Multiple Mechanisms for Wnt11-mediated Repression of the Canonical Wnt Signaling Pathway*

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The effect of a noncanonical Wnt, Wnt11, on canonical Wnt signaling stimulated by Wnt1 and activated forms of LRP5 (low density lipoprotein receptor-related protein-5), Dishevelled1 (Dvl1), and β-catenin was examined in NIH3T3 cells and P19 embryonic carcinoma cells. Wnt11 repressed Wnt1-mediated activation of LEF-1 reporter activity in both cell lines. However, Wnt11 was unable to inhibit canonical signaling activated by LRP5, Dvl1, or β-catenin in NIH3T3 cells, although it could in P19 cells. In addition, Wnt11-mediated inhibition of canonical signaling in NIH3T3 cells was ligand-specific; Wnt11 could effectively repress canonical signaling activated by Wnt1, Wnt3, or Wnt3a but not by Wnt7a or Wnt7b. Coculture experiments with NIH3T3 cells showed that the co-expression of Wnt11 with Wnt1 was not an essential requirement for the inhibition, suggesting receptor competition as a possible mechanism. Moreover, in both cell types, elevation of intracellular Ca²⁺ levels, which can result from Wnt11 treatment, led to the inhibition of canonical signaling. This result suggests that Wnt11 might not be able to signal in NIH3T3. Furthermore, P19 cells were found to express both endogenous canonical Wnts and Wnt11. Knockdown of Wnt11 expression using siRNA resulted in increased LEF-1 reporter activity, thus indicating that Wnt11-mediated suppression of canonical signaling exists in vitro.

The Wnt gene family encodes for a conserved class of secreted signaling molecules and is considered one of the major gene families essential for proper embryonic patterning and organogenesis. Genome sequencing projects have revealed that a large number of Wnt genes exist, including 7 Wnt genes in Drosophila and at least 19 Wnt genes in humans. Wnt proteins function as growth factors and morphogens modulating cell growth and specifying cell fate. Early segment polarity screens in Drosophila resulted in the identification of key components of a “canonical” Wnt signaling pathway (2). Since then, extensive research on the canonical pathway has identified several genes, the gene products of which interact in a complex series of mechanisms that ultimately regulate the canonical pathway.

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At the cell surface, Wnt proteins activate the pathway through binding a receptor complex consisting of Frizzled, a seven transmembrane receptor, and its co-receptor LRP-5/6 (low density lipoprotein receptor-related protein-5/6) (3–5). Intracellular mediators of the canonical pathway include Dishevelled and β-catenin, which promote pathway activation, and Axin and GSK-3β, which negatively regulate the pathway. In the absence of Wnt ligand, cytoplasmic levels of β-catenin remain low, as a result of its association with GSK-3β and Axin, which target it for proteolytic degradation (10). Upon Wnt ligand binding, GSK-3β is inhibited, and cytoplasmic levels of β-catenin protein increase (11) and subsequently translocate into the nucleus, where it functions as a transcriptional co-activator, commonly associating with members of the LEF/TCF family of transcription factors (12–14).

Several assays now exist that demonstrate the ability of Wnt proteins to activate canonical signaling. Injection of Wnt or β-catenin mRNAs into the ventral marginal zone of four cell stage Xenopus embryos results in axis duplication (15). Wnt-induced protein stabilization of β-catenin can be detected by Western blotting (11). Also, a LEF/TCF reporter gene construct can be used to measure pathway activation (16). Collectively, these assays reveal that not all Wnt proteins can trigger activation of the canonical pathway. As a result, this gene family has been subdivided into at least two classes (17). Members of the Wnt1 class (Wnt1, Wnt3, Wnt3a, Wnt7a, Wnt7b, Wnt8a) are effective activators of the canonical pathway, whereas members of the Wnt5a class (Wnt4, Wnt5a, and Wnt11), with few exceptions (18), are poor activators of the canonical pathway.

