proteins, which in turn activate phospholipase C (PLC). PLC then cleaves phosphatidylinositol–bisphosphate (PIP_2) to generate inositol–trisphosphate (IP_3) and diacylglycerol (DAG). While DAG activates protein kinase C (PKC), IP_3 induces the release of Ca^{2+} from the endoplasmic reticulum, which, in turn, binds to calmodulin to generate a Ca^{2+}/calmodulin complex. Ca^{2+}/calmodulin then activates kinases such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII).

Calmodulin has been shown to bind Smads1–4 *in vitro* and in transfected cells in a calcium-dependent manner (Zimmerman *et al*., 1998). Calmodulin can bind to the N-terminal MH1 domain of both Smad1 and Smad2, but the exact role of calmodulin in TGF-β-related signaling is still uncertain as there are some conflicting results. For instance, some data suggest a role of calmodulin in inhibiting activin signaling and stimulating BMP signaling (Fig. 3; Zimmerman *et al*., 1998; Scherer and Graff, 2000). In contrast, another group has reported a role of calmodulin in inhibiting Smad1-mediated BMP signaling (Xu *et al*., 1999). While the reason for this discrepancy is unclear, it could be attributed to differences in expression levels of the calmodulin protein in the experiments of the different groups.

The observation that calmodulin may stimulate BMP signaling by interacting with Smad1 is consistent with other findings suggesting that Ca^{2+}-signaling is important for ventral cell fate in embryonic development. For example, Kume *et al*. (1997) found that inhibiting Ca^{2+} release from the endoplasmic reticulum can suppress ventralization of *Xenopus* embryos after BMP4 overexpression. In addition, overexpression of CaMKII, which is normally activated by Ca^{2+}/calmodulin, promotes ventral cell fate specification in *Xenopus*, and endogenous CaMKII activity is highest on the prospective ventral side of blastula and gastrula stage *Xenopus* embryos (Kuhl *et al*., 2000a). Thus, some of the available evidence suggests synergistic crosstalk between Ca^{2+}/calmodulin and BMP–Smad signaling. This crosstalk may be conserved between vertebrates and invertebrates since a Ca^{2+}-gradient was found in *Drosophila* embryos with high levels on the dorsal side, coinciding with the peak Dpp activity (Créton *et al*., 2000). Importantly, suppression of dorsally elevated Ca^{2+} levels results in embryos missing dorsal structures, suggesting that in *Drosophila*, Ca^{2+} signaling is required for proper dorsal development and could enhance Dpp signaling (Créton *et al*., 2000).

Crosstalk between BMP–Smad and Wnt/Ca^{2+} Signaling

While Wnt/β-catenin signaling and BMP signaling may interact in the specification of ventral cell fates (Hoppler and Moon, 1998; Marom *et al*., 1999; Kazanskaya *et al*., 2000), there is also evidence that a noncanonical Wnt-pathway (Wnt/Ca^{2+} pathway; Fig. 3) may be involved in ventral cell fate specification.

The possibility of synergistic crosstalk of Ca^{2+}/calmodulin with BMP–Smad signaling, as discussed above, is intriguing considering the accumulating evidence that CaMKII is part of this Wnt/Ca^{2+} pathway (Fig. 3), which is thought to be active on the ventral side of early *Xenopus* embryos (reviewed in Kühl *et al*., 2000b). This pathway can be activated by certain members of the Wnt family (e.g., Wnt5A and -11), and their receptors of the Frizzled family. As Wnt5A and -11 do not induce a complete secondary axis in *Xenopus* embryos (Ku and Melton, 1993; Du *et al*., 1995), these Wnts presumably do not activate the canonical Wnt/β-catenin pathway, which stabilizes β-catenin to form

complexes with Lef/Tcf transcription factors to activate dorsal target genes. Instead, Wnt5A and -11 stimulate CaMKII activity (Kühl *et al*., 2000a). In addition, certain members of the frizzled family of Wnt-receptors have been shown to preferentially activate this Wnt/Ca^{2+} pathway by acting as G-protein-linked receptors (Sheldahl *et al*., 1999; Kühl *et al*., 2000a).

