Supplemental Data

Integrating Patterning Signals:

Wnt/GSK3 Regulates the Duration

of the BMP/Smad1 Signal

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Supplemental Experimental Procedures

Additional Cell Staining Methods

For immunostainings, Cos7 were grown on Lab-tek II 2-well chamber slides (Nalge Nunc). Cells were fixed in fresh paraformaldehyde (4% in PBS for 15 min), followed by permeabilization with 0.2% Triton X100 in PBS for 10 min. The antibodies also worked well after fixation in pure methanol at -20°C for 6 min followed by PBS washes and blocking solution (5% goat serum and 0.5% BSA in PBS). Primary antibodies were diluted in 1:5 blocking solution:PBS and 500 μl applied to each slide chamber, incubated overnight at 4°C, washed 2x in PBS, followed by 2x blocking solution washes, before applying secondary Alexa 488-conjugated anti-rabbit (1:1000; Molecular Probes) or Cy3-conjugated anti-mouse (1:500; Jackson Labs) for 1 hr at room temperature. After washing 3x in PBS, the slides chambers were removed, and the glass slides mounted on DAPI-containing Vectashield (Vector).

Supplemental Reference

Onai, T., Sasai, N., Matsui, M., and Sasai, Y. (2004). Xenopus XsalF: Anterior neuroectodermal specification by attenuating cellular responsiveness to Wnt signaling. Dev. Cell *7*, 95-102.

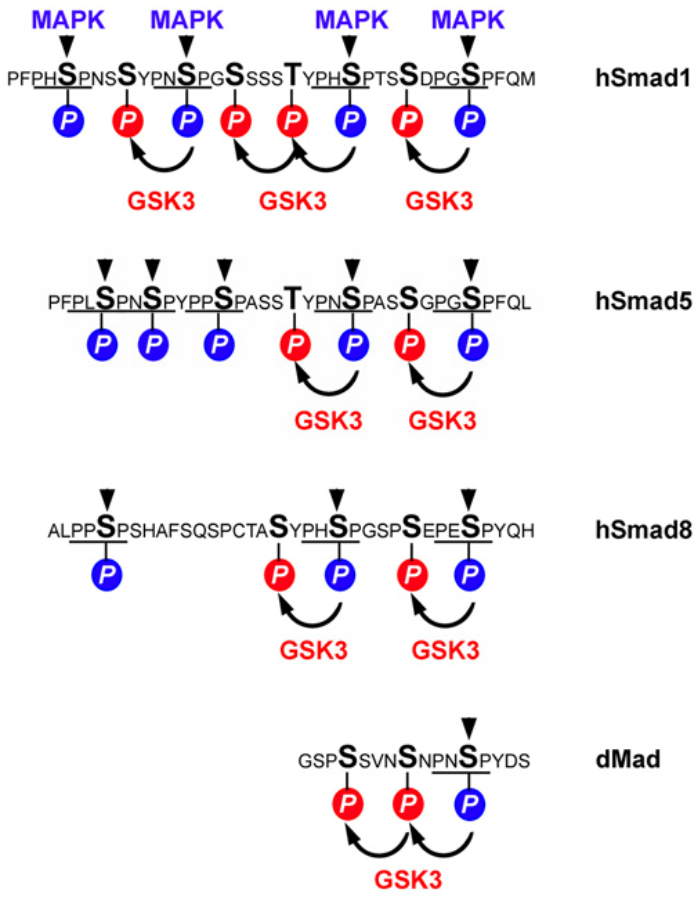

Figure S1. **Conserved Potential GSK3 Phosphorylation Sites**

GSK3 sites (in red) are present in Ser/Thr residues located four amino acids upstream of the MAPK sites (PXSP, in blue) in the linker (middle) region of human Smad1/5/8 as well as in their *Drosophila* homologue Mad. The GSK3 sites are conserved throughout vertebrate BMP-Smads, as well as in Smad4 (not shown). Note that *Drososphila* presents a particularly favorable situation for experimental analysis, since it has a single Mad transcription factor, instead of three as in vertebrates, with a single MAPK site and only two GSK3 phosphorylation sites. Studies in *Drososphila* Mad are currently being pursued (E.E., L.C.F., J. Clemens and E.M.D.R.).