Signal transduction mechanisms triggered by the Wnt5a class are less understood than the more extensively researched canonical pathway. Embryonic studies in Xenopus and Zebrafish indicate that overexpression of Wnt5a results in an increased release of intracellular calcium (19). Wnt5a overexpression can also lead to increased activation of classical protein kinase C and calmodulin-dependent protein kinase II (CamKII) (20, 21), two calcium-responsive serine threonine kinases. Further support for a Wnt5a-calcium pathway comes from the analysis of different Frizzled receptors, which show that a specific subset can trigger increases in intracellular calcium (20, 21). Also, recent work using the ΔDix mutant of Dishevelled demonstrates its ability to increase intracellular calcium as well as to activate protein kinase C and CamKII.

2 The abbreviations used are: LRP, low density lipoprotein receptor-related protein; GSK-3β, glycogen synthase kinase-3β; LEF, lymphoid enhancer factor; TCF, T cell factor; CamKII, calmodulin-dependent protein kinase II; DKK, Dickkopf; NLK, Nemo-like Kinase; siRNA, small interfering RNA.

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(22). Together, these data suggest that Wnt5a class members signal through Frizzled and Dishevelled, analogous to canonical Wnt signaling, but resulting in an intracellular calcium release.

There are multiple ways to inhibit Wnt canonical signaling. On the outer cell surface, there are at least four different gene families, the gene products of which have been shown to inhibit the canonical pathway. Specific members of the greased Frizzled-related protein (sFRP) family, Wnt inhibitory factor (WIF), and Cerberus/Dan family are believed to inhibit Wnt signaling by directly binding to Wnt ligands (23–25). Members of the Dickkopf (DKK) family, specifically DKK-1 and DKK-2, are also capable of inhibiting canonical Wnt signaling by targeting LRP5/6 at the receptor complex (26). There is evidence that members of the Wnt5a class can also inhibit canonical signaling. Early studies in Xenopus revealed that Wnt-triggered axis duplication could be blocked if mRNA from a member of the Wnt5a class was co-injected (27). Also, a recent microarray study identifies several canonical target genes up-regulated by Wnt1, which are repressed by Wnt5a (28). How this inhibition occurs is not entirely understood. Although some studies provide evidence that members of the Wnt5a class repress canonical signaling at the transcriptional level (29, 30), other reports suggest that inhibition may occur further upstream in the pathway (27, 31).

In this study, we investigated Wnt11-mediated repression of the canonical pathway in NIH3T3 cells and P19 embryonic carcinoma cells. We show that Wnt11 can effectively inhibit canonical signaling in both cell lines and provide evidence for more than one inhibitory mechanism. Although Wnt11 can effectively inhibit downstream of β-catenin in P19 cells, it can only inhibit canonical signaling upstream of LRP5/6 in NIH3T3 cells, suggesting a novel inhibitory mechanism located at the cell surface. Further analysis of this mechanism in NIH3T3 cells reveals that Wnt11 can only repress a specific subset of Wnt1 class ligands efficiently. Cell mixing experiments with NIH3T3 cells show that the co-expression of Wnt11 with Wnt1 is not essential for inhibition and suggests receptor competition as one possible mechanism. We also show that P19 embryonic carcinoma cells not only have an active endogenous Wnt canonical pathway, but also signal through Wnt11. siRNA-mediated knockdown of Wnt11 results in increased LEF/TCF reporter activity, suggesting the presence of an endogenous Wnt5a class signaling pathway that can repress Wnt canonical signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Reporter Gene Assays—**NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. P19 embryonic carcinoma cells were maintained on gelatin-coated plates in MEM-α medium containing 10% fetal calf serum. Various combinations of DNA constructs were transfected into cells using LipofectAMINE with Plus reagent as described by the manufacturer (Invitrogen). Unless otherwise noted, 75 ng/well LEF-1 reporter, 25 ng/well of LEF1, and 100 ng/well of green fluorescent protein were added to every transfection, which was carried out in a 24-well dish. For NIH3T3 cells and P19 cells, LacZ expression plasmid was added to equalize the total amount of DNA per well (0.5 μg/well). Transfections were stopped after 3 h, and lysates were assayed for luciferase activity after 24 h (Roche Applied Science, luciferase assay kit). In brief, lysates were initially measured for green fluorescent protein fluorescence (transfection control) followed by the addition of luciferase substrate and then measured for luciferase luminescence in a Wallac multiscatter. Luminescent intensity was normalized against fluorescent intensity. DNA concentrations were adjusted if significant differences were noted between normalized and non-normalized data. Experiments were carried out in duplicate and repeated twice to verify results. To quench endogenous Wnt signaling in P19 cells, Dkk-1 protein was added at the indicated concentrations after the transfection was stopped. Transfection studies utilized an N-terminal truncated form of mouse β-catenin, which was PCR-cloned using Pfu polymerase (Stratagene) with the following primers: 5′-Xbali-TCTCTGGACGA-GATGTTGAACACATGCAG-3′ and 5′-XhoI-CGTTCTGCAGACGATT-CAGTATCAAACCA-3′ (no stop codon in frame with c-terminal hemagglutinin tag). To address the TAK1-NLK pathway as a mechanism of inhibition, we constructed an NLK phosphorylation mutant of LEF-1 as discussed previously (30) by switching T155A and S166A in mouse LEF1. A back-to-back PCR strategy was used to construct the LEF-1 mutant. After 15 cycles, 1 μl of a 50-μl reaction was added to a second reaction for seven more cycles, using the following primer sets: reaction 1, HindIII 5′-TCTCAAGCTTGCACCTACGCGCGCATTGAGGTA-3′ and 5′-GCTCCTCTGCTAGTAGTGGTCTGAGTGCAGGCTTGGGA-3′, and reaction 2, HindIII primer and BamHI 5′-GTGGGGATCCCGGGCGTTGCTGTCGTATGTTGAGAGGTGCG-3′. For cell mixing experiments, 24 h after transfection in a 24-well dish, cells were trypsinized, resuspended, and added in various combinations in a new 24-well dish and were assayed 24 h after mixing.

