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MECHANISMS OF WNT SIGNALING IN DEVELOPMENT

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ABSTRACT

Wnt genes encode a large family of secreted, cysteine-rich proteins that play key roles as intercellular signaling molecules in development. Genetic studies in *Drosophila* and *Caenorhabditis elegans*, ectopic gene expression in *Xenopus*, and gene knockouts in the mouse have demonstrated the involvement of Wnts in processes as diverse as segmentation, CNS patterning, and control of asymmetric cell divisions. The transduction of Wnt signals between cells proceeds in a complex series of events including post-translational modification and secretion of Wnts, binding to transmembrane receptors, activation of cytoplasmic effectors, and, finally, transcriptional regulation of target genes. Over the past two years our understanding of Wnt signaling has been substantially improved by the identification of Frizzled proteins as cell surface receptors for Wnts and by the finding that β-catenin, a component downstream of the receptor, can translocate to the nucleus and function as a transcriptional activator. Here we review recent data that have started to unravel the mechanisms of Wnt signaling.

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INTRODUCTION

Together with other families of secreted factors such as FGF, TGF-beta, and Hedgehog proteins, Wnt proteins are implicated in a wide variety of biological processes. The first Wnt gene, mouse Wnt-1, was discovered in 1982 as a proto-oncogene activated by integration of mouse mammary tumor virus in mammary tumors (Nusse & Varmus 1982). Consequently, the potential involvement of Wnt genes in cancer was the main area of research in the 1980s (reviewed by Nusse & Varmus 1992). With the molecular identification of the *Drosophila* segment polarity gene *wingless* (*wg*) as the orthologue of Wnt-1 (Cabrera et al 1987, Rijsewijk et al 1987) and the phenotypic analysis of Wnt-1 mutations in the mouse (McMahon & Bradley 1990, Thomas & Capecchi 1990), it became clear that Wnt genes are important regulators of many developmental decisions (reviewed in Nusse & Varmus 1992, Parr & McMahon 1994, Cadigan & Nusse 1997) (Table 1). At this moment, close to 100 Wnt genes have been isolated from species ranging from human to the nematode *Caenorhabditis elegans* (a comprehensive and regularly updated list of Wnt genes can be viewed on the Wnt homepage at http://www.stanford.edu/∼rnusse/wntwindow.html). All these genes encode proteins with a signal sequence and a nearly invariant pattern of 23 cysteines. Presumably, all Wnt proteins are secreted from cells and act through cell surface receptors either on the producing or on adjacent cells to determine cell fate or other differentiation parameters.

Table 1 Phenotypes of Wnt mutations in mouse, *Drosophila*, and *C. elegans*[a]

Gene	Organism	Phenotype
Wnt-1	Mouse	Loss of midbrain and cerebellum
Wnt-2	Mouse	Placental defects
Wnt-3A	Mouse	Lack of caudal somites and tailbud
Wnt-4	Mouse	Kidney defects
Wnt-7A	Mouse	Ventralization of limbs
wingless	*Drosophila*	Segment polarity, limb development, many others
Dwnt-2	*Drosophila*	Muscle defects, testis development
lin-44	*C. elegans*	Defects in asymmetric cell divisions
mom-2	*C. elegans*	Defects in endoderm induction and spindle orientation

[a]A fully referenced version of this table can be found on the Wnt homepage: http://www.stanford.edu/~rnusse/wntwindow.html

GENETIC ANALYSIS OF WINGLESS/WNT SIGNALING IN *DROSOPHILA* AND *CAENORHABDITIS ELEGANS*

Drosophila

The *Drosophila wg* gene is one of the best-characterized Wnt family members. The function of *wg* as a segment polarity gene was uncovered in a genetic screen for zygotic lethal mutations that affect larval cuticle pattern (Nüsslein-Volhard & Wieschaus 1980). In the *Drosophila* embryo, *wg* is required for formation of parasegment boundaries and for maintenance of *engrailed* (*en*) expression in adjacent cells. The epidermis of *wg* mutant embryos shows only rudimentary segmentation, which is reflected in an abnormal cuticle pattern. While the ventral cuticle of a wild-type larva displays denticle belts alternating with naked regions, the cuticle of a *wg* larva is completely covered with denticles. Embryos mutant for the genes *porcupine* (*porc*), *dishevelled* (*dsh*), *armadillo* (*arm*), and *pangolin* (*pan*) show a very similar phenotype. By contrast, mutations in *zeste-white 3* (*zw3*) show the opposite phenotype, a naked cuticle (reviewed by Klingensmith & Nusse 1994, Perrimon 1994). Analysis of double mutants indicates that these genes cooperate in a pathway to transduce the Wg signal (Noordermeer et al 1994, Peifer et al 1994, Siegfried et al 1994, Brunner et al 1997, van de Wetering et al 1997) (Figure 1).

C. elegans

Wnt genes have recently been implicated in early *C. elegans* embryogenesis as well. In the *C. elegans* embryo, the EMS cell undergoes an unequal division to generate the MS cell, which gives rise to mesoderm, and the E cell, which is the founder cell of the complete gut lineage. An inductive signal from P2 to EMS is required at the four-cell stage for this unequal division to occur. In

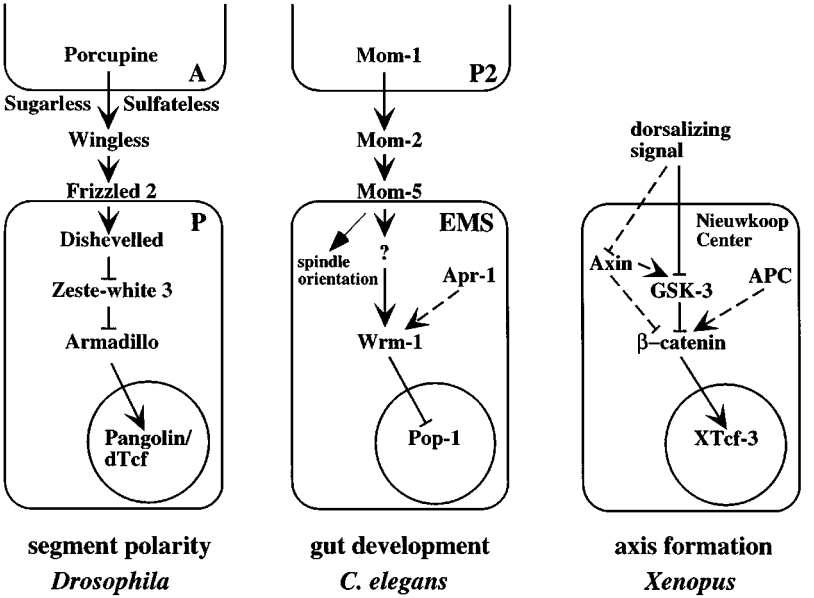

Figure 1 Wnt signaling pathways are conserved between *Drosophila*, *C. elegans*, and vertebrates. In *Drosophila*, anterior epidermal cells (*A*) express Wg and signal to adjacent posterior cells (*P*). This signal is required for establishment of parasegment boundaries and for maintenance of *engrailed* expression in posterior cells. In *C. elegans* a very similar pathway is used to induce the asymmetric division of the EMS cell. Note that only the first three components of this pathway, *mom-1*, *mom-2*, and *mom-5* are also involved in orientation of mitotic spindles during development. Formation of the primary body axis in *Xenopus* depends on a Wnt pathway comprising GSK3, β-catenin, and XTcf-3. Although overexpression of various Wnts and of Dsh leads to duplication of the body axis, they do not appear to be required for formation of the primary axis. Instead, the Wnt pathway may be triggered at the level of GSK3 by a hypothetical dorsalizing signal. Whether axin, APC, and its *C. elegans* counterpart *apr-1* are integral components of a common Wnt signaling pathway remains to be shown.

