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Report

Wnt11/5a Complex Formation Caused by Tyrosine Sulfation Increases Canonical Signaling Activity

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Summary

Wnt signaling plays important roles in embryonic development, tissue differentiation, and cancer [1–7]. In both normal and malignant tissue, Wnt family members are often expressed combinatorially [8–11], although the significance of this is not understood. We recently showed that Wnt11 and Wnt5a are both required for the initiation of embryonic axis formation and that the two proteins physically interact with each other [12]. However, little is known about the mechanism or biological significance of Wnt-Wnt protein interaction. Here we show in three assays, with *Xenopus* oocytes, mouse L cells, and human embryonic stem cells, that secreted *Xenopus* Wnt11/5a complexes have more canonical Wnt signaling activity than secreted Wnt11 or Wnt5a acting alone. We demonstrate that the sulfation activity of tyrosylprotein sulfotransferase-1 (TPST-1) is required for *Xenopus* dorsal axis formation and that O-sulfation of specific tyrosine residues is necessary for the interaction of Wnt11 with Wnt5a and for enhanced canonical signaling activity. These findings demonstrate a novel aspect of Wnt biology—Wnt family member interaction that depends on tyrosyl sulfation.

Results and Discussion

We showed previously that Wnt11 and Wnt5a interact with each other and that both maternal Wnt11 and Wnt5a are essential for the activation of both canonical and noncanonical Wnt signaling required for *Xenopus* dorsal axis formation [12]. Here, we first showed that the depletion of both *Wnt11* and *5a* mRNA (3 ng + 3 ng antisense oligo) caused a more extreme loss of axis formation and canonical Wnt target gene expression (*Xnr3* and *Xnr5* mRNA levels) than either *Wnt11* (3 ng) or *5a* (3 ng) mRNA depletion alone (Figure 1A). Six nanograms of either Wnt11 or 5a antisense oligonucleotide caused complete ventralization similar to 3 ng Wnt11 oligo + 3 ng Wnt5a oligo. Next, to answer the question of whether Wnt11 and 5a proteins are secreted together, we collected conditioned medium from Wnt11-HA or Wnt5a-Myc/Wnt11-HA-coexpressing *Xenopus* oocytes. We demonstrated by coimmunoprecipitation that Wnt11-HA is in a complex with Wnt5a-Myc protein in oocyte conditioned medium (CM) as well as in oocyte lysates (Figure 1C). We noted that immunoprecipitated Wnt proteins and Wnt proteins in conditioned medium have slightly higher molecular weight, presumably as a result of this fraction of synthesized Wnt proteins' undergoing posttranslational modification.

To determine whether secreted Wnt11/5a has biological signaling activity and to compare that activity with individual Wnt11 or 5a protein, we exploited the fact that canonical Wnt signaling has been shown to cause an increase in the formation of aggregates containing Dishevelled, axin, and LRP6 [13]. Here, we used paracrine assays [14, 15] in which *dishevelled-GFP* mRNA (Dvl2-GFP)-expressing *Xenopus* blastula animal caps acted as signal recipients and oocytes injected with *Wnt11-HA* (1 ng), *Wnt5a-Myc* (1 ng), or both (500 pg + 500 pg) acted as Wnt signal provider (Figure 2A). After 1 hr of coculture, animal caps were separated from the oocytes and analyzed for both live and fixed Dvl2-GFP localization. In three experiments, images from live animal caps showed that more Dishevelled protein was recruited to aggregates in the animal caps cocultured with oocytes expressing both Wnts than with either Wnt alone (Figure 2A, left panels). This was confirmed by repeating the experiment and immunostaining fixed animal caps for Dvl2-GFP (Figure 2A, right panels). Quantitation of the pixel intensity of immunostaining showed again that coculture with Wnt11-HA/Wnt5a-Myc-expressing oocytes caused a significant increase in cell membrane-localized Dvl2-GFP as compared to coculture with oocytes expressing either Wnt alone (Figure 2B).

Next, we addressed whether combinatorial signaling by these two family members might have broader biological importance by testing canonical signaling responsiveness in mouse L cells and in a human embryonic stem (hES) cell line (H9/WA09). We collected conditioned medium from batches of oocytes injected with *Wnt11-HA* mRNA, *Wnt5a-Myc* mRNA, or both and also examined whether mixing individual Wnt11 and Wnt5a conditioned medium together affected signaling activity. We used culture medium from uninjected oocytes [CM(blank)] as a negative control. After 3 hr incubation with conditioned medium, L cells were fixed and stained for β-catenin (red) and F-actin (green) (Figure 2C) or harvested for western blotting to analyze cytosolic and total β-catenin levels (Figure 2D). After excluding the membranous cadherin-bound β-catenin pool, we used the cytoplasmic β-catenin level as a readout of canonical Wnt signaling [16]. The cytosolic β-catenin pool was reproducibly increased in L cells treated with Wnt5a-Myc/Wnt11-HA conditioned medium compared to cells treated with either Wnt11-HA or Wnt5a-Myc conditioned medium alone or cells treated with Wnt11 medium mixed with Wnt5a medium (Figures 2C and 2D).

Similarly, when hES cells were incubated for 24 hr with conditioned medium, those exposed to the Wnt11-HA/Wnt5a-Myc complex showed enhanced β-catenin protein levels by immunostaining as compared to those treated with single-Wnt-containing conditioned medium (Figure 2E; β-catenin is green; DAPI-stained nuclei are red). The expression level of a known target of canonical Wnt signaling, *Brachyury* [17–19], was also increased in hES cells that were cultured in Wnt11-HA/Wnt5a-Myc complex-containing medium compared to nonconditioned medium or medium containing only Wnt11-HA or 5a-Myc (Figure 2F).