**Wnt11 siRNA Treatment—**For siRNA transfection, control siRNA and Wnt11 siRNA (AACATGCGCTCTACACAACA) were synthesized by Dharmacon (Lafayette, CO) and transfected into P19 cells in the presence of the LEF/TCF reporter using LipofectAMINE Plus reagent or Oligofectamine. To slow cell growth, P19 cells were grown in MEM-α containing 1% fetal calf serum and assayed 48 h after transfection.

**Western Analysis—**For Western blotting, HEK 293T cells were transfected with Wnt1α-FLAG and Wnt11-FLAG at the indicated dosages. 24 h after transfection, total cell lysates were separated by SDSPAGE on a 10% acrylamide gel, transferred to a nylon membrane, and analyzed by Western blotting using an anti-FLAG antibody. Super Signal (Pierce) horseradish peroxidase substrate was used to generate a chemiluminescent signal that was detected by a CCD camera.

**RESULTS**

**Suppression of Canonical Wnt1 Signaling by Wnt11 in NIH3T3 and P19 Cells—**Previous work in Xenopus demonstrated that members of the Wnt5a class could block axis duplication if co-injected with members of the Wnt1 class (27). Therefore, we tested the ability of Wnt11 to block canonical signaling in NIH3T3 and P19 cells. The activation of the canonical signaling pathway by Wnt1 in both cell lines was evaluated using the LEF-1 reporter gene assay as described previously (32, 33). Analogous to studies in Xenopus with members of the Wnt5a class, co-transfection of Wnt11 inhibited Wnt1-mediated canonical pathway activation in a dosage-dependent manner (Fig. 1, A and B). Although transfection of Wnt11, by itself, into NIH3T3 cells did not result in suppression of LEF-1 reporter activity (Fig. 1A), transfection of Wnt11 into P19 cells resulted in a significant reduction in LEF-1 reporter activity in the absence of Wnt1 (Fig. 1B). This result suggests that P19 cells may possess endogenous canonical signaling activity, which can be repressed by Wnt11 (Fig. 1B). Consistent with this hypothesis, treatment of P19 cells with Dkk-1 protein also resulted in inhibition of reporter activity (Fig. 1C). This hypothesis is further supported by a previous finding showing that members of the Wnt1 class, including Wnt3, are endogenously expressed in P19 cells (34).