the absence of signal, EMS divides symmetrically and gives rise to two MS-like daughters that form mesoderm but no gut. In a screen for mutants that interrupt signaling from P2 to EMS, five genes called *mom1-5* were identified. Molecular analysis of three revealed that they encode proteins similar to *porc* (*mom-1*), Wnt (*mom-2*), and *frizzled* (*fz*), a Wnt receptor (*mom-5*; Rocheleau et al 1997, Thorpe et al 1997). Mutation of *pop-1*, an HMG box protein similar to *pan*, has the opposite effect of *mom* mutations: Both EMS daughters adopt the E fate and produce exclusively gut (Lin et al 1995). These results indicate that a Wnt signaling cascade mediates induction of EMS by P2 (reviewed in Han 1997; see Figure 1).

ASSAYS FOR WNT SIGNALING

Axis Duplication in Xenopus

Among the various ways to test for function of Wnt signaling components, injection of RNA into *Xenopus* embryos is perhaps the most convenient and informative. These tests are based on the observation that injection of Wnt-1 mRNA into ventral blastomeres of early *Xenopus* embryos leads to duplication of the body axis (McMahon & Moon 1989). This property of Wnt-1 is shared with many, but not all, members of the Wnt family (Table 2). The apparent functional differences between Wnts have led to the hypothesis that Wnts can be divided into two distinct classes that might employ different signal transduction mechanisms; members of the Xwnt-8 class (among them Wnt-1) are active in the axis duplication assay, whereas members of the Xwnt-5A class are not. Instead, overexpression of members of the Xwnt-5A class affects morphogenetic movements and may antagonize members of the Xwnt-8 class (Moon et al 1993, Du et al 1995, Torres et al 1996, Moon et al 1997a). Experiments in zebrafish indicate that Xwnt-5A may signal through a pathway involving phosphatidylinositol, G proteins, and release of intracellular calcium (Slusarski et al 1997a,b). However, the inability of Xwnt-5A to induce a secondary axis can be overcome by coinjection of human Frizzled 5, suggesting that the availability of a specific receptor rather than a fundamental difference in its signal transduction

Table 2 Axis duplication and cell transformation by different Wnts[a]

Gene	Axis duplication in *Xenopus*	Transformation of C57 or RAC cells
Wnt-1	yes	yes
Wnt-2	yes	yes
Wnt-3A	yes	yes
Wnt-4	no	no
Wnt-5A	no	no
Wnt-5B	nd	yes
Wnt-6	nd	no
Wnt-7A	nd	yes
Wnt-7B	nd	yes
Wnt-8A	yes	nd
Wnt-8B	yes	nd
Wnt-11	yes/no[b]	yes

[a]A fully referenced version of this table can be found on the Wnt homepage: http://www.stanford.edu/~rnusse/wntwindow.html

[b]See references on the Wnt homepage.

mechanism determines whether a particular Wnt can induce a secondary axis (He et al 1997).

Duplication of the embryonic axis can not only be induced by Wnts but also by overexpression of downstream components of Wnt signaling. Overexpression of Dsh, β-catenin (the vertebrate homologue of Arm), and dominant-negative glycogen synthase kinase 3 (GSK3, the vertebrate homologue of Zw3) all lead to an ectopic axis (Dominguez et al 1995, Guger & Gumbiner 1995, He et al 1995, Sokol et al 1995). These results provide strong evidence that components of Wnt signaling are structurally and functionally conserved between *Drosophila*, *C. elegans*, and vertebrates (Figure 1).

WNT SIGNALING AND FORMATION OF THE PRIMARY BODY AXIS IN XENOPUS Despite the ability of injected Wnts to induce a secondary axis, it is not clear whether an endogenous Wnt protein is involved in the establishment of the primary body axis in *Xenopus*. So far there is no Wnt gene known to be expressed at the expected place and time during embryogenesis. Moreover, injection of a dominant-negative form of Xwnt-8 can block induction of a secondary axis by Wnts but does not block formation of the primary axis (Hoppler et al 1996). The same is true for a dominant-negative form of Dsh (Sokol 1996). By contrast, overexpression of wild-type GSK3, dominant-negative Xtcf-3 (an HMG box protein similar to Pan and Pop-1), depletion of β-catenin by antisense RNA injection, or overexpression of cadherins leads to ventralization (Heasman et al 1994, Dominguez et al 1995, He et al 1995, Molenaar et al 1996). It has been suggested that the Wnt signaling cascade may be triggered downstream of Dsh by an unknown dorsalizing signal unrelated to Wnts, possibly a stimulus provided by rotation of the egg cortex after fertilization (Moon et al 1997a).

Tissue Culture Assays for Wingless/Wnt Signaling

MAMMALIAN TISSUE CULTURE Although the genetic and developmental systems described above are invaluable for the identification of new components of Wnt signaling and for establishment of their epistatic relationship, their usefulness for biochemical analysis of the signaling mechanism is somewhat limited. Therefore, many laboratories have used cultured cells to study Wnt signaling. This approach has been hampered by a major problem: Most Wnts including Wnt-1 are difficult to obtain in a soluble, biologically active form and tend to stick to the extracellular matrix (Bradley & Brown 1990, Smolich et al 1993). Due to these limitations, most studies used cells transfected with Wnt cDNAs instead of soluble Wnt protein preparations to study effects of Wnts on cell morphology, proliferation, transformation, or gene expression.

When assayed for their ability to transform mammary epithelial cells, two functional classes of Wnts can be distinguished: Members of one class,

including Wnt-1, Wnt-3A, and Wnt-7A, readily transform cells at high frequency, whereas members of the other class, Wnt-4, Wnt-5A, and Wnt-6, do not (Wong et al 1994) (Table 2). Interestingly, the two functional classes defined by this assay are similar to the functional classes defined by the axis duplication assay in *Xenopus* (Du et al 1995) (Table 2).

DROSOPHILA TISSUE CULTURE In contrast to mammalian Wnts, Wg produced in transfected *Drosophila* Schneider S2 cells is secreted into the medium and is biologically active when added to Wg-responsive cells (van Leeuwen et al 1994). The assay used for Wg activity is derived from the observation that in the *Drosophila* embryo, Arm protein accumulates in the cytoplasm of cells that are exposed to Wg (Riggleman et al 1990, Peifer et al 1994). A *Drosophila* imaginal disc cell line, cl-8, shows the same response upon incubation with Wg-conditioned medium (van Leeuwen et al 1994). Overexpression of Dsh or dominant-negative Zw3 also leads to Arm accumulation (Yanagawa et al 1995, 1997). By contrast, S2 cells do not show increased Arm levels after exposure to Wg although they respond to overexpression of Dsh, which led to the suggestion that S2 cells lack a functional Wg receptor (Yanagawa et al 1995).

