Integration of Smad and MAPK pathways: a link and a linker revisited

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Cells develop by reading mixed signals. Nowhere is this clearer than in the highly dynamic processes that propel embryogenesis, when critical cell-fate decisions are made swiftly in response to well-orchestrated growth-factor combinations. Learning how diverse signaling pathways are integrated is therefore essential for understanding physiology. This requires the identification, in tangible molecular terms, of key nodes for pathway integration that operate in vivo. A report in this issue, on the integration of Smad and Ras/MAPK pathways during neural induction [Pera et al. 2003], provides timely insights into the relevance of one such node.

Pera et al. [2003] report that FGF8 and IGF2—two growth factors that activate the Ras/MAPK pathway—favor neural differentiation and mesoderm dorsalization in Xenopus by inhibiting BMP [Bone Morphogenetic Protein] signaling. Mesoderm is formed from ectoderm in response to Nodal-related signals from the endoderm at the blastula stage and beyond [Fig. 1; for review, see De Robertis et al. 2000]. BMP induces differentiation of ectoderm into epidermal cell fates at the expense of neural fates, and it ventralizes the mesoderm at the expense of dorsal fates [for review, see Weinstein and Hemmati-Brivanlou 1999; De Robertis et al. 2000]. Accordingly, neural differentiation and dorsal mesoderm formation are favored when BMP signaling is attenuated. Noggin, Chordin, Cerberus, and Follistatin, secreted by the Spemann organizer on the dorsal side at the gastrula stage, facilitate the formation of neural tissue by sequestering BMP [Weinstein and Hemmati-Brivanlou 1999; De Robertis et al. 2000]. Experimentally blocking BMP signaling with a dominant-negative BMP receptor has a similar effect of promoting ectoderm neuralization [Weinstein and Hemmati-Brivanlou 1999].

As it turns out, neural induction can also be achieved with FGF (fibroblast growth factor; Kengaku and Okayama 1993; Lamb and Harland 1995; Hongo et al. 1999; Hardcastle et al. 2000; Streit et al. 2000; Wilson et al. 2000) and IGF (insulin-like growth factor; Pera et al. 2001; Richard-Farpailion et al. 2002). Injection of transcripts encoding FGF8 or IGF2 into one animal-pole blastomere of a four- to eight-cell embryo results in an expanded neural plate at the injected side [Pera et al. 2003]. Surprisingly, expression of a dominant-negative FGF receptor prevents neuralization of ectoderm explants by the BMP blocker Noggin [Launay et al. 1996]. Likewise, the potent neuralizing effect of Chordin can be blocked by a dominant-negative FGF receptor or a morpholino oligonucleotide targeting the IGF receptor [Pera et al. 2003]. Thus, the neuralizing effect of BMP inhibitors is somehow tied to FGF and IGF signaling. The question is, how?

Because FGF8 and IGF2 activate MAPK, Pera et al. [2003] took heed from previous work showing that MAPK inhibits the BMP signal-transduction factor Smad1 [Kretzschmar et al. 1997a]. Smad1 is directly phosphorylated by the BMP receptor, resulting in Smad1 activation [Kretzschmar et al. 1997b], and by MAPK in response to EGF, resulting in Smad1 inhibition [Kretzschmar et al. 1997a; Fig. 2]. Smad transcription factors mediate gene responses to the entire TGFβ [Transforming Growth Factor-β] family, to which the BMPs belong [for review, see Massagueé 2000; Derynck and Zhang 2003]. Smads 1, 5, and 8 act primarily downstream of BMP receptors and Smads 2 and 3 downstream of TGFβ, Activin and Nodal receptors. Smad proteins have two conserved globular domains—the MH1 and MH2 domains [Fig. 2]. The MH1 domain is involved in DNA binding and the MH2 domain in binding to cytoplasmic retention factors, activated receptors, nucleoporins in the nuclear pore, and DNA-binding cofactors, coactivators, and corepressors in the nucleus [for review, see Shi and Massagueé 2003]. Receptor-mediated phosphorylation occurs at the carboxy-terminal sequence SXS. This enables the nuclear accumulation of Smads and their association with the shared partner Smad4 to form transcriptional complexes that are interpreted by the cell as a function of the context [Massagueé 2000].