To determine whether Wnt11-mediated inhibition of canonical signaling exist in vivo, we utilized siRNA to suppress Wnt11, which is expressed endogenously in the P19 cells (data not shown). Co-transfection of the LEF-1 reporter gene in the presence of the siRNA targeting Wnt11, but not the control siRNA, led to increases in the reporter activity (Fig. 1D). We further demonstrated the effectiveness of siRNA-mediated knockdown by co-transfecting P19 cells with Wnt11 and the two siRNAs. Wnt11 could only inhibit canonical signaling in the presence of the control siRNA (Fig. 1D).

**Wnt11 Represses Canonical Signaling through Multiple Mechanisms—**To further understand how Wnt11 inhibits canonical signaling, we tested its ability to inhibit downstream activated forms of LRP5 (LRPC2) and Dishevelled1 (Myri-Dvl1),...
Wnt11 was unable to repress reporter activity activated by the activated forms of LRP5, Dvl1, or β-catenin (Fig. 2A). This result suggests that Wnt11-mediated inhibition of canonical signaling in 3T3 cells lies far upstream in the pathway, possibly at the cell surface.

Analogous studies were carried out in P19 cells with a significantly different outcome. As mentioned previously, P19 cells retain endogenous levels of canonical signaling. Since the addition of any downstream activator would synergize with an already active pathway, the presence of endogenous canonical signaling initially complicated our attempts to demonstrate whether Wnt11 could block downstream pathway components. However, we could effectively quench endogenous signaling by adding purified Dkk-1 protein (Fig. 1C). Dkk-1 inhibits canonical signaling through binding to the LRP5/6 and Kremen1/2 proteins at the cell surface, where the receptor complex subsequently undergoes receptor-mediated endocytosis (26, 35, 36).

Transfection studies carried out in the presence of Dkk-1 revealed that Wnt11 could effectively repress canonical signaling downstream of both Dvl1 and β-catenin (Fig. 2B). In addition, Wnt5a, another noncanonical Wnt class member, could also effectively down-regulate canonical signaling activated by Dvl1 and β-catenin in a manner comparable with Wnt11 (data not shown). To demonstrate the ability of Dkk-1 to quench endogenous signaling far upstream in the pathway, the expression of Wnt1 in the presence of the Dkk1 protein hardly resulted in further stimulation of the reporter activity (compare Fig. 1B with Fig. 2B). Therefore, it is evident that Wnt11 may inhibit the canonical pathway via more than one mechanism.

**Elevation of intracellular Ca^{2+} Levels Inhibits Canonical Signaling in Both 3T3 and P19 Cells**—Although signaling mechanisms of the noncanonical Wnt class are poorly understood, previous studies indicate that some of the noncanonical Wnts can trigger the intracellular release of calcium (19–21). Furthermore, mediators of calcium signaling may be inhibitory to Wnt canonical signaling (29, 37). Consistent with these previous findings, increases in intracellular Ca^{2+} concentration by treatment with ionomycin or by transfection with constitutively active Goq (GqQ209L) in NIH3T3 cells and P19 cells led to the inhibition of the canonical pathway (Fig. 2, C and D). Interestingly, in NIH3T3 cells, the same treatments also led to the inhibition of canonical signaling activated by activated forms of RLP-5 and Dvl1 (Fig. 2C), which could not be inhibited by Wnt11 (Fig. 2A). Therefore, although NIH3T3 cells may retain the essential intracellular components necessary for the calcium-mediated inhibition of canonical Wnt signaling, Wnt11 does not seem to be able to activate these intracellular components in these cells. This further supports the idea that, in NIH3T3 cells, Wnt11 represses Wnt1 at the cell surface rather than through an intracellular signaling pathway.