WNT SIGNALING UPSTREAM OF THE RECEPTOR

A Role for Porcupine in Post-Translational Modification and Secretion of Wnts

Drosophila embryos mutant for the gene *porc* show a phenotype similar to that of *wg* mutants (van den Heuvel et al 1993, Kadowaki et al 1996). Clones of *porc* mutant cells display non-cell-autonomous effects, similar to *wg* clones, indicating that Porc is required in the cell that produces the signal rather than in the receiving cell (Kadowaki et al 1996). In *porc* embryos, Wg protein is confined to the narrow stripe of cells where the *wg* gene is transcribed, instead of spreading to adjacent cells as in wild-type embryos (van den Heuvel et al 1989, 1993). Several mutations in *wg* itself also cause retention of mutant Wg protein, indicating that changes of the protein structure can lead to misfolding and impair secretion (van den Heuvel et al 1993, Bejsovec & Wieschaus 1995, Hays et al 1997).

Molecular cloning of *porc* revealed that it encodes a multi-transmembrane protein predominantly found in the endoplasmic reticulum, consistent with a role in processing of Wg (Kadowaki et al 1996). Biochemical studies in cultured cells revealed increased N-linked glycosylation of Wg after coexpression with Porc (Kadowaki et al 1996). Glycosylation appears to be a common modification of Wnts and may be important for folding, secretion, and biological activity (Smolich et al 1993).

In *C. elegans*, the *porc* homologue *mom-1* is required in the P2 cell for signaling to the EMS cell by *mom-2* (a Wnt) (Rocheleau et al 1997, Thorpe et al 1997). Apart from this example, it is not known whether *porc* is essential for every Wnt protein to become secreted, mainly because there are few reagents to study the distribution of Wnt proteins in vivo.

Another ER-resident protein, the molecular chaperone BiP, associates with Wnt-1 (Kitajewski et al 1992). Although the functional significance of this interaction has not been tested, it is likely that BiP assists in proper folding of Wnts.

Proteoglycans Facilitate Wnt Signaling

The *Drosophila sugarless* (*sgl*) gene encodes UDP-glucose dehydrogenase, a key enzyme required for synthesis of proteoglycans. Mutants of *sgl* show a larval phenotype similar to *wg* (Binari et al 1997, Häcker et al 1997, Haerry et al 1997). Phenocopies of *sgl* can be generated by injection of heparinase, which selectively degrades heparin-like glycosaminoglycans, but not by chondroitinase, which degrades chondroitin sulfate, hyaluronic acid, and dermatan sulfate. Conversely, injection of heparan sulfate is sufficient to rescue the *sgl* mutant phenotype (Binari et al 1997). Mutations in *sulfateless* (*sfl*), a gene encoding heparan sulfate *N*-deacetylase/*N*-sulfotransferase, another enzyme required for heparan sulfate biosynthesis, also cause a *wg*-like phenotype, underpinning the importance of proteoglycans for Wg signaling (X Lin & N Perrimon, personal communication).

Sulfated glycosaminoglycans also affect Wg signaling in tissue culture. Treatment of cl-8 cells with heparinase, heparitinase, chondroitin ABC lyase, or perchlorate leads to reduced Wg-induced accumulation of Arm. This effect can be reversed by addition of chondroitin sulfate or heparin. Heparin binds to Wnt-1 (Bradley & Brown 1990) and Wg, and addition of heparin to Wg-conditioned medium leads to increased Wg activity (Reichsman et al 1996). However, heparin has also been reported to inhibit the activity of Wnt-1 in a cell transformation assay (Jue et al 1992). In *Xenopus* animal caps, removal of heparan sulfate proteoglycans blocks mesoderm induction by Xwnt-8, again suggesting that proteoglycans are important for the function of Wnts (Itoh & Sokol 1994).

Little is known about the role of proteoglycans in Wnt signaling. In analogy to FGF signaling, proteoglycans may be low-affinity coreceptors for Wnts, which would serve to increase the local concentration of ligand available for binding to high-affinity receptors. Alternatively, proteoglycans in the ECM may crosslink Wnts to induce clustering of Wnt receptors in the plasma membrane. In the absence of soluble, sufficiently pure, biologically active preparations of Wnts, this issue will be difficult to address.

INTERACTIONS BETWEEN WNTS AND PROTEINS OF THE FRIZZLED FAMILY

Frizzleds Are Receptors for Wnts

For many years a big question mark in models about Wnt signaling has been the nature of Wnt receptors. Recently, however, good evidence has emerged that seven-pass transmembrane proteins of the Frizzled family are involved in Wnt signaling: (*a*) Studies on tissue polarity in *Drosophila* reveal genetic interactions between *dsh* and *frizzled* (*fz*). Both genes act in a signaling pathway that controls correct orientation of hairs, bristles, and ommatidia in the epidermis and eyes of the adult fly (Adler 1992, Wong & Adler 1993, Krasnow et al 1995, Strutt et al 1997) (Figure 2). (*b*) Certain asymmetric cell divisions in *C. elegans* depend on the genes *lin-44* and *lin-17*. *lin-44* encodes a Wnt protein (Herman et al 1995), and *lin-17* encodes a Fz-like transmembrane protein, which led to the hypothesis that both proteins might interact in a ligand-receptor relationship (Sawa et al 1996).

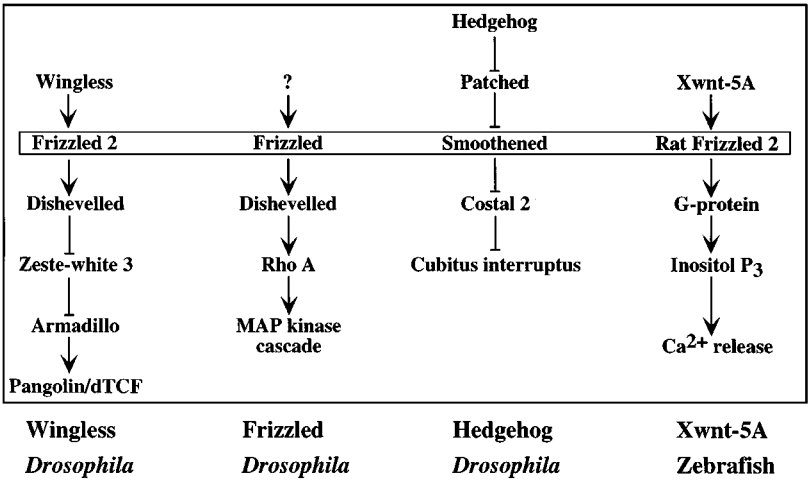

Figure 2 Frizzled receptors participate in different signaling pathways. Four examples are shown here. *Drosophila* Frizzled 2 functions in the classical Wg pathway, as shown in Figure 1. Frizzled is part of a pathway regulating planar polarity in the adult epidermis and in the eye of *Drosophila*. Downstream of Dsh, this pathway appears to be distinct from the Wg pathway. Whether Wg or another Wnt ligand is involved in planar polarity is unknown. Smoothened is a component of the Hedgehog signaling cascade and forms a complex with the Hedgehog receptor, Patched. Whether Smoothened has a ligand of its own is unknown. Rat Frizzled 2 cooperates with Xwnt-5A to activate a G protein–dependent pathway when overexpressed in zebrafish embryos. It is not known whether the interaction between rat Frizzled 2 and G proteins is direct.

