at 50 mM with the spectrometer set at a microwave frequency of 9.77 GHz with 20 mW of microwave power and a modulation amplitude of 0.5 G. Treatment with Cu-Zn SOD (Sigma), catalase (Sigma), DPI (Toronto Research), or *N*-acetyl-L-cysteine (Sigma) was for 20 min before recording of EPR spectra. Treatment with the FPTase inhibitor H-Ampamb-Phe-Met-OH (LC Laboratories) was for 48 hours before obtaining spectra. At this concentration and with this duration of treatment, no cellular toxicity was observed.

23. The empty expression plasmid pEXV and the expression plasmids pEXVracV12 (encoding myc epitope–tagged constitutively active Rac1), pEXVracN17 (dominant negative Rac1), pCMVrasN17 (dominant negative Ras), and pS3CAT (catalase) have been described (*15, 21*). Transient transfections were done according to the manufacturer's recommendations using 15 μl of Lipofectamine (Gibco) and 5 μg of DNA per 10^5 cells. EPR spectra were obtained 48 hours after transfection. For the LUCL assay, 1×10^5 transfected cells were replated onto each tissue culture insert 24 hours after transfection and the assay was performed 24 hours later. Protein immunoblot analysis was done on cells 48 hours after transfection.
24. Established A6, Neo, and Raf-transformed cell lines (*10, 11*) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Gibco) and G418 (250 μg/ml, Sigma). Expression of H-Ras^{V12} in A6 cells was confirmed by protein immunoblotting with an antibody to H-Ras (Oncogene Science). Serum starvation was carried out in 0.1% serum for 48 hours.
25. For immunoprecipitation and protein immunoblotting, cells were harvested in complete lysis buffer [145 mM NaCl, 0.1 mM $MgCl_2$, 15 mM Hepes, 10 mM EGTA (pH 7.0), 0.1% Triton X-100, 20 μg/ml each of chymostatin, pepstatin, antipain, and leupeptin, 1 mM 4-(2-aminoethyl)benzylsulfonyl fluoride, and 1 mM Na orthovanadate]. Immunoprecipitates or total cell lysates were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose filters. Blots were then probed with the indicated antibody and developed by ECL (Amersham).
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A Member of the Frizzled Protein Family Mediating Axis Induction by Wnt-5A

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In *Xenopus laevis* embryos, the Wingless/Wnt-1 subclass of Wnt molecules induces axis duplication, whereas the Wnt-5A subclass does not. This difference could be explained by distinct signal transduction pathways or by a lack of one or more Wnt-5A receptors during axis formation. Wnt-5A induced axis duplication and an ectopic Spemann organizer in the presence of hFz5, a member of the Frizzled family of seven-transmembrane receptors. Wnt-5A/hFz5 signaling was antagonized by glycogen synthase kinase-3 and by the amino-terminal ectodomain of hFz5. These results identify hFz5 as a receptor for Wnt-5A.

The *Drosophila melanogaster* tissue polarity gene *frizzled* (*fz*), which encodes a receptor-type protein (Fz) with seven putative transmembrane helixes (*1*), belongs to a family of related genes found in *Caenorhabditis elegans*, *Drosophila*, zebrafish, chicken, and mammals (*2–4*). DFz2, a Fz-type protein from *Drosophila*, functions as a receptor for the Wingless (Wg) protein (*4*), a member of the Wnt family of secreted signaling molecules essential for animal development (*5*). Upon transfection of *Drosophila* tissue culture cells, DFz2 confers both the ability to bind Wg at the cell surface and Wg responsiveness, as measured by accumulation of Armadillo protein (*4*). Certain mammalian Fz proteins also confer the Wg binding property to transfected cells (*4*) and can enhance the localization of Wnt-8 to the plasma membrane in *Xenopus laevis* embryos (*6*). Thus, the fz gene family may encode receptors for Wnt molecules, but the scarcity of soluble Wnt proteins complicates the study of ligand-receptor interactions and their specificity. Therefore, we used the *Xenopus* embryo to assay for such interactions and their specificity.

Mouse Wnt-1, *Xenopus* wnt-3A (Xwnt-3A), Xwnt-8, and *Drosophila* Wg induce dorsal axis duplication when small amounts, usually 1 to 10 pg, of their corresponding RNAs are injected into the ventral side of early *Xenopus* embryos (*7*). In contrast, Xwnt-5A RNA fails to do so even after ventral injection at higher doses (75 pg to 1 ng per embryo); instead, dorsal injection of Xwnt-5A RNA generates head and tail defects that may result from perturbation of cell movements during gastrulation (*8*). Xwnt-4 and Xwnt-11 behave similarly to Xwnt-5A (*9*). The Xwnt-8 dorsalizing function is observed before the mid-blastula transition when zygotic transcription begins, whereas the Xwnt-5A effect occurs after mid-blastula transition (*7, 8, 10, 11*). The difference between the effects of Xwnt-8 and Xwnt-5A may reflect the activation of distinct signaling pathways (*11*) or the lack of one or more functional Xwnt-5A receptors during axis formation.