The finding that conditioned medium containing Wnt11 mixed with medium containing Wnt5a does not have enhanced canonical signaling activity compared to Wnt11 medium alone suggests either that Wnt11/5a complex formation occurs

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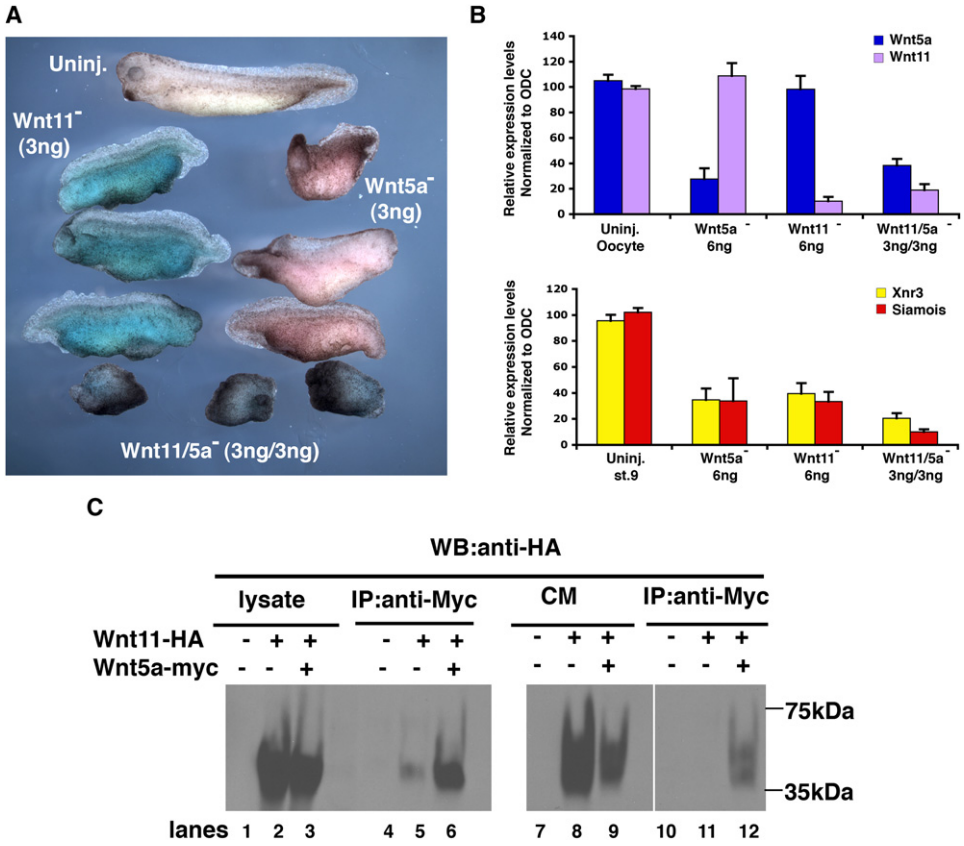

Figure 1. Wnt11/Wnt5a Biological and Physical Interaction

(A) Phenotype of tailbud-stage embryos derived from sibling control (Uninj.), Wnt11-depleted (blue, 3 ng antisense oligo), Wnt5a-depleted (red, 3 ng antisense oligo), or Wnt11/5a-depleted (3 ng Wnt11 + 3 ng Wnt5a antisense oligos, purple).

(B) Upper panel: real-time RT-PCR showing the relative expression of maternal *Wnt11* and *Wnt5a* mRNAs in mature uninjected oocytes and in oocytes injected with 6 ng of Wnt11 or Wnt5a antisense oligos or 3 ng of both oligos. Lower panel: real-time RT-PCR of late blastula-stage embryos, siblings of those shown above, analyzing the relative mRNA expression levels of the maternal canonical Wnt pathway target genes *Xnr3* and *siamois*. The results are means ± standard deviation (SD) from three independent experiments.

(C) Left: Wnt11-HA physically interacts with Wnt5a-Myc in oocyte lysates overexpressing *Wnt11-HA* mRNA (500 pg) and *Wnt5a-Myc* mRNA (500 pg). Right: secreted Wnt11-HA immunoprecipitated by Wnt5a-Myc from medium conditioned with *Wnt11-HA* mRNA (500 pg) + *Wnt5a-Myc* mRNA (500 pg)-injected oocytes. Secreted or immunoprecipitated Wnt proteins were slightly larger than Wnt proteins in the lysate.

intracellularly before secretion or that another interacting partner is involved that is not present when the two media are mixed together. We tested the latter hypothesis by assessing the roles of known components of the Wnt11 signaling complex involved in *Xenopus* dorsal axis formation. Frizzled 7, LRP6, and FRL1 are all necessary in canonical Wnt11-dependent axis formation [1, 20, 21], and the enzyme exostosin 1 (EXT1) is necessary for the addition of glycosaminoglycan chains on heparan sulfate proteoglycans required for Wnt signaling [1]. However, depletion of each of these mRNAs from *Xenopus* oocytes with antisense oligos did not prevent the formation of Wnt11/5a complexes by coimmunoprecipitation (see Figures S1A–S1D available online).

Next, we examined whether posttranslational modification of Wnt11/5a is required for their interaction. When Wnt11-HA and Wnt5a-Myc proteins were translated in vitro, no interaction was observed in coimmunoprecipitation experiments, suggesting that posttranslational modification is required (Figure S2A). The alteration of N-glycosylation of Wnt protein is known in some contexts to affect its signaling activity and secretion [22]. We generated single and double mutants of the known N-glycosylation sites of Wnt11-HA. Consistent with mouse Wnts [22], Wnt11 N88Q and N229Q single and double (N88,229Q) mutants exhibited lower molecular weight than wild-type protein, caused by loss of glycosylation, and could not be secreted into the medium (Figures S2B and S2C). However, all NQ mutants from cell lysates still formed complexes with Wnt5a-Myc (Figure S2D).

Several secreted proteins are known to interact via sulfated tyrosine residues, including IP-10 with CXCR3 and GPIbα with VWF [23, 24]. Via bioinformatics and previous work on Wnt5a [25], we confirmed that the Wnt11 and 5a proteins each possess two potential tyrosine sulfation sites near their C termini (Figure 3A). Protein alignment with other orthologs showed that one of these sites is conserved in both human and mouse Wnt11 and 5a. To determine whether Wnt11 and 5a are sulfated, we generated mRNAs encoding HA-tagged single- and double-mutant forms of Wnt11 and 5a in which the sulfatable tyrosine residues were replaced with phenylalanines (Wnt11 Y274F and Y281F; Wnt5a Y301F and Y308F; Figure 3A). By immunoprecipitation with anti-sulfotyrosine antibody (Figure 3B and Figure S2F for individual Wnts), we confirmed that wild-type Wnt11-HA (Wnt11-WT-HA) and Wnt5a-Myc (Wnt5a-WT-Myc) are sulfated proteins.