This issue has been directly addressed in tissue culture. Transfection of Dfz2, a member of the Fz family, from *Drosophila* into S2 cells (S2Dfz2) confers responsiveness to Wg to these cells. Like cl-8 cells, S2Dfz2 cells accumulate Arm when incubated with Wg-conditioned medium. Moreover, S2Dfz2 cells and human 293 cells transfected with Dfz2 bind Wg on their cell surfaces, whereas untransfected cells of both cell lines do not (Bhanot et al 1996).

Cadigan et al (1998) also showed that a dominant-negative form of the *Dfz2* gene blocks signaling by *wg* in the *Drosophila* imaginal disc and that overexpression of this receptor causes phenotypes similar to those brought about by ectopic *wg*, in a *wg*-dependent way.

Additional evidence for Frizzleds as Wnt receptors comes from studies in *Xenopus*. Coinjection of rat Frizzled 1 and Xwnt-8 into frog embryos results in recruitment of Xwnt-8 to the plasma membrane and in increased expression of Xwnt-8 target genes, compared with injection of Xwnt-8 alone (Yang-Snyder et al 1996). Also, as mentioned above, coinjection of rat Frizzled 5 and Xwnt-5A leads to axis duplication, whereas injection of either rat Frizzled 5 or Xwnt-5A does not (He et al 1997).

Genetic Analysis of Frizzled-Wnt Interactions

DROSOPHILA Genetic data on the requirement for Frizzleds in Wnt signaling are limited in *Drosophila*. Loss-of-function mutants of the original *fz* are viable and show misorientation of hairs and bristles in the adult epidermis (Vinson & Adler 1987). Rotation of ommatidia in the eye imaginal disc is also defective in *fz* mutants, resulting in a rough eye phenotype (Zheng et al 1995). Clones of *fz* mutant cells induced in wing imaginal discs show an interesting feature termed directional nonautonomy, which describes the fact that cells within the clone, as well as wild-type cells located outside the clone, have misoriented wing hairs. Those "shadows" are found in a distal direction (Vinson & Adler 1987), which led to the suggestion that Fz is required for not only reception of a polarity signal but also for generation or propagation of a signal. This signal, whose nature remains to be determined, would spread in a proximal-distal direction across the wing imaginal disc (Adler 1992).

Although a physiological ligand for Fz has not been identified, it is anticipated that this ligand may be a Wnt. It has been suggested that there is a gradient of Fz activity across the wing that is caused by a graded distribution of the ligand and that hair polarity follows the slope of that gradient. Support for this notion comes from the observation that creation of an artificial gradient of Fz expression with the high point at the distal tip of the wing leads to polarity reversal (Adler et al 1997).

Interestingly, some alleles of *fz* do not show directional nonautonomy in clones and instead behave strictly cell autonomously. Molecular analysis of four

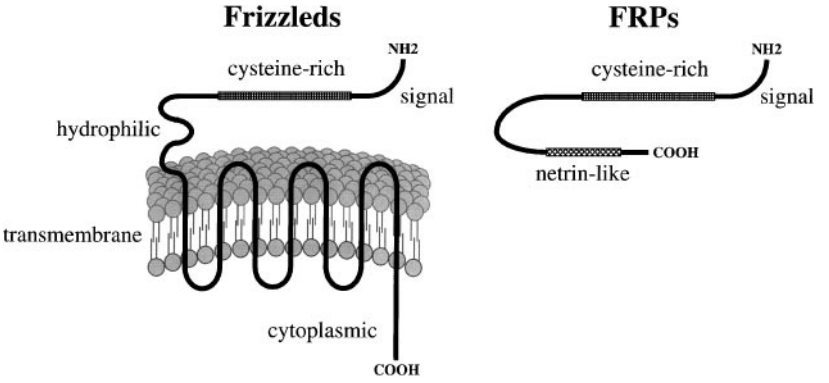

Figure 3 Structure of Frizzleds and FRPs. Frizzled receptors are characterized by an N-terminal signal peptide, a cysteine-rich ligand-binding domain (CRD) followed by a hydrophilic linker, seven transmembrane regions, and a cytoplasmic tail. FRPs are secreted proteins with a CRD similar to Frizzleds. In addition, they contain a region with similarity to netrins, secreted proteins involved in axon guidance.

of these alleles revealed that they all change the same proline residue in the first cytoplasmic loop (Jones et al 1996) (Figure 3). Because cell-autonomous and nonautonomous functions can be separated genetically, it has been speculated that *fz* interacts with at least two different effectors; one responsible for polarization of the cytoskeleton (cell-autonomous function) and the other one for relaying the signal to adjacent cells (nonautonomous function; Jones et al 1996).

In tissue culture, Fz binds Wg (Bhanot et al 1996). Whether Wg functions in tissue polarity is difficult to test because of the widespread effects of Wg on pattern formation. Nonetheless, ectopic expression of Wg in the eye imaginal disc leads to tissue polarity phenotypes (Tomlinson et al 1997). Conversely, overexpression of Fz in the embryonic epidermis causes phenotypes similar to those resulting from modest overexpression of Wg, which has been taken as evidence for Fz's involvement in Wg signaling (Tomlinson et al 1997).

Mutations in a second Fz gene, *Dfz2*, have not been isolated in *Drosophila*. Nonetheless, zygotic removal of Dfz2 by means of a large deficiency generated by chromosomal translocations does not block Wg signaling because the cytoplasmic increase of Armadillo protein in response to Wg is normal (HA Müller & E Wieschaus, personal communication). These results can be explained in several ways: *fz* and *Dfz2* act redundantly, and removal of both genes may be necessary to impair Wg signaling. Alternatively, maternally provided Dfz2 may be sufficient for Wg signaling during embryogenesis so that only removal of both maternal and zygotic expression would yield a phenotype. A third possibility would be the existence of additional Fz genes in *Drosophila*.

Besides *fz* and *Dfz2*, a third family member, *smoothened* (*smo*), has been identified in *Drosophila* (Alcedo et al 1996, van den Heuvel & Ingham 1996). Genetic analysis indicates that *smo* is required for Hedgehog (Hh) signaling rather than for Wg signaling (Figure 2). These findings, together with the predicted protein structure of Smo, led to the suggestion that Smo is an Hh receptor, implicating direct protein-protein interaction between Smo and Hh (Alcedo et al 1996, van den Heuvel & Ingham 1996). This hypothesis has been questioned by the finding that Hh and a vertebrate homologue, Sonic Hedgehog, can bind to Patched (Ptc), a multi-transmembrane protein, whereas no direct binding to Smo was detected (Chen & Struhl 1996, Marigo et al 1996, Stone et al 1996). However, Ptc can form a complex with Smo, suggesting that Hh activates Smo indirectly via Ptc (Chen & Struhl 1996, Stone et al 1996) (Figure 2).