To examine whether a particular Fz protein can function as an Xwnt-5A receptor, synthetic RNAs corresponding to Dfz2 (*4*) and six mammalian fz cDNAs—mfz3, mfz4, mfz6, mfz7, mfz8 (from mouse), and hfz5 [from human (*3*)]—were pooled into two groups and coinjected with 10 pg of Xwnt-5A RNA into the ventral side of embryos at the four-cell stage (*12*). Injection of Xwnt-5A alone, either fz group alone, or Xwnt-5A together with fz group 2 (mfz3, mfz4, mfz6, and mfz7) produced no phenotypic effects (Fig. 1A). However, coinjection of Xwnt-5A with fz group 1 RNAs (Dfz2, hfz5, and mfz8) induced extensive dorsal axis duplication (Fig. 1A) (*13*); in many cases, duplication was complete, as determined by the presence of anterior structures such as the eyes and the cement gland. When the three fz RNAs in group 1 were individually tested, Xwnt-5A plus hfz5 generated similar degrees of axis duplication, whereas Xwnt-5A plus Dfz2 or mfz8 did not (Fig. 1B). Thus, hFz5 alone among the Fz proteins tested is responsible for mediating axis induction by Xwnt-5A. Dorsal injection of the same concentration of Xwnt-5A plus hfz5 RNAs produced no axis duplication and injected embryos appeared normal.

The mature Wnt-5A proteins (after cleavage of the signal peptides) are 100% identical between mouse and human, and 95% identical between mouse and *Xenopus* (*8, 14*). Given this high degree of sequence identity, it is not surprising that murine wnt-5A RNA (*15*) also induced axis duplication when coinjected with hfz5 RNA, albeit less efficiently (Fig. 1C). The lower efficiency might be due to effects of untranslated regions in the murine wnt-5A construct (*15*) on RNA stability or translation efficiency or both.

Axis duplication by Xwnt-5A plus fz group 1 or hfz5, as described above, was observed in 14 of 20 embryo batches tested

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during the course of this study. In these cases, 90 to 100% of the injected embryos showed axis duplication, of which 17 to 82% included eyes (*16*). In the remaining 6 of 20 embryo batches, the same coinjection induced no or few axis duplications (<30%, none complete). The reason for this poor response in some batches is unknown. Possible explanations include variations in the stability of injected RNAs or translated proteins, or both, the efficiency of Wnt-5A secretion, the assembly or localization of hFz5 protein, or both, and the availability of unknown coreceptor molecules.

It should be noted that whereas hFz5 is more closely related to DFz2 and mFz8 than to any other known Fz proteins (*3*, *4*), neither DFz2 nor mFz8 cooperated with Xwnt-5A in axis induction. However, DFz2 appeared to be functional in *Xenopus* embryos, because the same concentration of Dfz2 RNA as used for coinjection with Xwnt-5A substantially enhanced axis induction by suboptimal amounts of wg RNA (*17*). The failure of the other Fz proteins to mediate Xwnt-5A function could be due to an inability to bind Xwnt-5A or an inability to signal in this context.

A dose-response curve illustrated that, in the presence of 0.4 ng of hfz5 RNA per embryo, 1 pg of Xwnt-5A RNA induced partial secondary axes whereas 20 pg of Xwnt-5A RNA sometimes hyperdorsalized embryos (Fig. 2A). Although the relative protein levels have not been determined, the dose of Xwnt-5A RNA required for axis induction was in a similar range to effective doses of Xwnt-3A, Xwnt-8, wnt-1, and wg RNAs (*7*). Together, these data suggest a high degree of specificity of the interaction between Xwnt-5A and hFz5.