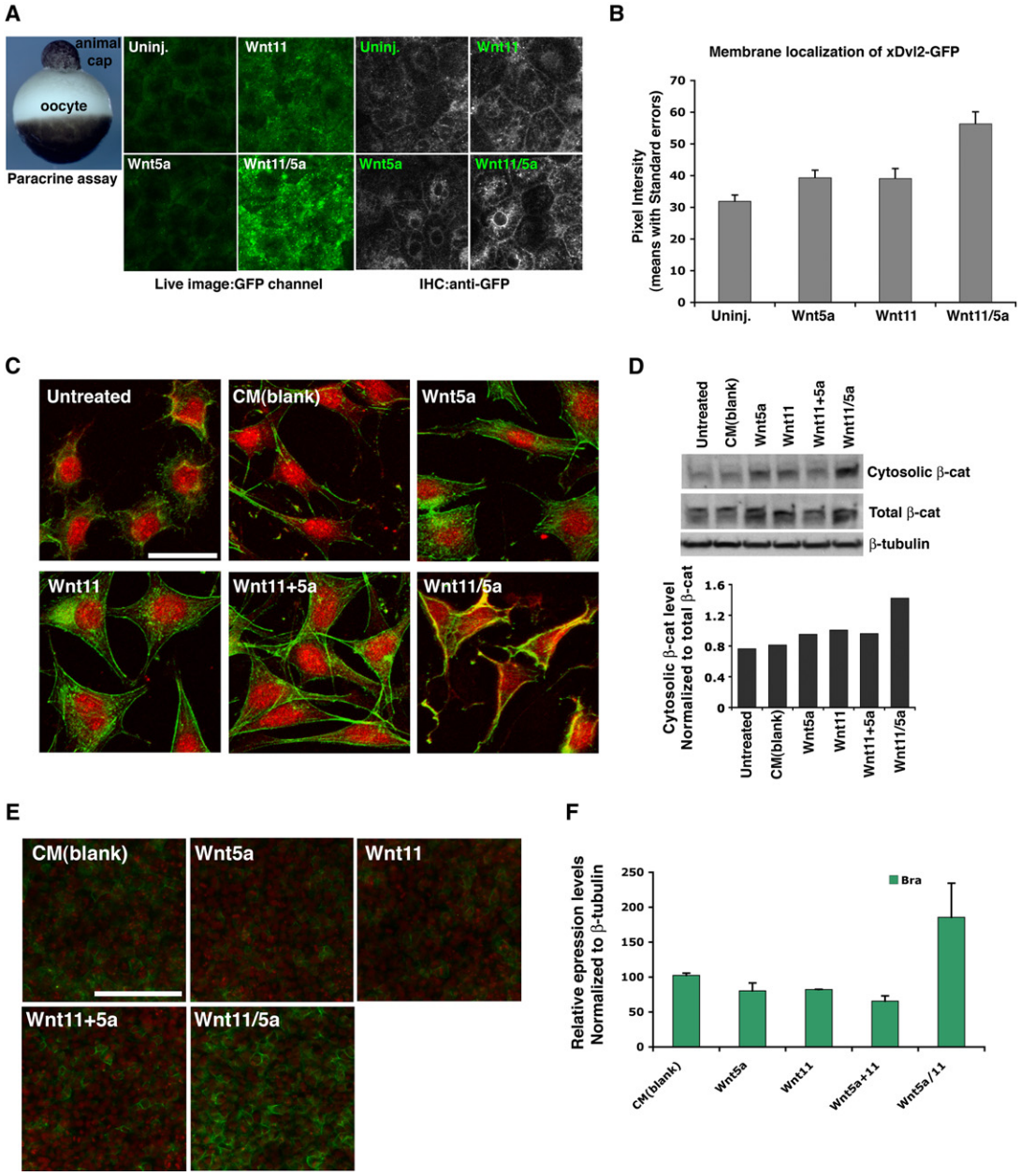

Figure 2. Cosynthesized and Secreted Wnt11 and 5a Have Enhanced Canonical Signaling Activity

(A and B) show *Xenopus* paracrine assays; (C and D) show mouse L cells; (E and F) show human ES (hES) cells.

(A) Leftmost image: example of a paracrine assay where a *dishevelled-GFP* mRNA (Dvl2-GFP)-expressing *Xenopus* blastula animal cap was cocultured for 1 hr with an oocyte injected with either *Wnt11-HA* mRNA (1 ng), *Wnt5a-Myc* mRNA (1 ng), or both (500 pg + 500 pg). Middle four images: exogenous Dvl2-GFP fluorescence was visualized in live animal caps stimulated with control uninjected oocytes (Uninj.) or with oocytes secreting Wnt11, Wnt5a, or both proteins. Rightmost four images: Dvl2-GFP was immunostained with anti-GFP antibody in fixed animal caps treated as above.

(B) Quantitation of Dvl2-GFP immunostaining measured across the cell membranes in animal caps. The results are means ± SD from three independent experiments.

(C) Immunostaining of fixed untreated mouse L cells; L cells treated with conditioned medium only [CM(blank)]; L cells treated with media conditioned by oocytes secreting Wnt5a, Wnt11, or both (Wnt11/5a) ; or L cells treated with a mixture of Wnt11 and 5a conditioned media (Wnt11+5a) stained with anti-β-catenin antibody (red) and phalloidin (green) for F-actin. Scale bar represents 50 μm.

(D) Upper panel: expression levels of cytosolic and total β-catenin protein measured by western blot of lysates of cells from (C). Lower panel: quantitation of cytosolic versus total β-catenin for this experiment. β-tubulin antibody was monitored as a loading control. The experiment was repeated with similar results.