C. ELEGANS In the worm, more genetic data are available for fz/Wnt interactions. The genes *lin-44* (a Wnt) and *lin-17* (an Fz) interact genetically in the control of certain asymmetric cell divisions. In *lin-44* mutants, the polarity of two asymmetric daughter cells is reversed, whereas in *lin-17* mutants, polarity is lost, resulting in two symmetric daughter cells (Herman et al 1995, Sawa et al 1996). *lin-44/lin-17* double mutants also produce symmetric daughter cells, indicating that *lin-17* acts downstream of *lin-44*. The fact that the phenotypes of *lin-44* and *lin-17* are not identical has led to the hypothesis that Lin-17 acts as a receptor for two signals coming from different directions—one Lin-44 and the other an unknown ligand X, possibly another Wnt (Sawa et al 1996).

Mutants in *mom-5*, a second Fz-like gene from *C. elegans*, also show some unexpected features. *mom-5* interacts with *mom-2* (a Wnt) to specify the gut precursor cell E, which is generated in the asymmetric division of EMS (Rocheleau et al 1997, Thorpe et al 1997; see above). The penetrance of the gutless phenotype of *mom-5* is low, $\approx$5%. Mutations in *mom-2* have a much higher penetrance, $\approx$40%. Surprisingly, the double mutant *mom-2/mom-5* shows a penetrance of only 8%, so the low penetrance of the *mom-5* phenotype is epistatic over the high penetrance *mom-2* phenotype, which is not compatible with the model that the receptor Mom-5 is simply activated by its ligand Mom-2 to specify the E fate. One explanation for these data would be that Mom-5 constitutively represses specification of E fate in the absence of the ligand Mom-2 and that this repression can be overcome by binding of Mom-2 (Rocheleau et al 1997).

In contrast to the low penetrance of endoderm defects in *mom-5* and *mom-2/mom-5* double mutant embryos, *mom-1* (Porc-like), *mom-2*, and *mom-5* mutants show a fully penetrant defect in orientation of the mitotic spindle in the ABar cell. This kind of defect is not observed in mutants for *apr-1* (APC), *wrm-1* (β-catenin), and *pop-1* (HMG box protein), indicating a branching of

the Wnt pathway downstream of *mom-5* (Rocheleau et al 1997, Thorpe et al 1997) (Figure 1). Interestingly, the function of *mom-1*, *mom-2*, and *mom-5* in spindle orientation may not require transcriptional regulation of Wnt target genes, since inhibition of the large subunit of RNA polymerase or α-amanitin treatment of embryos does not affect spindle orientation until the 26-cell stage (Rocheleau et al 1997 and references therein).

Together, these data indicate that Frizzled receptors can participate in several distinct signal transduction pathways (Figure 2). Moreover, there is evidence that an individual Frizzled can have more than one physiological ligand and, vice versa, that a Wnt may bind to more than one Frizzled. Regulation of Frizzleds may occur within the plane of the plasma membrane by association with other transmembrane proteins such as Ptc. In addition, Frizzleds may possess constitutive activity, allowing both activation and repression of downstream signaling components, depending on availability of ligand(s).

Structure of Frizzled Proteins

All members of the Fz family are characterized by the following features (beginning at the N terminus): a putative signal sequence, followed by a sequence of 120 amino acids (aa) containing 10 highly conserved cysteine residues (CRD), a highly divergent region of 40–100 aa predicted to form a flexible linker, seven transmembrane segments separated by short extracellular and cytoplasmic loops, and a cytoplasmic tail (Vinson et al 1989, Wang et al 1996) (Figure 3). The CRD appears to be the ligand-binding site of Frizzleds. Expression of the CRD of Dfz2 anchored in the membrane by a glycosyl-phosphatidylinositol (GPI) anchor is sufficient to provide binding sites for Wg on the surface of cells (Cadigan et al 1998). By contrast, expression of a mouse Fz4 construct lacking the CRD, but containing the signal sequence and all seven transmembrane domains, does not confer binding of Wg (Bhanot et al 1996).

The Wg-binding assay provides a means to test the specificity of ligand-receptor interactions. Several Fz proteins from human, mouse, and *Drosophila* have been tested for their ability to allow cell surface binding of Wg. Many of the Frizzleds tested, including the original Fz from *Drosophila*, do confer Wg binding, whereas others, e.g. mouse Fz3 and Fz6 and Smoothened, do not (Bhanot et al 1996, Nusse et al 1997, YK Wang et al 1997; Table 3). Although this assay does not provide measurement of binding affinities between Wg and different Frizzleds, it indicates that there is considerable promiscuity in the interaction of Wnts with their receptors.

The overall structure of Frizzleds resembles that of G protein-coupled receptors, which also have seven transmembrane regions. However, this similarity is mostly restricted to the membrane topology of both protein families, and there is little sequence identity between them. Nonetheless, expression of Xwnt-5A

Table 3 Interaction of Wnts with Frizzleds[a]

Frizzled Gene	Organism	Interaction with	Type of Interaction
Mfz3	Mouse	Not with Wg	Binding
Mfz4	Mouse	Wg	Binding
Mfz6	Mouse	Not with Wg	Binding
Mfz7	Mouse	Wg	Binding
Mfz8	Mouse	Wg	Binding
Hfz5	Human	Wg	Binding
		Xwnt-5A	Axis duplication in *Xenopus*
FZD3	Human	Wg	Binding
Rfz1	Rat	Xwnt-8	Recruitment to plasma membrane
Rfz2	Rat	Xwnt-5A	Activation of Ca^{2+}-signaling in zebrafish
Fz	*Drosophila*	Wg	Binding
Dfz2	*Drosophila*	Wg	Binding
Smo	*Drosophila*	Not with Wg	Binding
Lin-17	*C. elegans*	Lin-44	Genetic
		Egl-20	Genetic
Mom-5	*C. elegans*	Mom-2	Genetic

[a]A fully referenced version of this table can be found on the Wnt homepage: http://www.stanford.edu/~rnusse/wntwindow.html

in zebrafish embryos causes release of calcium from intracellular stores in a G protein-dependent fashion, whereas Xwnt-8 does not (Slusarski et al 1997a,b) (Figure 2). Expression of rat Frizzled 2 has a similar effect, whereas rat Frizzled 1 is inactive in this assay. Coinjection of Xwnt-5A and rat Frizzled 2 causes a more than additive increase in calcium release, indicating that both proteins may operate in a common pathway. Calcium release in response to Xwnt-5A or rat Frizzled 2 can be blocked by several inhibitors of G proteins, as well as by agents interfering with phosphatidylinositol signaling (Slusarski et al 1997a). While these data argue for an involvement of phosphatidylinositol and G proteins in Xwnt-5A signaling (Figure 2), it remains to be shown if they function as integral components of the pathway. Based on the available data (Slusarski et al 1997a,b), activation of G protein signaling by Xwnt-5A could also be a secondary effect.

Two downstream components of the Wnt-Fz pathway, Axin and Dishevelled, contain domains that are found in proteins involved in G protein signaling (Figure 4; see below). Thus the question whether G proteins play a role in Wnt signaling remains open and needs closer examination.