**The Cell Surface Mechanism Is Ligand-specific**—The possibility that Wnt11 inhibits Wnt1-mediated activation of canonical signaling at the cell surface in NIH3T3 cells intrigued us. We wanted to examine whether there is specificity in the antagonism of canonical Wnts by Wnt11 and Wnt5a. The following canonical Wnts were tested: Wnt1, Wnt3, Wnt3a, Wnt7a, and Wnt7b, which all resulted in significant LEF/TCF reporter activation in NIH3T3 cells (Fig. 3A). We also tested Wnt8a, which could only activate LEF-1 in P19 cells (data not shown) but not in NIH3T3 cells (Fig. 3A). Co-transfection of Wnt11 or Wnt5a with Wnt1, Wnt3, or Wnt3a resulted in greater than 70% inhibition of canonical signaling, whereas co-transfection of Wnt8a with Wnt1 or Wnt3 resulted in less than 20% inhibition (Fig. 3B and D). Interestingly, Wnt11 either poorly repressed or showed minor pathway stimulation when co-expressed with Wnt7a or Wnt7b (Fig. 3C and D). When compared with Wnt1, Wnt3, and Wnt3a, Wnt5a also showed a reduced ability to inhibit Wnt7a and Wnt7b (Fig. 3C and D). Surprisingly, Wnt8a substantially synergized with Wnt7a and Wnt7b to further activate LEF/TCF reporter activity (Fig. 3C and D).

To eliminate the possibility that Wnt11 or Wnt5a was being expressed at much higher levels than members of the canonical Wnts, resulting in nonspecific competition at the cell surface, we made FLAG-tagged versions of Wnt11 and Wnt3a and compared their expression levels. Both Wnt3a-FLAG and Wnt11-
FLAG showed comparable levels of expression (Fig. 4A) with Wnt3a showing slightly higher expression. The biological activity of the FLAG-tagged Wnt proteins were then compared with nontagged ones; no significant differences were observed (Fig. 4B). Also, similarly to Wnt11, Wnt11-FLAG could not effectively repress canonical signaling mediated by Wnt7a (Fig. 4C). Unfortunately, FLAG-tagged versions of Wnt7a at either end of the protein were not functional, and therefore, were not included in these studies. Comparison of the ability of Wnt11 to repress Wnt3a versus Wnt7a by percent of inhibition (Fig. 4B and C) further confirms our previous observation that inhibition appears to be ligand-specific.

Wnt11-mediated Inhibition Does Not Require Its Coexpression with Canonical Wnts—Collectively, the data in NIH 3T3 cells suggested that an inhibitory mechanism existed at the cell surface. Therefore, we speculated that Wnt co-expression may result in either the formation of a nonfunctional multimeric ligand complex or competition for common receptor components. To discriminate between these two possibilities, we car-
ried out a series of cell mixing coculture experiments. In the first coculture experiment (Fig. 5A, *Scheme 1*), two cell populations were mixed. One cell population co-expressed Wnts, whereas the other cell population was transfected with the reporter gene from which the response was measured. When these two cell populations were mixed, Wnt11 could still inhibit Wnt1 while having a mild stimulatory effect with Wnt7a (comparing Fig. 5A with Fig. 5B, white bars). In the second coculture experiment (*Scheme 2*), Wnt1 and Wnt11 were expressed in two distinct cell populations. Wnt11 was transfected into a cell population that was also transfected with the LEF-1 reporter gene. Under these conditions, Wnt11 still inhibited Wnt1 and moderately stimulated Wnt7a (comparing Fig. 5A with Fig. 5B, black bars). Because the first scheme favors ligand oligomerization, whereas the second scheme favors receptor competition as a potential mechanism, comparing the two separate mixing experiments suggests that receptor competition may play a more significant role as a mechanism for Wnt11-mediated inhibition (87% inhibition versus 67%). Consistent with *Scheme 2*, the ability of Wnt3a-conditioned media to trigger canonical signaling in NIH3T3 cells expressing Wnt11 was substantially reduced (data not shown). In addition, when we switched Wnts and expressed the canonical Wnt in the same cell containing the LEF-1 reporter gene and Wnt11 in a separate cell population, Wnt11 was unable to demonstrate significant repression (data not shown).