(E) Immunostaining of fixed hES cells treated with conditioned medium from oocytes expressing *Wnt11-HA* mRNA, *Wnt5a-Myc* mRNA, both (Wnt11/5a), or a mixture of conditioned media from Wnt11-HA- and Wnt5a-Myc-expressing oocytes (Wnt11+5a) stained with anti-β-catenin antibody (green) and DAPI to visualize nuclei (red). Scale bar represents 100 μm.

(F) Real-time RT-PCR of hES cells treated as in (E) for 24 hr and analyzed for relative expression levels of *Brachyury* (*Bra*). The experiment was repeated three times with similar results. The results are means ± SD from three independent experiments.

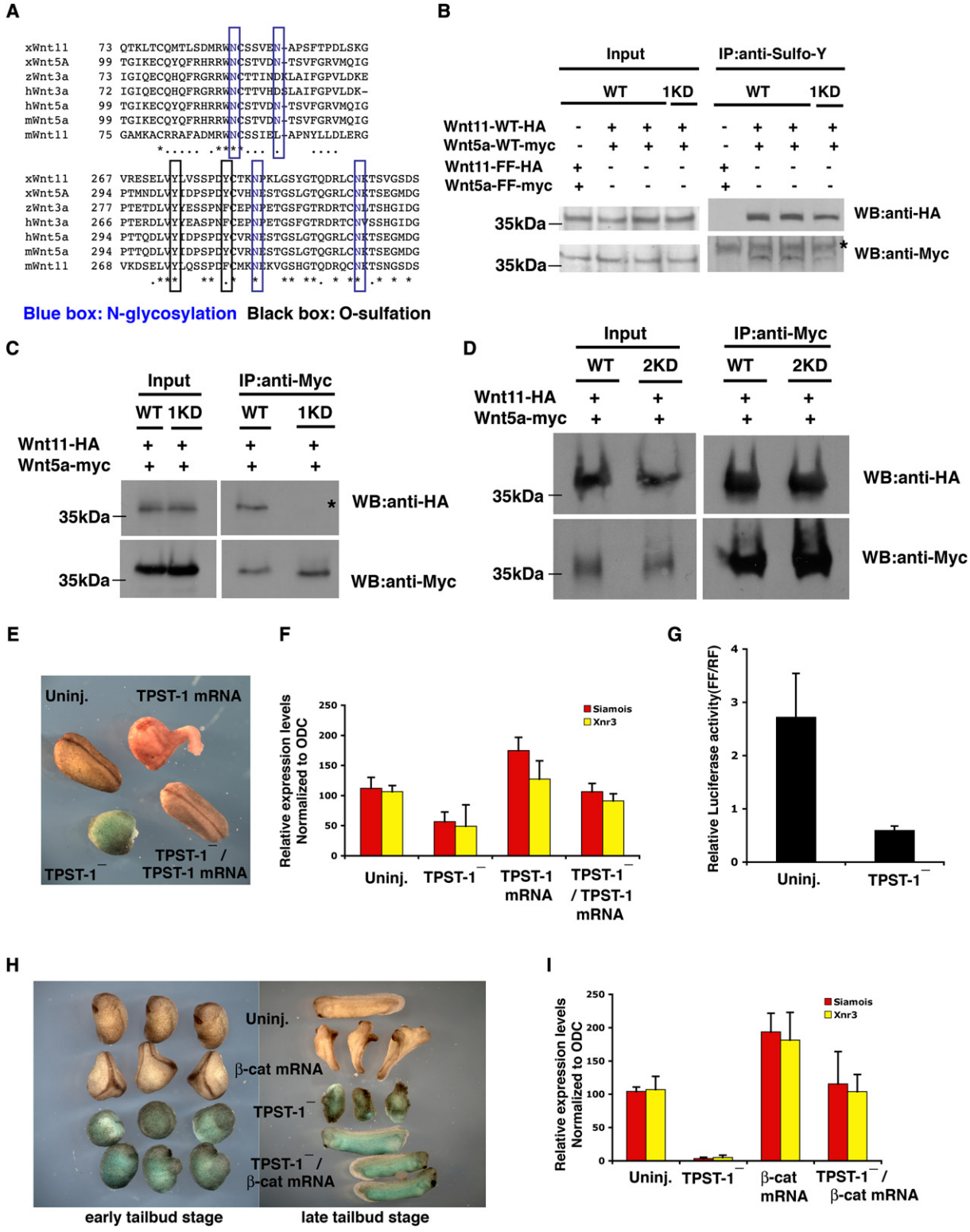

Figure 3. Effects of Tyrosylprotein Sulfotransferase-1 Depletion

(A) ClustalW alignment of partial protein sequences of Wnt family members showing the conserved potential sites of glycosylation (blue) and sulfation (black).

(B) Immunoprecipitation experiment with anti-sulfotyrosine antibody. No detectable Wnt11FF-HA or Wnt5aFF-Myc protein is coimmunoprecipitated by anti-sulfotyrosine antibody (lane 1 in right-hand blot). Asterisk indicates nonspecific IgG band.

(C and D) Immunoprecipitation experiments with lysates of wild-type oocytes and sibling tyrosylprotein sulfotransferase-1 (TPST-1)-depleted (1KD) oocytes (C) or wild-type and TPST-2-depleted (2KD) oocytes (D) injected with *Wnt11-HA* and *Wnt5a-Myc* mRNAs. Wnt11-HA is coimmunoprecipitated with Wnt5a-Myc in lysates from wild-type oocytes and TPST-2-depleted oocytes but is much reduced in TPST-1-depleted oocytes (*). Western blots of the input lysates (Input) show expression levels of the Wnt5a-Myc and Wnt11-HA proteins.