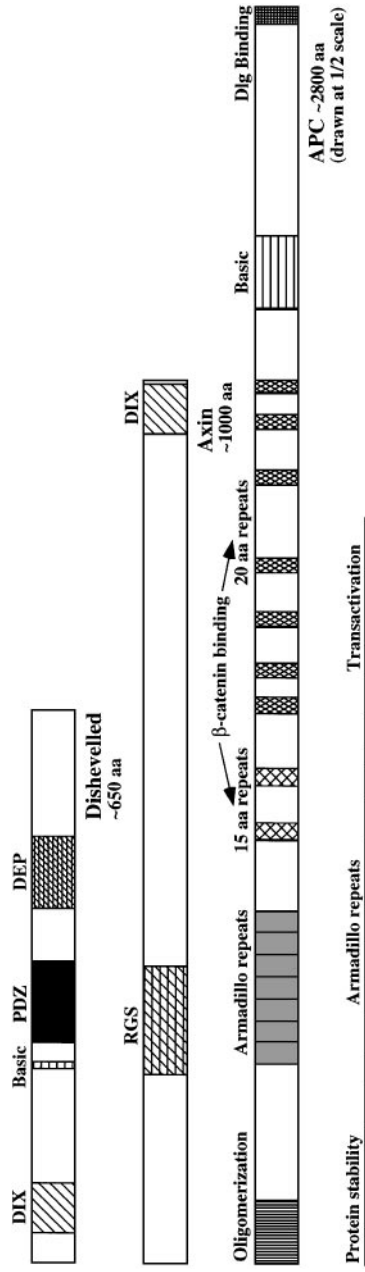

Figure 4 Components of Wnt signaling downstream of the receptor. Protein domains identified either by sequence similarity to other proteins or by functional assays are highlighted. For further explanations, see the respective paragraphs in the text.

The C terminus of many, but not all, Frizzleds has the sequence S/TXV, which has been described as a potential binding site for PDZ domains (Songyang et al 1997). Dsh is an obvious candidate for binding to the C terminus of Frizzleds because genetically it is most proximal to the receptor and contains a PDZ domain. However, attempts to show such a direct interaction have been unsuccesful (Nusse et al 1997). Moreover, the PDZ domain of Dsh lacks crucial amino acid residues predicted to interact with the S/TXV motif and therefore probably binds to a different sequence motif (Doyle et al 1996). The functional importance of the C terminus of Frizzleds is also questioned by an in vivo experiment in *C. elegans*: A Lin-17-GFP fusion protein lacking the 8 most C-terminal residues of Lin-17 rescues the *lin-17* mutant phenotype (Sawa et al 1996).

Frizzled/Wnt Interactions: How Many Pathways Are There?

One of the most important questions arising from the data summarized above is the following: How can individual Wnts elicit specific biological responses that are distinct from the effects of other members of the Wnt family? One way to achieve specificity is provided by restricting the expression pattern of different Wnts to discrete, nonoverlapping regions of the organism. This is often the case, but there are many examples for overlapping expression patterns of Wnts. The next level to discern between Wnts may be the specificity of ligand-receptor interactions. While the experiments described above reveal that not every Wnt can bind to every Frizzled, they also show that there is a high degree of promiscuity in Wnt/Frizzled interactions. However, many Frizzleds show highly specific expression patterns themselves (Bhanot et al 1996, Wang et al 1996), which further restricts the number of potential ligand-receptor interactions in any given tissue.

Once a Wnt has bound to a suitable Frizzled receptor, what happens next? Several scenarios are possible:

1 Every Wnt/Frizzled interaction leads to activation of the same downstream signaling cassette, comprising Dsh, GSK3, β-catenin, and HMG box proteins.

2 There are different signaling pathways downstream of Frizzleds, but each member of the Frizzled family can couple to only one of these pathways. If an individual Frizzled can bind different Wnts, binding of any one will activate the same pathway.

3 There are different signaling pathways downstream of Frizzleds, and a given Frizzled can couple to several of these pathways. Which pathway is activated depends on the Wnt ligand bound to the receptor.

4 There are different signaling pathways downstream of Frizzleds, but only some can be triggered by Wnts, while others are ligand independent or are triggered by ligands unrelated to Wnts.

This list can of course be extended, and the true situation may not be reflected correctly by any of these statements. Fortunately, we now have an increasing number of experimental tools to study these questions in various systems.

FRPs Are Structurally Related to the CRD of Frizzleds and Act as Secreted Antagonists of Wnts

Recently, a family of secreted proteins containing a CRD similar to that of Frizzleds has been identified in vertebrates (Hoang et al 1996, Finch et al 1997, Leyns et al 1997, Mayr et al 1997, Rattner et al 1997, S Wang et al 1997). In addition to the CRD, these frizzled-related proteins (FRPs) contain a C-terminal region with similarity to netrins, secreted proteins involved in axon guidance (Leyns et al 1997, S Wang et al 1997) (Figure 3). Some evidence has been obtained that FRPs can bind Wnts directly and that the CRD is necessary and sufficient for this interaction (Leyns et al 1997, Lin et al 1997, Rattner et al 1997, S Wang et al 1997) (Table 4). Coinjection of different FRP mRNAs together with Xwnt-8 mRNA into ventral blastomeres in *Xenopus* blocks axis duplication by Xwnt-8 (Finch et al 1997, Leyns et al 1997, Mayr et al 1997, S Wang et al 1997). In the case of Frzb-1 (also called FRP-3 or Fritz), this inhibition is also observed when Frzb-1 and Xwnt-8 are injected into different blastomeres, consistent with Frzb-1's being a secreted, diffusible antagonist of

Table 4 Interaction of Wnts with FRPs[a]

FRP Gene	Organism	Interaction with	Type of Interaction
FRP-1	Human	Wg, Wnt-1, Xwnt-8	Inhibition of axis duplication in frogs
FRP-2 (SDF-5)	Mouse	Wg	Binding
FRP-3 (Frzb-1)	Mouse	Wg, Xwnt-8	Binding, inhibition of axis duplication in frogs
	Xenopus	Xwnt-8, Wnt-1	Binding, coimmuno-precipitation, inhibition of axis duplication in frogs
	Bovine	Wnt-1, Wnt-5A	Coimmuno-precipitation, inhibition of Wnt-1-induced accumulation of β-catenin in cells, no effect on Wnt-5A in frog assay
Sizzled	*Xenopus*	Xwnt-8	Antagonism to Xwnt-8 in several frog assays

[a]A fully referenced version of this table can be found on the Wnt homepage: http://www.stanford.edu/~rnusse/wntwindow.html

Xwnt-8 (Leyns et al 1997, Mayr et al 1997, S Wang et al 1997). A similar conclusion was drawn from tissue culture experiments with mammalian cells, where Frzb-1 inhibits Wnt-1-mediated accumulation of β-catenin (Lin et al 1997). Interestingly, although Frzb-1 can also bind to Wnt-5A, as shown by coimmunoprecipitation, it does not block the activity of Wnt-5A in a *Xenopus* assay (Lin et al 1997) (Table 4).