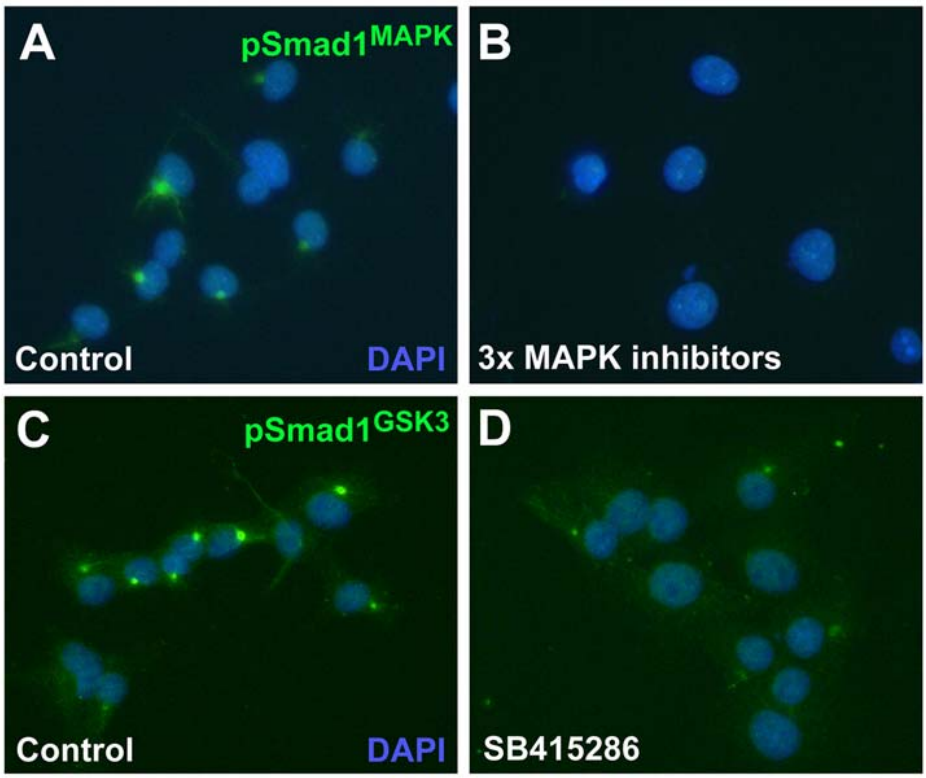

Figure S2. Phospho-Smad1^{MAPK} and Phospho-Smad1^{GSK3} Antigen Localization in the Centrosome Requires MAPK and GSK3 Activities

(A) pSmad1^{MAPK} antigen immunostains a single bright spot corresponding to the centrosome in Cos7 cells. Note that microtubule-like fibers converge on the centrosome and are also stained. Centrosomal staining is strongest in sparse, non-confluent cell cultures. DAPI stains the nuclear DNA in blue.
(B) Antibody staining of pSmad1^{MAPK} was blocked by triple inhibition of cellular MAPK enzymes. A cocktail of three inhibitors targeting: 1) MEK, the MAPKK that activates Erk (10 μM UO126), 2) p38 (10 μM CFPD p38 inhibitor) and 3) JNK (25 μM SP600125) when added to Cos7 cells for 2 hours. All inhibitors were obtained from Calbiochem. This experiment demonstrates that the pSmad1^{MAPK} antigen centrosomal localization requires MAPK activity.
(C-D) Centrosomal localization of pSmad1^{GSK3} is inhibited by treatment with the chemical GSK3 inhibitor SB415286 (BIOMOL) at 40 μM for 2 hours.

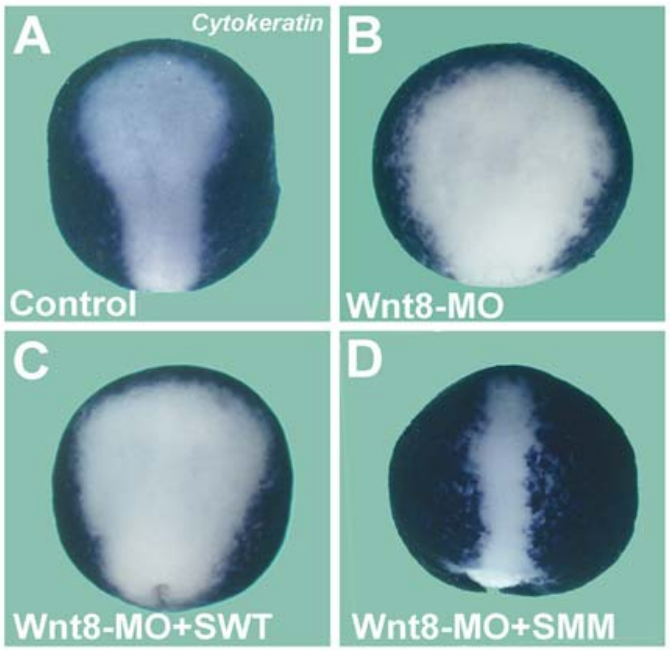

Figure S3. Phosphorylation-Resistant Mutation of the MAPK Sites in Smad1 Are Able to Counteract the Dorsalizing Effects of Wnt8 Depletion in *Xenopus*