Sulfation on tyrosine is mediated by tyrosylprotein sulfotransferase enzymes in the *trans*-Golgi network, and these enzymes have different target protein sets for tyrosyl sulfation [26, 27]. Because *Xenopus* has two maternally expressed *tyrosylprotein sulfotransferase* genes, we designed antisense oligos to deplete maternal *tyrosylprotein sulfotransferase-1* and *-2* mRNAs without affecting *Wnt11* and *Wnt5a* mRNA levels (Figure S2G). To test whether either tyrosylprotein sulfotransferase-1 or -2 (TPST-1 or -2) is required for Wnt11 and 5a to form complexes, we injected *Wnt11-HA* and *Wnt5a-Myc* mRNAs into TPST-1- or TPST-2-depleted oocytes and performed coimmunoprecipitation experiments. In TPST-1-depleted oocytes, the interaction between Wnt5a-Myc and Wnt11-HA proteins was reproducibly very low compared to that in control sibling oocytes (Figure 3C), suggesting that TPST-1 enzyme activity might be important for this interaction. We confirmed that TPST-1 depletion reduced the ability of Wnt11 and 5a to bind to anti-sulfotyrosine antibody (Figure 3B, lane 4 in right-hand blot; KD = TPST-1 depletion). This suggests that Wnt11 and 5a sulfation is mediated by TPST-1 and that this posttranslational modification is required for their interaction. TPST-2 antisense oligo did not affect the interaction between Wnt11 and 5a (Figure 3D), although the efficiency of the depletion of TPST-2 was only 50%, leaving the possibility that sufficient protein might remain to carry out TPST-2 function. Nevertheless, the fact that TPST-1 depletion reduced Wnt11 and 5a sulfation suggests that it does not act redundantly with TPST-2.

To determine whether the reduction of Wnt11/5a interaction in TPST-1-depleted oocytes correlated with reduced activity of Wnt11/5a in dorsal axis formation, we fertilized control and TPST-1-depleted oocytes and assayed dorsal axis formation. TPST-1-depleted embryos developed the classical ventralized phenotype, similar to Wnt11/5a-depleted embryos (compare Figures 3E and 3H with Figure 1A) and could be rescued by injection of *TPST-1* mRNA. TOPflash analysis and real-time polymerase chain reaction (PCR) analysis of Wnt target gene expression confirmed that TPST-1-depleted embryos had reduced canonical Wnt signaling activity (Figures 3F and 3G). To determine whether the TPST-1 depletion phenotype resulted from the loss of canonical Wnt activity, we tested that whether β-*catenin* mRNA could rescue TPST-1-depleted embryos. Injection of β-*catenin* mRNA into TPST-1-depleted oocytes rescued both the levels of the Wnt target genes *siamois* and *Xnr3* and the ventralized phenotype (Figures 3H and 3I). This suggests that the tyrosine sulfation activity of TPST-1 is critical for normal axis formation, which is regulated by maternal Wnt signaling.

To determine the importance of tyrosine sulfation for Wnt11 and 5a complex formation, we used Wnt11-HA and Wnt5a-Myc mutants in which residues Y274 and Y281 in Wnt11 and Y301 and Y308 in Wnt5a were mutated to phenylalanine (F) (Wnt11FF and Wnt5aFF, respectively). We also generated single mutations of the individual tyrosines, Y281F (Wnt11YF) for Wnt11 and Y308F (Wnt5aYF) for Wnt5a. We first confirmed that the FF forms of Wnt11 and 5a were no longer immunoprecipitated by anti-sulfotyrosine antibody (Figure 3B; Figure S2F). We also showed that these mutants were secreted as efficiently as their wild-type equivalents (Figure 4A). In fact, more Wnt5aFF-Myc protein was detectable than Wnt5a-WT-Myc protein in conditioned medium, suggesting that the sulfation moiety of Wnt5a also affected its secretion profile. Furthermore, the FF forms of Wnt11 and 5a formed homodimers as efficiently as wild-type Wnt11 and 5a did (Figure 4B), and the mutant form of Wnt5a-Myc coimmunoprecipitated with the Xfz7 receptor, suggesting that Wnt sulfation is not a prerequisite for receptor binding (Figure S2G). However, in coimmunoprecipitation analyses, although wild-type Wnt5a-Myc complexed efficiently with Wnt11-HA, all mutant forms showed reduced binding to each other, with single mutants showing less inhibition than double mutants (Figure 4C; Figures S2H and S2I). Under nonreducing conditions, the interaction between Wnt11 and 5a was almost completely abolished when both tyrosines on both proteins were converted to phenylalanines (Figure 4D).

Finally, we investigated whether this mutation affected biological signaling activity by monitoring TOPflash reporter activity in *Xenopus* embryos expressing wild-type Wnt11-HA, Wnt5a-Myc, or both and in embryos expressing FF mutant forms of Wnt11-HA, Wnt5a-Myc, or both. Control TOPflash reporter-injected embryos exhibited activation of TOPflash by endogenous Wnt signaling at the blastula stage (Figure 4E, Uninj). Injection of wild-type *Wnt5a* together with *Wnt11* mRNAs caused activation of canonical Wnt signaling above control levels (Figure 4E), as shown previously [12]. In contrast, embryos coexpressing double sulfation mutants of Wnt11 and Wnt5a exhibited no ability to activate TOPflash above control levels (Wnt5aFF + Wnt11FF, Figures 4E and 4F). Moreover, combinations of wild-type and double sulfation mutants (Wnt5aWT + Wnt11FF or Wnt5aFF+ Wnt11WT) exhibited no ability to activate the TOPflash reporter (Figure S2J). Thus, loss of sulfated tyrosine reduces both the interaction of Wnt11 and 5a and their canonical Wnt signaling activity.

We have demonstrated here that posttranslational tyrosine sulfation of Wnt11 and 5a is responsible for the formation of Wnt11/Wnt5a complexes and that Wnt11/Wnt5a complex formation is required for efficient signaling in the context of *Xenopus* axis formation. We show that such complexes enhance canonical Wnt signaling as compared to individual Wnts when tested on *Xenopus*, mouse, and human cell types. It will be important to determine whether mouse and human Wnt11 and 5a, as well as other Wnt family members, share the ability of *Xenopus* Wnt11 and 5a to interact with each other.