Between the MH1 and MH2 domains lies a linker region of variable sequence and length. Attention was drawn to this region when it was found that EGF [epidermal growth factor], a classical activator of the Ras/MAPK pathway, causes phosphorylation of the Smad1 linker at four MAPK sites [FXS sequences; Kretzschmar et al. 1997a]. This prevents the nuclear localization of Smad1 and inhibits BMP signaling. Mutation of these
serines to alanine rendered Smad1 resistant to EGF-induced phosphorylation and inhibition. In the Ras pathway, MEK1 activates the MAPKs Erk1 and Erk2. A MEK1 inhibitor prevented Smad1 phosphorylation in response to EGF and HGF (hepatocyte growth factor). In vitro, recombinant Erk2 specifically phosphorylated Smad1 at the MAPK sites in the linker region but not at the carboxy-terminal receptor phosphorylation sites (Kretzschmar et al. 1997a). These results led to the proposal that the BMP and EGF/Ras/MAPK pathways converge on Smad1 by phosphorylating the carboxy-termin- nal tail and the linker region, respectively, with opposite effects (Kretzschmar et al. 1997a, Fig. 2). The balance of these two inputs would determine the level of Smad1 activity in the nucleus, and so, the participation of BMP signaling in the control of cell fate.

This work revealed a direct link between the Smad1 and MAPK pathways, but, because it was done in cell culture, it did not establish the physiological relevance of this link. Pera and colleagues now show that the MAPK-Smad1 link may be critical for neuralization and dorsal-ventral differentiation during *Xenopus* gastrulation (Pera et al. 2003). Overexpression of Smad1 in the embryo results in a surprisingly mild ventralization phenotype and a small decrease in CNS neuronal markers. However, a Smad1 mutant protein lacking the MAPK sites in the linker potently induced a ventralized phenotype and an almost complete absence of head structures and CNS (Pera et al. 2003). Furthermore, in various neural induction assays, this Smad1 mutant blocked the neuralizing effects of FGF8 and IGF2, whereas the wild-type Smad1 did not. To be active, this MAPK-resistant Smad1 mutant still requires endogenous BMP signaling, as inferred from the mild phenotype of a Smad1 double mutant lacking both MAPK sites and BMP-receptor kinase sites (Pera et al. 2003).

**Figure 2.** A linker in more ways than one. The linker region between the MH1 and MH2 domains also links Smad signaling to the Ras/MAPK pathway. Growth factors that activate the Ras/MAPK pathway induce MAPK-mediated phosphorylation of Smad1 and Smad2. MAPK-mediated silencing of Smad1 favors neural differentiation of the ectoderm (Pera et al. 2003), and MAPK-mediated phosphorylation of Smad2 depletes the ectoderm of its competence to become mesoderm (Grimm and Gurdon 2002). Opportunities for interaction between MAPK* and Smads exist at the blastula stage. However, MAPK-mediated phosphorylation and inhibition of Smad1 (Pera et al. 2003) and Smad2 (Grimm and Gurdon 2002) are principally observed during early gastrulation. Scheme adapted from Scohöf and Fagotto (2002).

**Figure 1.** Moments of Smad silence. Schemes representing mid-blastula and early gastrula stages in *Xenopus* development, showing the three germ layers, the regions of neural and epidermal differentiation during gastrulation, and the areas of immunoreactivity with antibodies against MEK-phosphorylated Erk1 and Erk2 [Erk*], receptor-phosphorylated Smad1 (Smad1*, blue), or receptor-phosphorylated Smad2 (Smad2*, orange). The differences in the level of intensity of the blue or orange represent the varying concentrations of Smad1* and Smad2*, respectively. The area represented by orange and blue lines has both receptor phosphorylated Smad1 and Smad2. MAPK-mediated silencing of Smad1 favors neural differentiation of the ectoderm (Pera et al. 2003), and MAPK-mediated phosphorylation of Smad2 deprives the ectoderm of its competence to become mesoderm (Grimm and Gurdon 2002). Opportunities for interaction between MAPK* and Smads exist at the blastula stage. However, MAPK-mediated phosphorylation and inhibition of Smad1 (Pera et al. 2003) and Smad2 (Grimm and Gurdon 2002) are principally observed during early gastrulation. Scheme adapted from Schohlof and Fagotto (2002).
FGF8 and IGF2 induce MAPK-dependent phosphorylation of Smad1 at the linker region in cell culture [Pera et al. 2003], as do EGF and HGF [Kretzschmar et al. 1997a]. Furthermore, Pera and colleagues show that Smad1 can undergo linker phosphorylation by endogenous MAPK in Xenopus embryos [Pera et al. 2003]. Using whole-embryo extracts, they go on to show that the onset of Smad1 linker phosphorylation occurs in the early-to-mid gastrula stage, along with an increase in endogenous Erk activation. This body of evidence suggests that FGF- and IGF-like factors cause MAPK-dependent Smad1 phosphorylation during gastrulation [Fig. 2]. This would desensitize ectoderm and mesoderm cells to BMP, allowing the emergence of neural tissue and dorsal mesoderm [Fig. 1]. Why the cocktail of BMP-sequestering factors from the organizer alone is not enough to fully silence BMP remains unknown.