We also tested the ability of Wnt11 to repress canonical signaling under conditions in which three cell populations are mixed (Fig. 5, C and D, *Scheme 3*). In this scheme, Wnt1, Wnt11, and the LEF/TCF reporter are transfected into three separate cell populations. In these mixing experiments, Wnt11 or control transfected (LacZ) cells were added in either an equal number (one) or twice the number (two) of Wnt1 cells. Although we detected some inhibition with Wnt11, its ability to repress canonical signaling was substantially reduced when compared with *Schemes 1* and 2. One likely reason for reduced inhibition with this scheme is that Wnt proteins normally stay highly associated with the cell surface and are poorly released into the media. Therefore, the function of Wnts is likely to be limited at the interfaces of producer and target cells.

**DISCUSSION**

An increasing body of evidence suggests that members of the noncanonical Wnt class, Wnt11 and Wnt5a, can function as repressors of canonical Wnt signaling (27, 29, 30). Work presented in this investigation further substantiates these previous findings. In addition, the results from this study indicate that there is more than one mechanism of repression by noncanonical Wnt proteins, as depicted in Fig. 6; one mechanism occurs extracellularly, and at least one occurs intracellularly.

*The Intracellular Mechanisms*—Although the biochemical
mechanisms by which Wnt5a class proteins inhibit canonical Wnt signaling are poorly understood. Studies have indicated the existence of a Wnt5a class intracellular pathway that can repress canonical signaling far downstream at the transcriptional level (29, 30). Consistent with these studies, we showed that Wnt11 and Wnt5a in P19 cells could repress canonical signaling activated by β-catenin, strongly implying that the inhibition occurs downstream of β-catenin and probably at the transcriptional level. How this transcriptional repression occurs is not entirely clear. Previous work on CamKII, a calcium-responsive serine threonine kinase, the activity of which increases upon Wnt5a signaling, showed that this kinase could phosphorylate LEF-1 in vitro, suggesting that it may be directly involved in transcriptional regulation (29). Another study suggests that CamKII may activate the TAK1-NLK pathway (30), which has been shown previously to inhibit β-catenin-mediated activation of LEF-1 (38). However, we have been unable to demonstrate any significant effect on canonical Wnt signaling in either P19 cells or NIH3T3 cells using an activated form of CamKII or a pharmacological inhibitor of CamKII (KN-62) (data not shown). Furthermore, there were no differences in the ability of Wnt11 to repress canonical signaling in P19 cells when comparing the wild-type form of LEF-1 versus an NLK phosphorylation mutant, suggesting that Wnt11 does not utilize the TAK1-NLK pathway to inhibit canonical signaling (data not shown). Despite this, we do observe inhibition of canonical signaling by the activation of Ca²⁺ signaling via ionomycin treatment or the expression of activated GoQ. A previous study has shown that GoQ-induced repression of canonical signaling was calcium-mediated (37).

In addition to transcriptional repression of Wnt canonical signaling, there is also evidence that the inhibition may occur upstream in the pathway. Early Xenopus embryo studies demonstrated that when members of the Wnt5a class were co-expressed with members of the Wnt1 class, axis duplication could be blocked. However, members of the Wnt5a class could not block axis duplication mediated through β-catenin or a kinase-dead form of GSK-3β (27). Similar studies with DWnt4, which has been shown to repress Wg signaling in Drosophila (39), also suggest that inhibition does not occur at the transcription level but upstream of β-catenin (31). Our observation that the inhibition by ionomycin treatment or transfection with activated GoQ in NIH3T3 cells became progressively less effective with downstream pathway activators (Fig. 2C) also suggests that repression through calcium may target different points of the canonical Wnt pathway.

**The Extracellular Mechanism**—In addition to the intracellular mechanisms, we also discovered that Wnt11 can inhibit canonical Wnts via an extracellular mechanism. This conclusion is mainly based on the finding that in NIH3T3 cells, although Wnt11 could effectively inhibit Wnt1-activated canonical signaling, it could not inhibit signaling activated by activated forms of LRP5, Dishevelled1, and β-catenin. Two additional pieces of evidence further support this conclusion. First, the activation of endogenous calcium signaling in NIH3T3 cells results in the inhibition of downstream pathway components, suggesting that Wnt11 is unable to trigger the release of intracellular calcium in these cells. Second, the Wnt11 inhibition of Wnt1 class members is selective. If Wnt11 targeted canonical pathway repression through an intracellular mechanism, it most likely would be common to all members of the Wnt1 class.