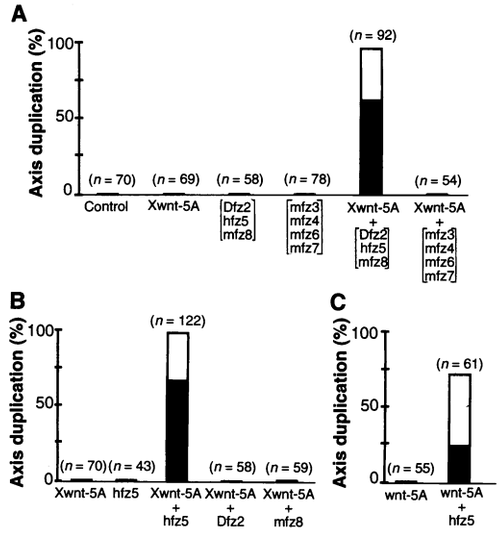
Embryos ventralized by ultraviolet (UV) irradiation (*18*) can be rescued by the Wg/Wnt-1 subclass of Wnt molecules (*7*). Xwnt-5A plus hFz5 could also restore dorsal development in UV-ventralized embryos, whereas neither Xwnt-5A nor hFz5 alone did (Fig. 2B). Many embryos rescued by Xwnt-5A plus hFz5 had a dorsoanterior index (DAI) (*19*) of 5, signifying normal development.

Histological examination (*20*) of embryos with duplicated axes revealed that Xwnt-5A plus hFz5 RNAs induced a full array of dorsal tissues, including notochord, neural tube, and somites (Fig. 3B). There is one notable difference between axes induced by Xwnt-8 and those induced by Xwnt-5A plus hFz5: whereas the ectopic axes induced by Xwnt-8 are often indistinguishable from the endogenous ones, the axes induced by Xwnt-5A and hFz5 are shorter in most cases, even when eyes and the cement gland are present (Fig. 3A). This might reflect the previously described ability of Xwnt-5A to inhibit cell movements during gastrulation (*8*).

We tested whether Xwnt-5A plus hFz5 induce an ectopic Spemann organizer, as does Xwnt-8 or Wnt-1, by examining the expression of the organizer-specific gene *goosecoid* (*gsc*) (*21*). Embryos injected with Xwnt-5A or hfz5 RNA alone expressed *gsc* only dorsally, as did uninjected controls; in contrast, embryos coinjected with Xwnt-5A and hfz5 RNAs exhibited two opposing domains of *gsc* expression, indicating the formation of an ectopic organizer (Fig. 3C).

Drosophila Wg function is mediated by inhibition of the *zeste-white 3* (*shaggy*) gene

Fig. 1. Dorsal axis induction by Xwnt-5A mediated via hFz5; *n* represents the total number of embryos scored from two to six independent experiments; each bar represents the average percentage of axis duplication; the solid portion within each bar represents the average percentage of extensive axis duplication, which is defined by the presence of the cement gland and at least one eye in the duplicated axis. Unless otherwise specified, 10 pg of wnt RNA or 400 pg of each fz RNA, or both, were injected per embryo. (**A**) Ventral injection of Xwnt-5A RNA together with RNA for fz group 1 (Dfz2, hfz5, and mfz8) induced axis duplication. Xwnt-5A RNA alone, fz group 1 or group 2 (mfz3, mfz4, mfz6, and mfz7) alone, and Xwnt-5A RNA plus fz group 2 did not. (**B**) Xwnt-5A RNA induced axis duplication in the presence of hfz5 RNA, but not with Dfz2 or mfz8 RNA. (**C**) Murine wnt-5A RNA plus hFz5 also induced axis duplication.



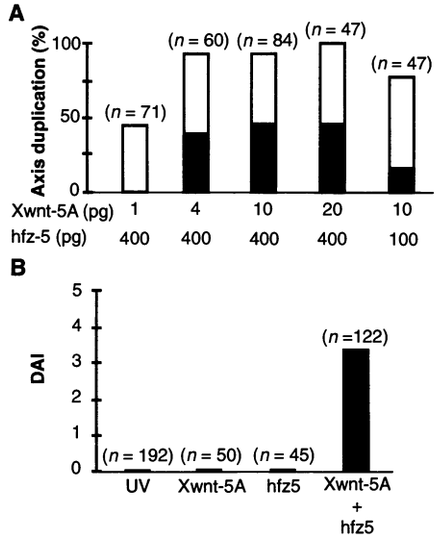


Fig. 2. (**A**) Dose-response curve of Xwnt-5A and hfz5 RNAs for axis duplication. The bar legend is as in Fig. 1. (**B**) Xwnt-5A plus hFz5 rescued UV-ventralized embryos. All embryos were UV-irradiated and were either left uninjected (UV), or injected with RNA for Xwnt-5A or hfz5 alone, or a combination of both (Xwnt-5A + hfz5). The DAI is defined in (*19*), with a DAI of 0 representing complete ventralization and a DAI of 5 equivalent to normal embryos. The average DAI from two to four experiments was shown.