(E–G) TPST-1-depleted embryos show reduced canonical Wnt signaling responses.
(E) Phenotype of sibling control and TPST-1-depleted embryos at the early tailbud stage. All 35 of 35 control embryos were normal (brown), 47 of 49 TPST-1-depleted embryos had a ventralized phenotype (green), 23 of 27 *TPST-1* mRNA-injected embryos had a dorsalized phenotype (red, upper right), and 21 of 34 TPST-1-depleted embryos injected with *TPST-1* mRNA were normal (purple, lower right).
(F) Real-time RT-PCR of late blastula-stage embryos, analyzing relative mRNA expression levels of the maternal canonical Wnt pathway target genes *Xnr3* and *siamois*. The results are means ± SD from three independent experiments.
(G) TOPflash analysis measuring the activation of injected *Tcf3-luciferase* reporter in wild-type versus TPST-1-depleted sibling embryos at the late blastula stage (means ± SD).
(H and I) β-*catenin* mRNA rescues TPST-1 depletion.
(H) Nine of ten control embryos were normal; 23 of 24 TPST-1-depleted embryos had a ventralized phenotype. All 5 of 5 β-*catenin* mRNA-injected embryos had secondary axis; 5 of 11 TPST-1-depleted embryos injected with β-*catenin* mRNA were normal.
(I) Real-time RT-PCR of late blastula-stage embryos, analyzing relative mRNA expression levels of the maternal canonical Wnt pathway target genes *Xnr3* and *siamois*. The results are means ± SD from three independent experiments.

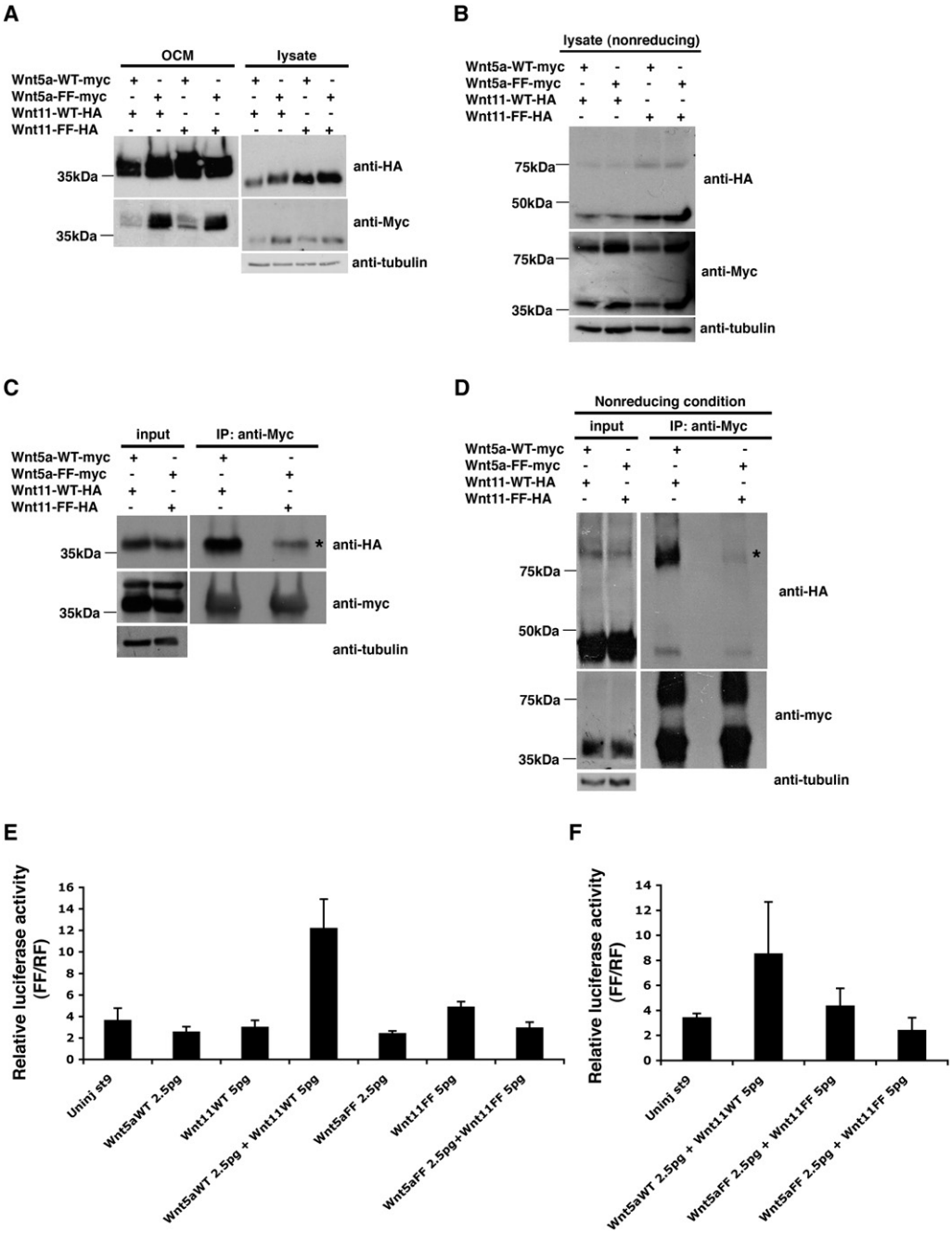

Figure 4. Y-to-F Mutations of Wnt11 and 5a Block Their Physical and Biological Interaction

(A) Western blot of oocyte conditioned medium (OCM) and lysates from oocytes injected with mRNA for wild-type and tyrosine-to-phenylalanine (FF) mutated forms of Wnt11-HA and Wnt5a-Myc.

(B) Western blot under nonreducing conditions showing that Wnt11FF-HA and Wnt5aFF-Myc form homodimers as efficiently as wild-type Wnt11-HA and 5a-Myc do.

(C) Coimmunoprecipitation analyses showing that wild-type Wnt5a-Myc complexes efficiently with Wnt11-HA, whereas Wnt11FF-HA and Wnt5aFF-Myc exhibit reduced interaction (*). Expression levels of the wild-type and mutant proteins are shown by western blotting (Input).

(D) Coimmunoprecipitation as in (C) run under nonreducing conditions, showing the loss of binding between Wnt11FF and Wnt5aFF homodimers (*) compared to wild-type Wnt11-HA and Wnt5a-Myc.