Several pieces of evidence suggest that the Wnt/Ca^{2+} pathway may be involved in the specification of ventral cell fates in *Xenopus* embryos. First, inhibition of either CaMKII or Xwnt11 can dorsalize embryos and induces dorsal marker gene expression (Kuhl *et al*., 2000a). Second, ventral expression of a Wnt antagonist derived from a Frizzled receptor, ECD8 (extracellular domain of Xfz8), can induce dorsal structures in a β-catenin-independent manner (Itoh and Sokol, 1999). Third, Wnt5A and -11 antagonize Xwnt8-mediated axis induction, which is mediated by the canonical Wnt/β-catenin pathway (Torres *et al*., 1996). Lastly, Ca^{2+} appears to be required for the ventralizing effect of BMP4 overexpression in *Xenopus* embryos (Kume *et al*., 1997).

In conclusion, this evidence together with the possibility of synergistic crosstalk between Ca^{2+}/calmodulin and BMP–Smad signaling suggests that there may be crosstalk between the Wnt/Ca^{2+} pathway and BMP signaling in the specification of ventral cell fates in *Xenopus*. It should be noted, however, that Wnt5A and -11 themselves do not ventralize embryos (Itoh and Sokol, 1999; Kühl *et al*., 2000a), suggesting that there may be as yet unidentified ventralizing Wnts.

Crosstalk between the BMP–Smad and the Erk–MAPK Pathway

The Erk–MAP kinase pathway, which mediates the effects of certain receptor tyrosine kinases (RTKs), can also modulate the BMP–Smad pathway by regulating Smad activity (Fig. 3). Activation of RTKs by ligands such as epidermal growth factor (EGF) or hepatocyte growth factor (HGF) subsequently activates the Erk (extracellular signal regulated kinase) subfamily of mitogen-activated protein kinases (MAPK). *In vitro* and cell culture studies have revealed that Erk kinases, in turn, phosphorylate serine residues in consensus PXSP motifs which are located within the region linking the MH1 and MH2 domains of Smad1 (Kretzschmar *et al*., 1997a). As a consequence of this phosphorylation, nuclear accumulation of Smad1 is inhibited in cultured cells (Kretzschmar *et al*., 1997a), although the mechanism for this inhibition is unclear. Thus, it appears that the Erk–MAPK pathway can crosstalk with BMP–Smad signaling by differentially phosphorylating Smad1 to affect its nuclear localization. This mechanism may underlie the observed opposing effects of mitogenic factors and BMPs during vertebrate development. For example, EGF can oppose the BMP2-dependent induction of osteogenic differentiation (Bernier and Goltzman, 1992), and FGF opposes BMP4's ability to induce interdigital apoptosis during digit formation (Gañan *et al*., 1996). It should be noted, however, that BMP and RTK signaling do not only antagonize each other's activity, but also cooperate in certain contexts, such as tooth morphogenesis in vertebrates (Vainio *et al*., 1993; Neubüser *et al*., 1997; Kettunen and Thesleff, 1998; reviewed in Peters and Balling, 1999) and *Drosophila* eggshell patterning (Deng and Bownes, 1997; Dobens *et al*., 2000; Peri and Roth, 2000; reviewed in Dobens and Raftery, 2000).

The observed antagonistic effect of Erk kinases on Smad1 is in contrast with the activity of Ca^{2+}/calmodulin, which seems to augment the activity of BMP–Smad signaling, at least according to one study (Scherer and Graff, 2000). In addition, calmodulin inhibits Smad1 and -2 phosphorylation by Erk2, while Smad1 and -2 phosphorylation by Erk2 inhibits calmodulin binding *in vitro* (Scherer and Graff, 2000). Taken together, these results raise the possibility that the Ca^{2+}/calmodulin and Erk–MAPK pathways not only have opposing effects on BMP signaling, but may inhibit each other's influence on BMP signaling (and TGF-β signaling in general; see Fig. 3).

In *Drosophila* midgut formation, BMP and RTK signaling has been shown to synergistically regulate the genes *Ubx* and *labial*. This synergism has been correlated to the presence of adjacent Mad binding sites and CREs (cAMP response elements) in the promoters of these genes (Szüts *et al*., 1998). It will be interesting to see whether similar interactions on the promoter level exist for BMP and RTK signaling in vertebrates.