One question that the report by Pera et al. (2003) leaves unanswered is whether linker phosphorylation causes nuclear exclusion of Smad1 in the embryos as it does in cell lines [Kretzschmar et al. 1997a]. However, work published by Grimm and Gurdon last year sheds light into this issue, by inference at least [Grimm and Gurdon 2002]. Grimm and Gurdon were trying to explain how, at the mid-gastrula stage, the ectoderm abruptly looses competence to generate mesoderm in response to Activin-like signals. This loss of competence may serve to spare ectoderm for differentiation into other tissues [Grainger and Gurdon 1989]. To investigate the mechanism, Grimm and Gurdon (2002) allowed animal cap ectoderm explants from mid-blastula embryos to mature into gastrula-like stages in culture. The explants were dissociated into cell suspensions and their ability to differentiate into mesoderm in response to Activin was tested. At the mid-gastrula stage, the explants suddenly lost responsiveness to Activin. This loss was so complete, that it raised the suspicion that a general block in the Activin pathway was at play. Confirming this suspicion, it was found that Smad2 in these cells no longer translocates to the nucleus in response to Activin. Furthermore, this was correlated with an increase in Smad2 phosphorylation [Grimm and Gurdon 2002].

Previous work, also by Kretzschmar and colleagues, had shown that activation of the Ras/MAPK pathway leads to Smad2 phosphorylation at several sites in the linker region, resulting in nuclear exclusion of Smad2 and resistance to TGFβ signaling [Kretzschmar et al. 1999]. To investigate the involvement of this mechanism in the loss of mesoderm competence, Grimm and Gurdon used explants expressing a mutant Smad2 construct lacking MAPK sites in the linker. When expressed in ectoderm explants, this mutant Smad2 was able to translocate into the nucleus in response to Activin at the gastrula stage. Not only that, this mutant extended the ability of ectoderm cells to undergo mesoderm differentiation by Activin. Thus, loss of mesoderm competence in the ectoderm explants is mediated by linker phosphorylation and nuclear exclusion of Smad2 [Grimm and Gurdon 2002].

These insights suggest that during early gastrulation, there are periods of MAPK-mediated Smad desensitization that allow the emergence of Smad-suppressed cell fates. Gratifying as this progress is, it also raises a number of important questions. The first is regarding when and where these events may be taking place in the embryo. During Xenopus embryogenesis, MAPK becomes progressively activated in the mesoderm [marginal zone] dorsally and then ventrally at the blastula stage, engulfing the posterior neuroectoderm and the dorsal endoderm during early gastrulation and receding to the blastopore lips later in gastrulation [Schohl and Fagotto 2002, Fig. 1]. The activated MAPK overlaps areas of receptor-phosphorylated Smad1 and Smad2 in the mesoderm and endoderm, and to a lesser extent, receptor-phosphorylated Smad1 in the ectoderm [Schohl and Fagotto 2002]. As Smad2 signaling is important for mesoderm formation [De Robertis et al. 2000], questions arise about how Smad2 may be protected from MAPK-mediated inhibition in the mesoderm of the blastula stage [Fig. 1]. By necessity, functional studies were performed with embryos ectopically overexpressing Smad proteins [Pera et al. 2003] or with embryo explants in culture [Grimm and Gurdon 2002]. The experimental conditions in both systems are likely to distort the temporal and quantitative aspects of these processes. Therefore, we do not know whether Smads are always accessible to activated MAPK, or whether MAPK-phosphorylated Smads are always barred from the nucleus in vivo. In situ analysis of MAPK-phosphorylated Smads is needed in order to address these questions.