(A) *Cytokeratin* probe delineates the neural plate at stage 13 (n= 30).
(B) xWnt8 MO (Lee et al., 2006) expands the neural plate at the expense of epidermis (n= 24).
(C) Smad1-WT mRNA is unable to inhibit the expansion of the neural plate caused by xWnt8 depletion (100%, n=16).
(D) Co-injection of Smad1-MM mutated at the four MAPK sites is able to overcome the neural plate expansion caused xWnt8 depletion (100%, n= 40). This result shows that the inactivation of the sites that prime GSK3 phosphorylations in Smad1 produce identical phenotypes to those caused by Smad1-GM mRNA (Figure 6P).

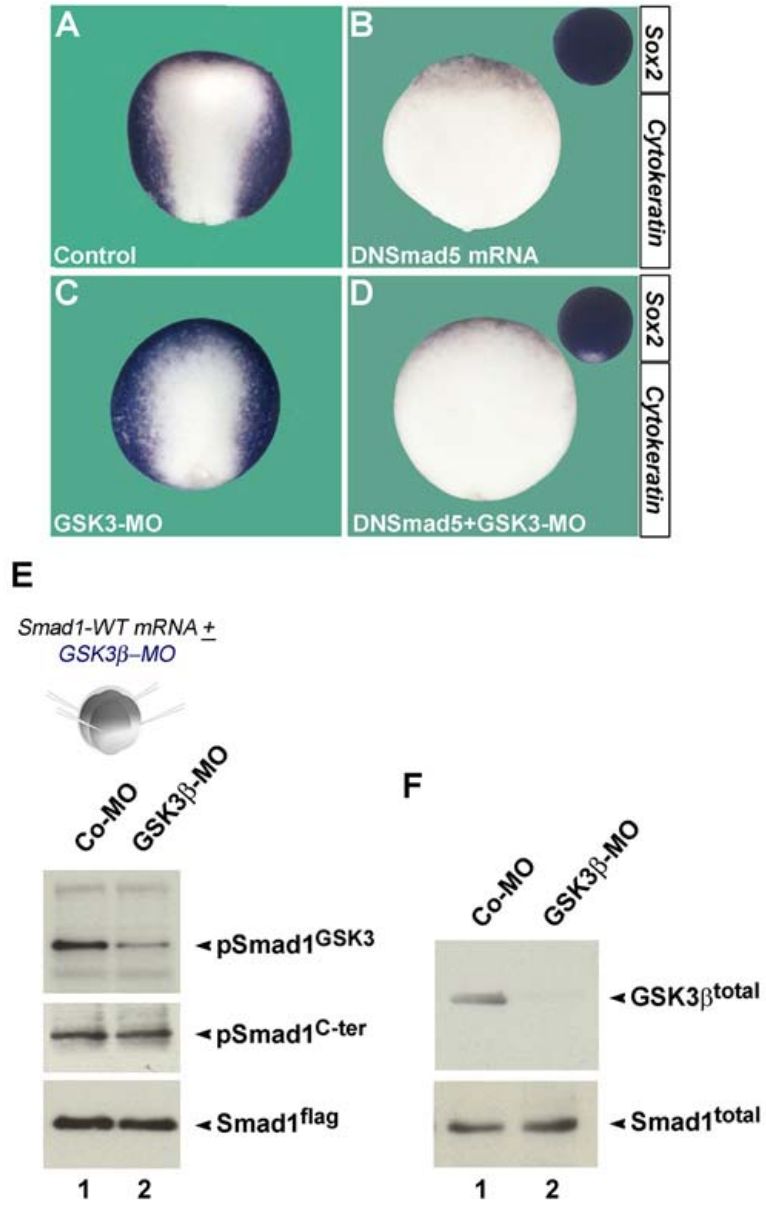

Figure S4. GSK3β MO Reduces the Neural Plate and Expands Epidermis in Wild-Type Embryos but It Is without Effect in Embryos Dorsalized by Dominant-Negative-Smad5; GSK3β Phosphorylates Smad1 In Vivo

(A, B) DN-Smad5 prevents epidermal differentiation (n=18) except for some very weak *Cytokeratin* staining (which can be eliminated by coinjection of ADMP MO as in Figure 7D). The entire ectoderm stains positive for the neural marker *Sox2* (100%, n=20).