Although a function for FRPs during normal development has not been rigorously shown owing to the absence of mutants, Frzb-1 shows an intriguing expression pattern during *Xenopus* embryogenesis. Expression is restricted to the region of the Spemann organizer and thus is complementary to the pattern of Xwnt-8, which is expressed in the ventral marginal zone (Leyns et al 1997, S Wang et al 1997). Because Xwnt-8 appears to specify ventrolateral mesoderm, it is tempting to speculate that Frzb-1 expression in the Spemann organizer specifies dorsal cell fates by antagonizing Xwnt-8 (Christian & Moon 1993, Hoppler et al 1996, Leyns et al 1997, Moon et al 1997b, S Wang et al 1997).

Are Frizzleds the Only Receptors for Wnts?

Based on genetic interaction studies in *Drosophila*, it has been speculated that Notch (N), a transmembrane protein involved in many developmental processes, might be a receptor for Wg (Couso & Martinez-Arias 1994, Hing et al 1994). However, in the complete absence of N, Wg signaling appears unaffected in the embryo (Cadigan & Nusse 1996). In the wing imaginal disc, the effect of N on Wg signaling may be indirect. Removal of N by means of a temperature-sensitive mutation or in clones leads to loss of Wg expression, providing an explanation for the genetic interaction between these two genes. Nonetheless, clones of N mutant cells show a normal response to Wg produced by wild-type cells adjacent to the clone (Rulifson & Blair 1995). Transfection of S2 cells with N does not lead to significant cell surface binding of Wg and does not confer responsiveness to Wg (Bhanot et al 1996). Taken together, these data do not support a direct role for N in reception of the Wg signal.

WNT SIGNALING DOWNSTREAM OF THE RECEPTOR

Dishevelled

Genetic analysis in *Drosophila* reveals an absolute requirement for *dsh* in reception of the Wg signal (Klingensmith et al 1994, Theisen et al 1994). *dsh* acts cell autonomously and has been placed genetically between *wg* and *zw3* in the Wg signaling cascade (Klingensmith et al 1994, Noordermeer et al 1994, Siegfried et al 1994, Theisen et al 1994) (Figure 1). *dsh* is also part of a tissue

polarity signaling pathway, where it is required cell autonomously downstream of *fz* and upstream of *rhoA* (Theisen et al 1994, Krasnow et al 1995, Strutt et al 1997) (Figure 2). Furthermore, Dsh has been reported to suppress N signaling when overexpressed and to directly bind to the cytoplasmic domain of N in a yeast two-hybrid assay (Axelrod et al 1996).

Knockout mice lacking *dvl-1*, a mouse homologue of *dsh*, do not have any obvious anatomical abnormalities and are viable and fertile. However, on closer examination, these animals show abnormal social behavior and several neurological defects (Lijam et al 1997). Similar defects are commonly seen in human patients suffering from certain neurological disorders. The somewhat surprising lack of gross morphological defects in the *dvl-1* knockout mice may be explained by a redundant function provided by at least two other *dsh* homologues in the mouse.

dsh encodes a ubiquitously expressed cytoplasmic protein containing four domains that are highly conserved among all known Dsh homologues, ranging from *C. elegans* to human (Figure 4). At the N terminus, Dsh contains a stretch of 50 aa similar to a region in axin, a protein implicated in Wnt signaling in vertebrates (Zeng et al 1997) (Figure 4). The three other conserved regions are a short basic domain, a centrally located PDZ domain, and a more C-terminal DEP-domain, which is also found in several proteins interacting with protein kinase C (PKC) (Klingensmith et al 1994, Theisen et al 1994, Ponting & Bork 1996). The function of Dsh is unknown, but the presence of two domains implicated in protein-protein interactions suggests that Dsh may be an adaptor protein required for assembly of a signaling complex, analogous to Grb-2 in the Ras pathway.

Dsh is a phosphoprotein that becomes more highly phosphorylated on serine and threonine residues when Wg signaling is activated. Moreover, the most highly phosphorylated form of Dsh is enriched in the membrane fraction, suggesting that Wg signaling leads to recruitment of Dsh to a membrane compartment. Overexpression of Dsh in the absence of Wg also leads to hyperphosphorylation and to accumulation of Arm and thus mimics activation of Wg signaling (Yanagawa et al 1995). The latter conclusion has also been confirmed in vivo (Axelrod et al 1996, Cadigan & Nusse 1996).

Affinity purification of a complex containing Dsh and several associated proteins revealed the association of Dsh with casein kinase 2 (CK2), a serine-threonine-specific protein kinase. CK2 efficiently phosphorylates Dsh in vitro and in vivo, but the functional significance of this phosphorylation is not clear (Willert et al 1997).

Frizzled expression can affect the phosphorylation status of Dsh (Willert et al 1997) and alter its subcellular localization. In *Xenopus*, overexpressed

Xdsh-GFP is localized in the cytoplasm in a punctate pattern. By contrast, overexpression of Xdsh-GFP together with Rat Frizzled 1 results in relocalization of Xdsh-GFP to the plasma membrane (Yang-Snyder et al 1996).

Zeste-White 3/Glycogen Synthase Kinase 3

Mutations in *zeste-white 3* (*zw3*), a gene encoding the *Drosophila* homologue of vertebrate glycogen synthase kinase 3 (GSK3), a serine-threonine protein kinase, have the opposite phenotypes of the mutations in *wg*, *dsh*, and *arm* (Siegfried et al 1992). By genetic epistasis analysis, *zw3* has been placed between *dsh* and *arm* in the Wg signaling pathway (Peifer et al 1994, Siegfried et al 1994) (Figure 1). The consensus model is that *zw3* acts as a constitutive repressor of the signaling activity of Arm, further implying that Wg signaling leads to repression of this constitutive activity of Zw3, thus allowing activation of Arm.

Results consistent with this model were obtained after injection of RNAs encoding wild-type and dominant-negative forms of GSK3 into *Xenopus* embryos, supporting the view that GSK3 is an integral component of Wnt signaling in vertebrates (Dominguez et al 1995, He et al 1995) (Figure 1).

These findings raise two major questions: (*a*) What is the mechanism for suppression of Zw3/GSK3 activity by Wg/Wnt, and (*b*) what are the targets for Zw3/GSK3 activity responsible for suppressing the signaling activity of Arm/β-catenin?

In cultured mouse 10T1/2 fibroblast cells, the enzymatic activity of GSK3 is inhibited by incubation of cells with Wg-conditioned medium (Cook et al 1996). Several inhibitors of established signal transduction pathways were tested for their ability to block Wg-induced inhibition of GSK3 activity. Treatment of cells with wortmannin, an inhibitor of insulin-mediated repression of GSK3 activity, had no effect on inhibition by Wg. By contrast, treatment of cells with different inhibitors of PKC blocked the effect of Wg on GSK3, indicating an involvement of PKC signaling in Wg-mediated inhibition of GSK3 (Cook et al 1996). Because Dsh is required for transduction of the Wg signal and contains a potential PKC-binding site in its DEP domain, there is increasing evidence for PKC playing a role in Wg signaling. Interestingly, several PKC isoforms can inhibit GSK3-β by direct phosphorylation in vitro (Goode et al 1992).