Crosstalk between the BMP–Smad and the JAK–STAT Pathway

The Smads are sometimes referred to as "fast-track" molecules because they can directly transduce a signal from the plasma membrane to the gene. Another pathway, the JAK–STAT pathway, also uses "fast-track" molecules, namely the STATs (signal transducers and activators of transcription), as signal transducers from the plasma membrane to the gene (reviewed in Williams, 2000). Recently, evidence for crosstalk between these two pathways has been reported (Fig. 3). Leukemia inhibitory factor (LIF), which acts through the gp130 receptor and STAT3, can act synergistically with BMP2 in inducing astrocyte differentiation in cell culture (Nakashima *et al*., 1999). This synergism has been shown to be due to the formation of a Smad1 and STAT3 complex bridged by the general transcriptional coactivator p300 on the promoter of the *glial fibrillary acidic protein* (GFAP) gene, a marker of astrocyte differentiation (Nakashima *et al*., 1999).

In *Xenopus*, both gp130 and Stat3 are expressed throughout early development (Nishinakamura *et al*., 1999). In addition, activation of gp130 signaling ventralizes embryos, and inhibits the induction of a secondary axis in embryos where BMP signaling has been reduced by injection of a dominant negative BMP receptor or the BMP antagonist noggin (Nishinakamura *et al*., 1999). This suggests that the Smad1–STAT3 synergism may also work in early dorsal–

ventral mesoderm patterning of *Xenopus* embryos. However, it appears that the gp130–STAT3 pathway acts independent of BMP signaling, since gp130 cannot rescue expression of the BMP target gene *Xvent-2* in embryos where BMP signaling has been reduced by injection of a dominant negative BMP receptor. On the other hand, gp130 inhibits axis duplication mediated by Smad2 and activation of an activin-responsive reporter gene. This suggests that the gp130-STAT3 pathway may act by inhibiting TGF-β/activin signaling and/or through a signaling pathway for ventralization which is independent of the BMP pathway. Currently, the exact role of the gp130–STAT3 pathway in early *Xenopus* BMP signaling is unclear.

CONCLUSIONS AND PERSPECTIVES

In the past several years, we have seen major advances in understanding the BMP signal transduction pathway. One emerging conclusion is that there exist many negative feedback loops in modulating the expression levels and activity of essential components of the BMP signaling pathway, both at the extracellular and intracellular levels. Intracellular factors that inhibit BMP signaling as part of a negative feedback loop include BAMBI at the cell surface, Smad6 and -7 in the cytoplasm, and Tob in the nucleus. This mechanism of negative feedback regulation ensures that BMP signaling is tightly regulated during various stages of embryonic development and the maintenance of tissue stasis. Another major advance is the recent identification of nuclear cofactors that act in regulating the transcription of BMP target genes. Ski and Tob act as transcriptional corepressors, and OAZ has been implicated in positively regulating the transcription of a BMP target gene, *Xvent-2*. The induction of the BMP target gene *Osteopontin* is unusual in that it involves dislodging of the transcriptional repressor Hoxc-8 by the Smad1/4 complex. This suggests that nuclear cofactors cooperate with the Smads in regulating specific target genes depending on the cellular context.

Recent work has also revealed a considerable degree of crosstalk between the BMP pathway and TGF-β/activin, Wnt, Ca^{2+}/calmodulin, Erk–MAPK, and JAK–STAT pathways. Furthermore, BMP signals may be transduced by MAP kinases, in addition to Smads. This adds considerable complexity to our ever-expanding knowledge of the networks of regulation of the BMP and other signaling cascades. Perhaps, considering the diverse roles BMPs play in many different biological processes, and the limited number of signaling components involved in developmental processes, this complexity is not surprising, but rather expected to provide a greater diversity of cellular responses during animal development. Hence, in the future, we will likely witness additional unsuspected interactions of BMP signaling components with other signaling pathways.

While these interactions are likely to reveal many important biological processes, the *in vivo* biological significance of such interactions needs to be examined more closely. This is because many interaction studies to date are performed by using overexpression assays for various technical reasons, and only a handful of experiments have demonstrated the presence of such interactions *in vivo*. However, this *in vivo* criterion is the most challenging and important criterion to show biological relevance of the findings. This criterion could be met by demonstrating the natural interaction of given molecules in nonoverexpression systems and use of cell lines or embryos deficient in the molecules of interest.

In addition to such studies, we envision that DNA microarray technology is likely to provide a tremendous opportunity for unraveling various signal transduction processes. The genome-wide view provided by the microarrays could reveal the involvement of genes previously not thought to be involved in the signal transduction network, assisting in the dissection of these pathways. Some of these "new" players could be part of already-characterized gene networks, others could produce new information on gene relationships.

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Note added in proof. Since writing this review a relevant paper by Goswami *et al.* (2001) has been published.

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