(C) Microinjection of 8.5 ng of GSK3β MO reduces the anterior neural plate and correspondingly expands the epidermal territory (82%, n=11), as previously reported by Onai et al. (2004).

(D) DN-Smad5 is epistatic over GSK3β MO (100%, n=18). The entire ectoderm remains neural.

(E) GSK3β MO specifically inhibits the phosphorylation of Ser-210 in microinjected flag-Smad1-WT in stage 10 *Xenopus* embryos. This experiment demonstrates that Smad1 is indeed phosphorylated by GSK3β in vivo.

(F) GSK3β MO inhibits the translation of endogenous *Xenopus* GSK3β protein (assayed with anti-GSK3β antibody from BD Transduction Laboratories at 1:1000 dilution), but not that of total endogenous Smad1 protein which serves as a loading control. Embryos were injected with GSK3β MO at 4-cell and cultured until stage 12 (late gastrula).

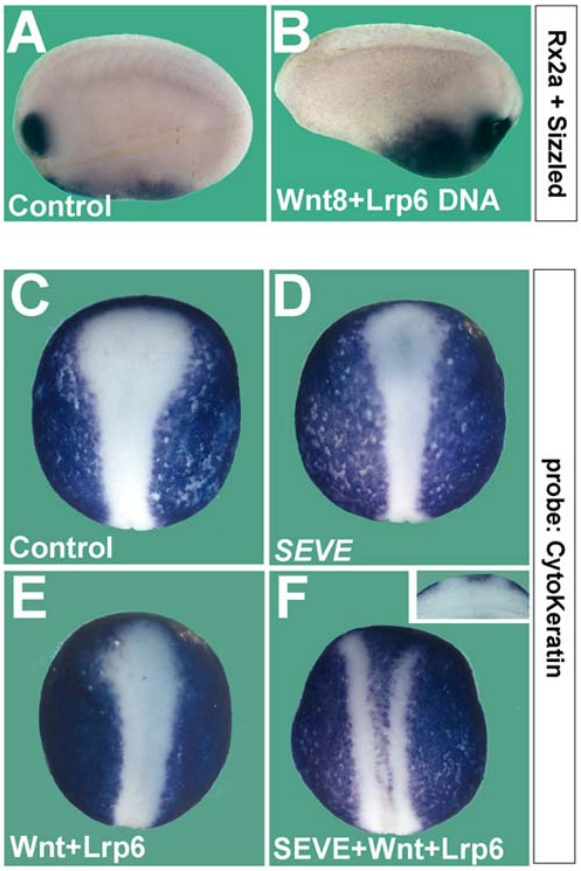

Figure S5. Coinjection of Wnt8 and LRP6 DNA Causes Ventralization of the *Xenopus* Embryo and Synergizes with CA-Smad1-EVE to Induce Epidermis in the Floor Plate Region

(A,B) Microinjection of 30 pg pCSKA-Wnt8 (Christian and Moon, 1993) together with 10 pg of pCS2-LRP6 (Tamai et al., 2004) DNA strongly ventralized the *Xenopus* embryo (95%, n=19). Note that expression of the forebrain/eye marker *Rx2a* is decreased or absent, while the ventral center gene *Sizzled* is increased. This phenotype is typical of *Xenopus* embryos with increased BMP signaling levels (as seen, for example, in Chordin or Sizzled depleted embryos, Lee et al., 2006). Preliminary titrations showed that the response to Wnt8 DNA expressed at the gastrula stage was stronger when its receptor LRP6 is also co-expressed (data not shown).

(C-E) The size of the neural plate, outlined by the epidermal marker *cytokeratin*, is decreased by injection of the constitutively active Smad1 phospho-mimetic mutant Smad1^{EVE} (SEVE) mRNA or Wnt8 plus LRP6 DNA (n=10, 5 and 10 respectively).

(F) When SEVE, Wnt8 and LRP6 mRNAs were coinjected, the embryos were strongly ventralized and the floor plate expressed ectopic cytokeratin in the neural midline (n=12, of which 50% had ectopic epidermis in the midline of the neural plate, see inset). This result provides strong evidence that Wnt8 signals through Smad1 to induce ectopic epidermis in the neural plate midline. This is because Wnt/LRP6 does not induce epidermis in the floor plate in the absence of injected BMP-independent activated Smad1 (SEVE). The only other case in which we have observed this very unusual phenotype of epidermis in the floor plate was in embryos injected with SEVE mRNA mutated either in the MAPK or GSK3 sites (data not shown).