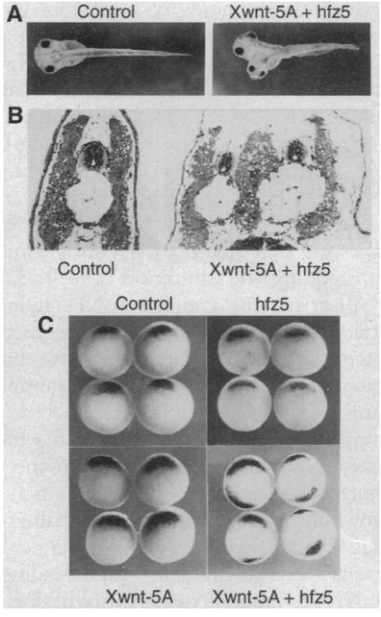


Fig. 3. Induction of anteriormost structures, dorsal axial tissues, and the Spemann organizer by Xwnt-5A plus hfz5. (**A**) Xwnt-5A plus hfz5 induced axis duplication. An example of complete axis duplication with eyes in both axes is shown (stage 41). (**B**) Xwnt-5A plus hfz5 induced a complete set of dorsal tissues. Cross section in the trunk region of an embryo at stage 41 reveals the presence of a neural tube, notochord, and somites in both axes. (**C**) Xwnt-5A plus hfz5 induced ectopic *gsc* expression in embryos at stage 10.5, as visualized by whole mount in situ hybridization.

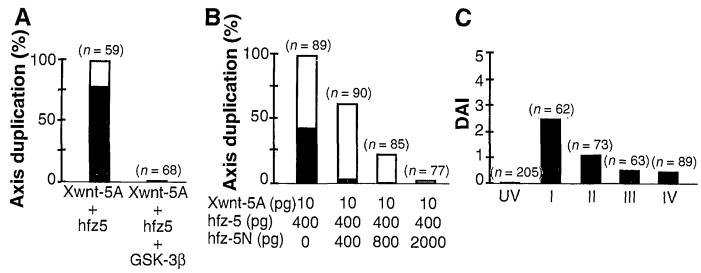


Fig. 4. GSK-3β and the secreted NH_2-terminal ectodomain of hFz5 (hFz5N) antagonize axis induction by Xwnt-5A plus hfz5. The bar legend for (A) and (B) is as in Fig. 1. (**A**) GSK-3β suppressed axis duplication. RNA for human GSK-3β was injected at 1 ng per embryo. (**B**) hFz5N suppressed axis duplication by Xwnt-5A plus hfz5 in a dose-dependent manner. Dorsal injection of the same doses of hfz5N RNA did not affect the endogenous dorsal development. At higher doses (0.8 to 2 ng RNA per embryo) some embryos with gastrulation defects were observed. (**C**) hFz5N inhibited Xwnt-5A/hFz5 signaling in neighboring cells. hfz5N RNA and RNAs for Xwnt-5A plus hfz5 were injected into two separate neighboring blastomeres at the four-cell stage. This experiment was done in UV-ventralized embryos because of the semi-quantitative nature of the DAI score. Embryos were either left uninjected (UV) or injected with the following RNA combinations: I, Xwnt-5A (10 pg) plus hFz5 (400 pg) in one blastomere; II and III, Xwnt-5A plus hFz5 in one blastomere, then with 400 pg (II) or 800 pg (III) of hfz5N RNA in a neighboring blastomere; or IV, Xwnt-5A, hfz5, and hfz5N (800 pg of RNA) were coinjected into a single blastomere. The average DAI from three independent experiments is shown. Note that the overall inhibitory effect of hFz5N via coinjection (IV) and the separate injection (III) are similar.

product, the homolog of vertebrate glycogen synthase kinase–3 (GSK-3) (*22*), and the dorsalizing function of Xwnt-8 RNA in *Xenopus* is mimicked by dominant-negative mutant forms of GSK-3 and antagonized by overexpression of wild-type GSK-3 (*23*, *24*). Coinjection of wild-type GSK-3β RNA (*24*) blocked dorsal axis duplication by Xwnt-5A plus hfz5 RNAs (Fig. 4A), suggesting that Xwnt-5A signaling through hFz5 requires the inhibition of GSK-3.

