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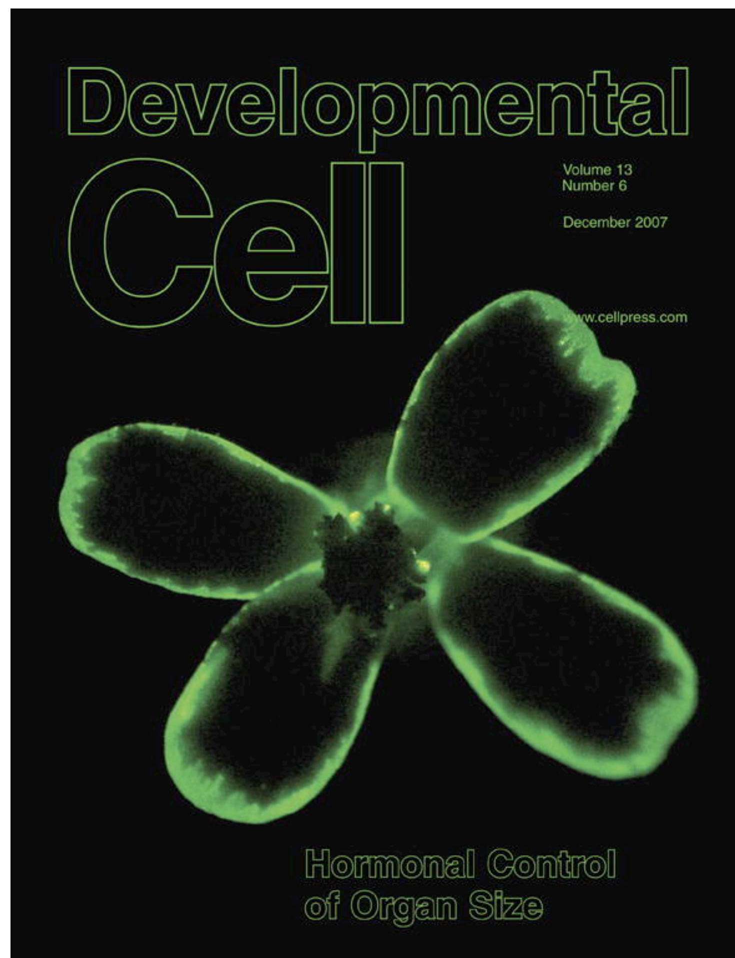

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Opposing Effects of Wnt and MAPK on BMP/Smad Signal Duration

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Signaling downstream of BMP receptors relies on activated nuclear Smad proteins. Recent studies, one of which is published in the November 30 issue of *Cell*, shed light on a mechanism that balances inputs from both activated MAPK and Wnt pathways to regulate the proteasomal degradation of Smad1 and thus modulate the extent of BMP signaling.

It has long been appreciated that multiple signaling pathways coexist within cells during development. Many examples have been presented in which these pathways can influence each other's behavior through crosstalk. The puzzle that remains for investigators is to determine the extent and relevance of such crosstalk.

The bone morphogenetic proteins (BMPs) play essential roles during embryogenesis, tissue patterning, proliferation and apoptosis. Abnormal BMP activity contributes to numerous abnormalities including cancers (Waite and Eng, 2003). With most essential signaling pathways, multiple levels of regulation have evolved to ensure adequate signaling at appropriate times and the cessation of signaling when required. Upon BMP ligand stimulation, the BMP receptor kinases phosphorylate R-Smads, such as Smad1 (Figure 1). This C-terminal activating phosphorylation (pSmad1^{Cter}) promotes complex formation with the Co-Smad Smad4, subsequent nuclear retention, and DNA binding, leading to expression of BMP target genes. Recent studies revealed the presence of nuclear phosphatases that can remove the C-terminal receptor phosphorylation and inactivate Smads, leading to them being shuttled out of the nucleus, yet also allowing possible reactivation (Chen et al., 2006). The cycle of phosphorylation and dephosphorylation provides a mechanism by which Smads monitor BMP receptor activity.

In addition, phosphorylation of Smads by MAP kinases (MAPKs) such as Erk, Jnk, and p38 can inhibit Smad function in vitro and in vivo (Kretzschmar et al., 1997; Aubin et al., 2004). MAPKs can phosphorylate conserved serine and threonine sites (pSmad1^{MAPK}) in the linker regions connecting the MH1 and MH2 domains (Figure 1). Linker phosphorylation strongly decreases nuclear accumulation of Smads and therefore inhibits expression of BMP target genes. Thus, the activity of the BMP pathway can be tempered by activated MAPKs downstream of FGF or Ras signaling. Recently, the MAPK-like Nemo kinase (Nlk) was also shown to phosphorylate a *Drosophila* Smad (known as Mad), promoting its nuclear export and inhibiting BMP pathway activity (Zeng et al., 2007). These studies reveal that phosphorylation events, distinct from pSmad^{Cter}, can have a negative impact on BMP signaling.

Another mechanism to regulate Smads involves Smurf1, a member of the E3 ubiquitin ligase family that targets proteins for ubiquitination and proteasomal degradation (Zhu et al., 1999). Smurf1 can target BMP Smads for degradation in a manner that is independent of receptor activity. Earlier this year, Sapkota et al. (2007) found that Smurf1 selectively binds pSmad1^{MAPK} and polyubiquitinates the protein, leading to its degradation (Figure 1). Smurf1 was also found to interfere with nuclear entry of Smads, resulting in BMP signal attenuation through either degradation or cytoplasmic retention of Smads.