How phosphorylation of the linker region may inhibit nuclear import, or stimulate export, is an enigma as well. Linker phosphorylation does not seem to prevent receptor-mediated Smad phosphorylation [Kretzschmar et al. 1997a; Wicks et al. 2000], although this needs to be analyzed in more detail. An interference of the phosphorylated linker with nuclear translocation is possible. Smads 2 and 3 can undergo nuclear import and export on their own by directly binding to the nucleoporins Nups215 and Nups153 [Xu et al. 2002, 2003] and Smad3 binds importin β [Xiao et al. 2000, Kurisaki et al. 2001]. Smad subcellular localization is controlled by interaction with cytoplasmic and nuclear retention factors [Shi and Massagué 2003]. Therefore, a phosphorylated linker region could cause cytoplasmic retention by blocking Smad interactions with the nuclear pore complex, or by increasing the affinity of Smads for a cytoplasmic anchor or a nuclear export molecule.

There are also questions about the selectivity of MAPK action on Smad1 and Smad2. In the context of Xenopus gastrulation, it may be important to silence both BMP/Smad1 signaling and Nodal/Smad2 signaling for the purpose of giving neural and dorsal tissues a chance to emerge. However, silencing both pathways simultaneously may not always be the goal. In fact, the configuration of phosphorylation sites in the linker region of Smad1/5/8 and Smad2/3 are quite different. The Smad1-type linkers contain a cluster of four PXSP sequences, which are classical MAPK sites, whereas the Smad2-type linkers contain one PXTP followed by three
XXSP sequences. Therefore, the two Smad subtypes may differ in their recognition by different members of the MAPK family, and this might lead to the silencing of one Smad subtype while sparing the other. A hint of this was initially reported in *Xenopus* (Grimm and Gurdon 2002), but it subsequently could not be confirmed (Pera et al. 2003). Nonetheless, it remains a valid possibility. Furthermore, Smad inhibition by nuclear exclusion may occur via other protein kinases acting on selective sites in the linker region, as in the case of Smad2 phosphorylation by Ca-calmodulin-dependent kinase II (Wicks et al. 2000).

Finally, additional factors may be controlling the workings of the MAPK–Smad link. Extensive variability has been observed in the extent of Smad nuclear exclusion caused by the Ras/MAPK pathway in different cell types. In some cases, Ras/MAPK can inhibit Smad2 nuclear accumulation only against submaximal doses of TGFβ (Kretzschmar et al. 1999); in other cases, it is not effective at all (Lehmann et al. 2000). In *Xenopus*, Smad2-dependent formation of mesoderm in the blastula stage appears to occur in regions of peak MAPK activation (Schohl and Fagotto 2002). This raises the interesting possibility that MAPK access to Smads, or the access of MAPK-phosphorylated Smads to nuclear exclusion mechanisms, may be controlled by rate-limiting factors (X and Y in Fig. 2). The paradox that Ras/MAPK and Smad signaling may cooperate in some instances (Whitman 1998; Lehmann et al. 2000) while acting in opposition in others, could be eventually resolved by answering the above questions.

Although much remains to be learned about this subject, the new evidence suggests that MAPK-dependent phosphorylation of the Smad linker region and nuclear exclusion are critical for cell fate determination during *Xenopus* gastrulation. As pointed out by Pera et al. (2003), Smad linker phosphorylation could be playing a similar role in other confrontations between the BMP and FGF pathways, such as in the antagonism between FGF10 and BMP4 during lung morphogenesis (Weaver et al. 2000), FGF8 and BMP4 during tooth development (Neubuser et al. 1997), FGF2 and BMP4 during cranial suture closure (Warren et al. 2003), and in the classical case of FGF4 and BMP2 antagonism during limb bud formation (Niswander and Martin 1993). MAPK-mediated linker phosphorylation of Smads 2 and 3 may confer resistance to TGFβ-mediated growth inhibition in cancer cells harboring a hyperactive Ras/MAPK pathway (Kretzschmar et al. 1997a; Siegel and Massagué 2003). At any rate, the new work validates the concept that this region of Smad acts as a linker in more ways than one. The stage, therefore, is set for a further exploration of this important link in the future.

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