(E and F) TOPflash analysis measuring the activation of injected *Tcf3-luciferase* reporter in sibling embryos at the late blastula stage, injected as oocytes with different doses of wild-type (WT) and/or FF mutant *Wnt11-HA* and *Wnt5a-Myc* mRNAs. The experiment was repeated with similar results (means ± SD).

A second question is whether Wnt11/Wnt5a complex formation increases canonical Wnt signaling activity by increasing the stability of Wnts or by altering their affinity for a receptor or coreceptor. These findings also reveal a novel avenue to explore with regard to Wnt signaling regulation—whether, when two different Wnt family members are expressed together, their interaction plays a role in determining the level of Wnt signaling activity. For example, the potential significance for human

disease processes is that, if gene expression is altered such that new Wnt family members are overexpressed, as occurs in mammary epithelial cells undergoing carcinogenesis [11], the new coexpression and Wnt/Wnt interaction might increase canonical Wnt signaling and alter cell behavior as a result.

Experimental Procedures

Oocytes and Embryos

Oocytes and embryos were cultured and oocyte culture medium (OCM) was generated as described previously [28]. Antisense oligos were injected on day 1, and oocytes were matured on day 3 and fertilized on day 4. Antisense oligos were TPST-1 AS1, 5′-TTGAGTTTTCCAATCATC-3′; TPST-1 AS3, 5′-ACTCCATTGCATGCTGCC-3′; and TPST-2 AS3, 5′-GCTTCATAACACCTCGGT-3′. Antisense oligos for Wnt11, Wnt5a, Lrp6, Frl1, Xfz7, and EXT1 were as described previously [1, 12, 20, 21].

Generation of NQ and YF Mutants

For Wnt11NQ, asparagine (N) 88, N94, and N299 residues were mutated to glutamine (Q). For Wnt11FF, Y274 and Y281 residues were mutated to F from pCS2+/Wnt11-HA. For Wnt5aFF, Y301 and Y308 residues were mutated to F from pSP64T/Wnt5a-Myc. All mutants were generated by PCR-based site-directed mutagenesis (Stratagene). For mRNA, Wnt11 variants were linearized with NotI and transcribed with SP6 polymerase, and Wnt5a variants were linearized with EcoRI and transcribed with SP6 polymerase.

Paracrine Assays

Xenopus Dvl2-GFP (200 pg) was injected into the animal pole at the two-cell stage, and animal caps were dissected at blastula stage. *Wnt5a* and *Wnt11* mRNAs were injected into oocytes, which were cultured overnight. Dissected animal caps were then placed on the Wnt-expressing oocytes [15]. After 1 hr incubation, animal caps were separated from oocytes and immediately examined under a Zeiss LSM 510 confocal microscope for live imaging or fixed for immunostaining.

Cell Lines

The mouse L cell line and the hES cell line (H9/WA09) were maintained according to the suppliers' instructions. Cells were washed three times with phosphate-buffered saline and incubated with conditioned media from Wnt-expressing oocytes. After 3 hr treatment, cells were fixed in 4% paraformaldehyde for immunostaining or harvested for western blotting.

Generation of Conditioned Media

Wnt mRNAs and control oocytes were incubated for 16 hr in heparin-supplemented (final concentration 50 μg/μl) OCM for secretion or dimerization assays (4 ml of OCM was concentrated 100 times by membrane filtration [Ultra-4, Millipore]). For use on mammalian cell lines, CM was prepared from batches of 100 *Wnt* mRNA-expressing or control oocytes by culturing in bovine serum albumin-supplemented Dulbecco's modified Eagle's medium (DMEM) (for L cells) or DMEM/F12 (for hES cells) as above. CM was used immediately.

Quantitative RT-PCR

Total RNA from oocytes, explants, and early embryos was isolated via a previously described protocol [1]. Real-time RT-PCR was performed with a LightCycler (Roche). Water blank and RT-minus controls were included in all runs. All RT-PCR results are presented as percentages of the level in uninjected embryos after normalization to the expression of ornithine decarboxylase (ODC) for *Xenopus* or β-tubulin for hES cells.

Luciferase Assay

TOPflash DNA (50 pg, Upstate) together with pRLTK DNA (25 pg) was injected into two dorsal vegetal cells at the eight-cell stage. Three replicate samples of three embryos each were frozen for each group at the early blastula stage and assayed via the Promega Luciferase Assay System.

Western Blot and Immunostaining

Western blots were performed as described previously [29]. Antibodies used were mouse anti-sulfotyrosine (sulfo-C1-A2, 1:1000, Millipore) and mouse anti-tubulin (DM1B, 1:5000, NeoMarkers). Dimerization assays for YF mutants and coimmunoprecipitations were performed as described previously [12]. β-catenin and GFP immunostaining were performed with 1:200 anti-β-catenin antibody (H-102, Santa Cruz) or 1:250 anti-GFP antibody (sc-8334, Santa Cruz) followed by Cy5-conjugated anti-rabbit secondary antibody (Molecular Probes).

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01488-2.