What is the biochemical mechanism of repression at the cell surface? There are the following possibilities: 1) nonspecific aggregation of overexpressed Wnt proteins. Wnt proteins, which are cysteine-rich, glycosylated, and lipid-modified (40–42), can be highly retained in the endoplasmic reticulum, most likely as misfolded protein aggregates (42). Thus, overexpression of two Wnts in the same cell may cause a problem in either protein synthesis or the transport pathway, resulting in pathway repression. However, we feel that this is unlikely for four reasons. First, Wnt8a, a member of the Wnt1 class, which could not trigger pathway activation in NIH3T3 cells, had little to no effect on pathway inhibition. Second, Wnt11 is unable to repress Wnt7a or Wnt7b, demonstrating a ligand-specific nature to repression. Problems in protein synthesis or secretion, as a result of overexpression, most likely would not be so specific. Third, data obtained from various cell mixing coculture experiments indicate that co-expression is not essential for the inhibition. Last but not least, Wnt11-mediated suppression occurs in a nontransfected endogenous system. Suppression of endogenous Wnt11 expression by Wnt11-specific siRNA led to increases in canonical signaling (Fig. 1D). 2) Repression may occur through ligand oligomerization. Although Wnt proteins are active in their monomeric form (42), early studies have implied that Wnt proteins may form multimeric structures (40). A hetero-oligomer ligand may not function well. We think that this is unlikely to be the major mechanism (see below). 3) Repression may occur through competition, most likely to receptors. Two different Wnts may compete for the same receptor components, although the ligands trigger two different pathways. One explanation for this may have to do with the multifunctional nature of Frizzled receptors. Genetic studies in Drosophila have shown that DFz1 and DFz2, which are developmentally redundant for canonical signaling, distinctly function in planar cell polarity (43, 44) and cell motility, respectively (43, 45). Because of this multifunctionality, the same Frizzled receptor may bind more than just one class of Wnt ligands. Consistent with this thinking, ligand-receptor binding studies in Drosophila indicate that although certain Wnts, such as DWnt8, are very selective in what Frizzled it binds, other Wnts, such as Wg, Dwnt2, and DWnt4, appear to be more promiscuous in their specificity for Frizzled proteins (46).

The data from the coculture experiments suggest that the competition model may be the predominant mechanism, if not the sole one, for Wnt11-mediated inhibition of canonical Wnts in NIH3T3 cells. If oligomers were formed, it would have been more efficient for them to form when two Wnt proteins are coexpressed in the same cells. Thus, Wnt11 should have shown stronger inhibition in the scheme in which Wnt1 and W11 were coexpressed in the same cell population than the scheme in which Wnt11 was cotransfected with the reporter gene. In addition, as Wnt is usually membrane-associated, cells produc-
ing them would have better access to the Wnt proteins than cells that do not produce them. In fact, Wnt11 showed stronger expression in a population different from the one expressing both Wnt1 and the reporter gene.

Localized repression of the Wnt canonical pathway by members of the Wnt5a class is an essential part of embryonic development. Previous work in Xenopus implicated that both canonical signaling and Wnt5a class noncanonical signaling antagonistically control convergent extension (29). Two recent studies, carried out in Zebrafish and mice, now provided genetic evidence that Wnt5a actively represses canonical signaling in vivo. Analysis of the Zebrafish Wnt5 mutant, pipetail, during dorsal/ventral axis specification and the Wnt5a−/− mouse during limb bud formation revealed ectopic activation of the canonical pathway (47, 48). Further confirming the antagonistic role of Wnt5a, patterning defects in the limb bud, as a result of Wnt5a disruption, could be partially rescued by transplanted cells expressing SFRP-2, a different type of secreted Wnt inhibitor (1). Future studies on the molecular pathways of the Wnt5a class will be an important aspect of Wnt research and may also provide significant insight into treating cancers that result from a dysfunctional canonical pathway.

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