All Fz proteins contain an NH_2-terminal extracellular domain composed of a conserved cysteine-rich domain and a variable linker region before the first putative transmembrane helix (*1*, *3*). The NH_2-terminal ectodomain of DFz2, when anchored to the membrane, promoted binding of Wg to the cell surface, and deletion of the cysteine-rich domain from mFz4 abolished Wg binding (*4*). Thus, the NH_2-terminal extracellular domain appears to be involved in ligand binding. If the NH_2-terminal ectodomain of hFz5 is involved in binding Xwnt-5A, its overexpression as a secreted molecule might prevent Xwnt-5A from binding to and signaling through hFz5. Indeed, coinjection of RNA coding for the hFz5 NH_2-terminus (hFz5N), without provision of a membrane anchor (*25*), antagonized axis induction by Xwnt-5A plus hFz5 in a dose-dependent manner (Fig. 4B). Dorsal injection of hfz5N RNA alone did not affect endogenous dorsal axis formation (although gastrulation defects were seen in some embryos); the injected embryos appeared to have an intact neural groove at the neurula stage, and the anteriormost structures (eyes and the cement gland) were present at the tadpole stage (*17*). These results rule out the possibility that the effect of hFz5N in blocking axis duplication by Xwnt-5A plus hFz5 is due to nonspecific toxicity on dorsal development.

To further eliminate the possibility that the expression of hFz5N causes a generalized defect in protein secretion, thus preventing Xwnt-5A or hFz5 or both from reaching the cell surface, we injected hfz5N RNA and RNAs for Xwnt-5A plus hfz5 into separate neighboring blastomeres. Under these conditions, hFz5N inhibited axial development induced by Xwnt-5A plus hFz5 to an extent similar to that observed after coinjection of a single blastomere (Fig. 4C). This result demonstrates that secreted hFz5N can diffuse to neighboring cells to inhibit Xwnt-5A/hFz5 signaling.

In summary, our data indicate that in the presence of hFz5, Wnt-5A can transduce a signal similar to that of Xwnt-8 or Wnt-1, mediated by a pathway involving down-regulation of GSK-3. The simplest interpretation of these results is that hFz5 functions as a receptor for Wnt-5A. Our experiments also provide a general assay to address the relationships among 14 Wnt and eight Fz proteins thus far identified in mammals.

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12. All fz cDNAs were subcloned in the pRK5 vector [C. Gorman, D. R. Gies, G. McCray, *DNA Prot. Eng. Techn.* **2**, 3 (1990)] with an optimal Kozak consensus sequence for translation at the initiator ATG (CCACCATG, preceded by different restriction sites for subcloning) and with different lengths of 3′ untranslated regions. The details of each construct are available upon request. Xwnt-5A was in pSP64 vector as described (*8*). All RNAs for injection were synthesized as capped transcripts in vitro with SP6 RNA polymerase (Ambion Megascript). Unless otherwise specified, RNAs were injected into the two cells near the equatorial midline region at the four-cell stage. Embryo preparation and staging were performed as described (*24*).
13. Percentages of axis duplication were obtained by dividing the total number of embryos with a duplicated axis by the total number of embryos, scored from two to six independent experiments. Extensive axis duplication is defined by the presence of the cement gland and at least one eye in the duplicated axis.
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15. Murine wnt-5 cDNA (*14*) was cloned in pCS2+ vector [D. L. Turner and H. Weintraub, *Genes Dev.* **8**, 1434 (1994)] as the Eco RI and Xba I fragment, which contains about 60 bp 5′ and 360 bp 3′ untranslated regions.
16. Some variability exists in the completeness of duplicated axes or axial rescue in UV-treated embryos among different batches of embryos. One possibility is that the coinjection of Xwnt-5A and hfz5 RNAs inevitably causes more variable results than does injection of a single RNA species, because the two proteins derived from the injected RNAs must be translated, processed, and localized properly to function together. In injection of a single RNA, such as one for Xwnt-8, the variables are only associated with the Xwnt-8 protein, because one or more receptors are provided endogenously. Another possibility is that Xwnt-5A inhibits gastrulation movements in gastrula stages (*8*); this later function of Xwnt-5A might affect the completeness of the axis to a variable degree.
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25. hfz5N was generated by introduction of a stop codon just before the first putative transmembrane helix (changing the preceding amino acids 237 and 238 from phenylalanine and tryptophan to threonine and arginine, respectively). The corresponding DNA fragment was subcloned in pCS2+.

26. The authors are grateful to R. Moon for Xwnt-5A plasmid. We thank B. Huang for technical help and B. Williams, J. Debnath, P. Schwartzberg, and M. Rebagliati for discussion and comments on the manuscript. X.H. is supported by the U.S. Army Breast Cancer Research Program. J.N. is an investigator of the Howard Hughes Medical Institute.

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