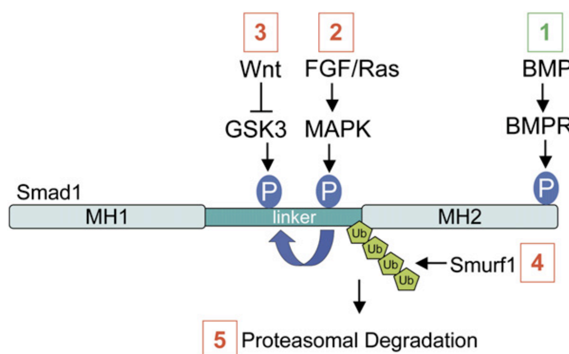

Figure 1. Sequential Events in Smad1 Degradation
Activation of BMP receptor kinase activity leads to C-terminal phosphorylation of Smad1 (1). Activated MAPKs downstream of multiple inputs such as FGF or Ras can phosphorylate the linker domain (2), which provides a primed substrate for GSK3, which in turn is active in the absence of Wnt signaling (3). Smurf1 recognizes the doubly phosphorylated linker domain of Smad1 and polyubiquitinates (Ub) it (4), targeting Smad1 for degradation in centrosomes (5).

Another layer of complexity in Smad regulation is provided in a new report in *Cell*, in which De Robertis and colleagues propose that Smad1 degradation requires not only pSmad1^{MAPK} phosphorylation but subsequent GSK3 phosphorylation (pSmad1^{GSK3}) in the linker domain (Fuentealba et al., 2007) (Figure 1). Both modifications appear to be required for Smad1 ubiquitination and degradation, and consequently, downregulation of Smad1 activity. A version of Smad1 in which

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either MAPK or GSK3 target sites were mutated resulted in gain-of-function phenotypes upon mRNA injections into *Xenopus* embryos, and showed higher gene expression in BMP reporter assays. Inhibitors specific to MAPK, GSK3, or the proteasome all stabilized pSmad^{Cter}. Antibodies that recognize the pSmad^{Cter}, pSmad^{MAPK}, and pSmad^{GSK3} forms of Smad1 proved to be particularly useful in following the dynamics and subcellular localization of Smad1. Fuentealba et al. propose that BMP stimulation triggers three sequential phosphorylation events, thereby limiting the duration of Smad1 activation. While pSmad^{Cter} was detected in the nucleus after BMP4 treatment, pSmad^{MAPK} was seen in the nucleus and centrosomes, where the proteasomal machinery is enriched. pSmad^{GSK3} strongly stained cytoplasmic microtubule structures that converged on the centrosomes. Upon treatment with a proteasome inhibitor, all pSmad forms were enriched in centrosomes, supporting the model that Smad1 is targeted to the centrosomal proteasome, where it is degraded. Whether pSmad^{Cter} is a required event for subsequent phosphorylations remains to be seen, as C-terminal mutants resistant to receptor phosphorylation can still be targeted for degradation by Smurf1 (Fuentealba et al., 2007; Sapkota et al., 2007).

Fuentealba et al. (2007) also demonstrate that activation of Wnt signaling or inhibition of GSK3 can abrogate Smad1 inhibition. GSK3 is a constitutively active, multifunctional kinase that plays roles in Wnt and Hedgehog signaling, insulin signaling through PI3K, and numerous other processes (Forde and Dale, 2007). In the Wnt pathway it participates in the destruction complex that targets β-catenin for ubiquitination and degradation. Wnt signaling inhibits GSK3, allowing β-catenin stabilization and activation of target genes with Tcf/Lef transcription factors. Treatment of cells with Wnt3a can inhibit GSK3 and lead to stabilized pSmad1^{Cter}, with a decrease in pSmad1^{GSK3}. In *Xenopus* embryos neural tissue differentiates when BMP signaling is inhibited, while epidermis forms upon high BMP signaling. Expression of the Wnt pathway inhibitor Dickkopf1 (Dkk1) causes neuralized phenotypes like those caused upon inhibition of BMP (Niehrs et al., 2001). Fuentealba et al. showed that injection of either Wnt morpholinos (MO) or Dkk1 mRNA resulted in expansion of neural tissues, and that this effect required intact GSK3 phosphorylation sites in Smad1. Furthermore, elevated Wnt or Dkk1 MO could induce epidermis, and this effect required active BMP/Smad signaling.

While the studies of Fuentealba et al. (2007) and Sapkota et al. (2007) report comparable findings, there are some discrepancies that highlight unresolved questions. Fuentealba et al. propose that GSK3 phosphorylation is a critical step toward degradation, while Sapkota et al. find that GSK3 phosphorylation is not essential, but acts to enhance Smurf1 binding and ubiquitination of Smad1. While it appears that pSmad1^{Cter} phosphorylation is not essential for subsequent MAPK and GSK3 phosphorylation, the nuclear enrichment of Smad1 seen upon pSmad1^{Cter} phosphorylation may enable interaction with nuclear MAPKs, facilitating subsequent degradation. Interestingly, both groups find that total amounts of Smad1 are unchanged in experimental conditions, showing that the pools of degraded Smad1 represent only a small proportion of total protein. It remains to be seen how the MAPK/GSK3 induced degradation integrates with the phosphatase-mediated downregulation of Smads.

These findings shed light on a mystery that has puzzled *Xenopus* embryologists—namely, how loss of either BMP or Wnt can trigger the neural program, even though they have distinct requirements in patterning. The integration of negative regulation by MAPK and GSK3 with the positive effects of BMP and Wnt illustrates the interplay of multiple pathways regulating Smad1 during development.

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