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References

1. Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C.C., Lin, X., and Heasman, J. (2005). Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. Cell *120*, 857–871.
2. De Calisto, J., Araya, C., Marchant, L., Riaz, C.F., and Mayor, R. (2005). Essential role of non-canonical Wnt signalling in neural crest migration. Development *132*, 2587–2597.
3. Ouji, Y., Yoshikawa, M., Shiroi, A., and Ishizaka, S. (2006). Wnt-10b promotes differentiation of skin epithelial cells in vitro. Biochem. Biophys. Res. Commun. *342*, 28–35.
4. Castelo-Branco, G., Wagner, J., Rodriguez, F.J., Kele, J., Sousa, K., Rawal, N., Pasolli, H.A., Fuchs, E., Kitajewski, J., and Arenas, E. (2003). Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. Proc. Natl. Acad. Sci. USA *100*, 12747–12752.
5. Kurayoshi, M., Oue, N., Yamamoto, H., Kishida, M., Inoue, A., Asahara, T., Yasui, W., and Kikuchi, A. (2006). Expression of Wnt-5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion. Cancer Res. *66*, 10439–10448.
6. Whangbo, J., and Kenyon, C. (1999). A Wnt signaling system that specifies two patterns of cell migration in C. elegans. Mol. Cell *4*, 851–858.
7. Cohen, E.D., Tian, Y., and Morrisey, E.E. (2008). Wnt signaling: An essential regulator of cardiovascular differentiation, morphogenesis and progenitor self-renewal. Development *135*, 789–798.
8. Gregorieff, A., Pinto, D., Begthel, H., Destrée, O., Kielman, M., and Clevers, H. (2005). Expression pattern of Wnt signaling components in the adult intestine. Gastroenterology *129*, 626–638.
9. Saitoh, A., Hansen, L.A., Vogel, J.C., and Udey, M.C. (1998). Characterization of Wnt gene expression in murine skin: Possible involvement of epidermis-derived Wnt-4 in cutaneous epithelial-mesenchymal interactions. Exp. Cell Res. *243*, 150–160.
10. Reddy, S., Andl, T., Bagasra, A., Lu, M.M., Epstein, D.J., Morrisey, E.E., and Millar, S.E. (2001). Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a target of Sonic hedgehog in hair follicle morphogenesis. Mech. Dev. *107*, 69–82.
11. Benhaj, K., Akcali, K.C., and Ozturk, M. (2006). Redundant expression of canonical Wnt ligands in human breast cancer cell lines. Oncol. Rep. *15*, 701–707.
12. Cha, S.W., Tadjuidje, E., Tao, Q., Wylie, C., and Heasman, J. (2008). Wnt5a and Wnt11 interact in a maternal Dkk1-regulated fashion to activate both canonical and non-canonical signaling in Xenopus axis formation. Development *135*, 3719–3729.
13. Bilic, J., Huang, Y.L., Davidson, G., Zimmermann, T., Cruciat, C.M., Bienz, M., and Niehrs, C. (2007). Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. Science *316*, 1619–1622.
14. Yamanaka, H., and Nishida, E. (2007). Wnt11 stimulation induces polarized accumulation of Dishevelled at apical adherens junctions through Frizzled7. Genes Cells *12*, 961–967.

15. Lustig, K.D., and Kirschner, M.W. (1995). Use of an oocyte expression assay to reconstitute inductive signaling. Proc. Natl. Acad. Sci. USA *92*, 6234–6238.
16. Young, C.S., Kitamura, M., Hardy, S., and Kitajewski, J. (1998). Wnt-1 induces growth, cytosolic beta-catenin, and Tcf/Lef transcriptional activation in Rat-1 fibroblasts. Mol. Cell. Biol. *18*, 2474–2485.
17. Vijayaragavan, K., Szabo, E., Bossé, M., Ramos-Mejia, V., Moon, R.T., and Bhatia, M. (2009). Noncanonical Wnt signaling orchestrates early developmental events toward hematopoietic cell fate from human embryonic stem cells. Cell Stem Cell *4*, 248–262.
18. Beqqali, A., Kloots, J., Ward-van Oostwaard, D., Mummery, C., and Passier, R. (2006). Genome-wide transcriptional profiling of human embryonic stem cells differentiating to cardiomyocytes. Stem Cells *24*, 1956–1967.
19. Nakanishi, M., Kurisaki, A., Hayashi, Y., Warashina, M., Ishiura, S., Kusuda-Furue, M., and Asashima, M. (2009). Directed induction of anterior and posterior primitive streak by Wnt from embryonic stem cells cultured in a chemically defined serum-free medium. FASEB J. *23*, 114–122.
20. Sumanas, S., Strege, P., Heasman, J., and Ekker, S.C. (2000). The putative wnt receptor Xenopus frizzled-7 functions upstream of beta-catenin in vertebrate dorsoventral mesoderm patterning. Development *127*, 1981–1990.
21. Kofron, M., Birsoy, B., Houston, D., Tao, Q., Wylie, C., and Heasman, J. (2007). Wnt11/beta-catenin signaling in both oocytes and early embryos acts through LRP6-mediated regulation of axin. Development *134*, 503–513.
22. Kurayoshi, M., Yamamoto, H., Izumi, S., and Kikuchi, A. (2007). Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. Biochem. J. *402*, 515–523.
23. Dong, J., Ye, P., Schade, A.J., Gao, S., Romo, G.M., Turner, N.T., McIntire, L.V., and López, J.A. (2001). Tyrosine sulfation of glycoprotein Ib(b)alpha. Role of electrostatic interactions in von Willebrand factor binding. J. Biol. Chem. *276*, 16690–16694.
24. Gao, J.M., Xiang, R.L., Jiang, L., Li, W.H., Feng, Q.P., Guo, Z.J., Sun, Q., Zeng, Z.P., and Fang, F.D. (2009). Sulfated tyrosines 27 and 29 in the N-terminus of human CXCR3 participate in binding native IP-10. Acta Pharmacol. Sin. *30*, 193–201.
25. Clark, C.C., Cohen, I., Eichstetter, I., Cannizzaro, L.A., McPherson, J.D., Wasmuth, J.J., and Iozzo, R.V. (1993). Molecular cloning of the human proto-oncogene Wnt-5A and mapping of the gene (WNT5A) to chromosome 3p14-p21. Genomics *18*, 249–260.
26. Ouyang, Y., Lane, W.S., and Moore, K.L. (1998). Tyrosylprotein sulfotransferase: Purification and molecular cloning of an enzyme that catalyzes tyrosine O-sulfation, a common posttranslational modification of eukaryotic proteins. Proc. Natl. Acad. Sci. USA *95*, 2896–2901.
27. Corbeil, D., Thiele, C., and Huttner, W.B. (2005). Tyrosine O-sulfation. In Current Protocols in Protein Science, J.E. Coligan, B.M. Dunn, D.W. Speicher, P.T. Wingfield, and H.L. Ploegh, eds. (Hoboken, NJ: John Wiley & Sons).
28. Mir, A., and Heasman, J. (2008). How the mother can help: Studying maternal Wnt signaling by anti-sense-mediated depletion of maternal mRNAs and the host transfer technique. Methods Mol. Biol. *469*, 417–429.
29. Birsoy, B., Kofron, M., Schaible, K., Wylie, C., and Heasman, J. (2006). Vg 1 is an essential signaling molecule in Xenopus development. Development *133*, 15–20.