Axin

A novel and unexpected player in Wnt signaling has emerged from studying a classical mouse mutation, *fused* (now called *axin*). Mutations in this gene, which encodes a protein with sequence similarity to the conserved N-terminal region of Dsh and to regulators of G protein signaling (RGS) proteins result in axial duplications in mouse embryos (Zeng et al 1997). Ectopic expression of wild-type axin in dorsal blastomeres of *Xenopus* embryos causes ventralization,

indicating an inhibitory function of axin in normal axis formation. Conversely, ventral injection of RNA encoding a mutant axin lacking the RGS domain leads to axis duplication, suggesting that this mutant is a dominant-negative form of axin. Coinjection of *axin* RNA together with RNAs encoding components of Wnt signaling revealed that axin acts downstream of Xwnt-8, Dsh, and GSK3 and upstream of β-catenin (Zeng et al 1997; Figure 1). Indeed, it was recently shown that the axin protein can bind to GSK3 and to β-catenin directly, promoting phosphorylation of β-catenin in this complex (Ikeda et al 1998, Sakanaka et al 1998). Loss of axin would therefore lead to lack of GSK3-mediated β-catenin phosphorylation, up-regulation of β-catenin activity, and axis duplication, which is the phenotype of the mouse-fused mutation, the gene encoding axin.

Beside the fact that deletion of the RGS domain creates a dominant-negative protein, the role of this domain for regulation or function of axin is unclear. Whether the RGS domain of axin can bind to G_α subunits of heterotrimeric G proteins is unknown. Nonetheless, together with the similarity of Frizzleds to G protein-coupled receptors, the presence of this domain in axin provides an additional hint to the potential involvement of G proteins in Wnt signaling.

A Role for Zw3/GSK3 and APC in Regulation of Arm/β-Catenin Turnover

As discussed above, Zw3/GSK3 acts as a constitutive inhibitor of the signaling activity of Arm/β-catenin. Zw3/GSK3 appears to shorten the half life of Arm/β-catenin by promoting phosphorylation of several sites in the N-terminal portion of Arm/β-catenin (Munemitsu et al 1996, Yost et al 1996, Pai et al 1997). Although these sites fit the consensus for phosphorylation by Zw3/GSK3, Arm/β-catenin appears to be a poor substrate for Zw3/GSK3 (Stambolic et al 1996, Pai et al 1997). However, phosphorylation of β-catenin by GSK3 is promoted in the ternary complex between these two proteins and axin (Ikeda et al 1998, Sakanaka et al 1998).

How does phosphorylation affect the stability of Arm/β-catenin? A common pathway for regulated degradation of short-lived proteins involves ubiquitination of the protein, followed by degradation in the proteasome. In line with this mechanism, β-catenin is ubiquitinated, and its turnover is slowed after incubation of cells with specific proteasome inhibitors. Ubiquitination is reduced in Wnt-1 transfected cells, resulting in stabilization of β-catenin (Aberle et al 1997). The potential GSK3 phosphorylation sites mentioned above are required for ubiquitination to occur, providing an explanation for the observed stabilization of mutant β-catenin lacking these sites (Aberle et al 1997).

Moreover, mutations in the *Drosophila* gene *slimb*, which encodes an F-box WD40-repeat protein, reveal that this gene negatively regulates wg signaling (Jiang & Struhl 1998). These F-box proteins have been shown to target other

proteins for degradation by the ubiquitin/proteasome pathway, and in the case of wg signaling, the target of *slimb* could be the Arm protein (Jiang & Struhl 1998).

The turnover of Arm/β-catenin can be regulated not only by Zw3/GSK3, but also by the adenomatous polyposis coli (APC) protein (Figure 4). APC can bind to Arm/β-catenin and promotes its degradation in tissue culture (Rubinfeld et al 1993, Su et al 1993, Munemitsu et al 1995). Loss of APC function is observed in the majority of human colon cancers and leads to elevated β-catenin levels (Korinek et al 1997, Morin et al 1997, Rubinfeld et al 1997). Mutations in β-catenin that lead to stabilization of the protein are also frequently found in colon cancers and melanomas, suggesting that the main function of APC as a tumor suppressor is the control of β-catenin levels (Morin et al 1997, Rubinfeld et al 1997). In this function APC may be regulated by GSK3, because APC, GSK3, axin, and β-catenin can associate in a complex. APC is a good substrate for GSK3 in vitro and association of β-catenin with APC appears to depend on phosphorylation of APC by GSK3 (Rubinfeld et al 1996; reviewed in Barth et al 1997, Cavallo et al 1997, Willert & Nusse 1998).

WHAT IS THE ROLE OF APC IN WNT SIGNALING? Despite the impressive amount of data implicating APC in β-catenin regulation, its role in Wnt signaling is not firmly established: (*a*) Mutation of a *Drosophila* homologue of APC does not interfere with Wg signaling (Hayashi et al 1997). (*b*) Based on its role in promoting degradation of β-catenin, APC would be predicted to act antagonistically to Wnts or β-catenin. Exactly the opposite has been found in *Xenopus* and *C. elegans*. Injection of RNAs encoding various forms of APC capable of promoting degradation of β-catenin in tissue culture into *Xenopus* embryos leads to axis duplication, as does injection of Xwnt-8 or β-catenin (Vleminckx et al 1997). Axis duplication by APC requires β-catenin, but it is not correlated with downregulation of β-catenin, indicating that APC and β-catenin may signal together in a complex (Vleminckx et al 1997). In *C. elegans*, inhibition of *apr-1*, an APC-related gene, by RNA interference has a phenotype similar to that of mutants in *mom-2* (Wnt) or *wrm-1* (β-catenin) (Rocheleau et al 1997). Together, these data indicate that APC may have additional functions in Wnt signaling independent of its role in downregulation of β-catenin (reviewed in Barth et al 1997, Cavallo et al 1997, Willert & Nusse 1998).

Wnt Signaling Regulates Transcription of Target Genes Through a Complex of Arm/β-Catenin with HMG-Box Proteins

Significant progress has been made in understanding how Wnt signals are transduced into the nucleus to alter cell fate and transcription of target genes. The most downstream component of Wnt signaling identified by genetics

was Arm/β-catenin, a cadherin-associated protein required for assembly of adherens junctions (Noordermeer et al 1994, Peifer et al 1994, Siegfried et al 1994) (Figure 4). Studies in *Drosophila* reveal that Wg signaling leads to post-transcriptional stabilization of Arm in the cytoplasm of embryonic and cultured cells (Riggleman et al 1990, Peifer et al 1994, van Leeuwen et al 1994). Similar observations were made for β-catenin in Wnt-1-transfected mammalian cells (Hinck et al 1994). Upon closer examination it became clear that Wg/Wnt signaling predominantly stabilizes a soluble, cytoplasmic form of Arm/β-catenin that is not associated with cadherins (Peifer et al 1994, Papkoff et al 1996; A Wodarz, DB Stewart, WJ Nelson & R Nusse, unpublished data). Genetically, the functions of Arm in Wg signaling and in cadherin-mediated cell adhesion can be separated, arguing that they are, to some extent, independent of each other (Orsulic & Peifer 1996, Sanson et al 1996). These findings led to the conclusion that the non-cadherin bound form of Arm/β-catenin, stabilized by Wg/Wnt signaling, is crucial for transduction of the signal to the nucleus.

According to recent data, the stabilized form of β-catenin forms a complex with the HMG-box transcription factor LEF-1 and acts as a transcriptional activator in the nucleus (Behrens et al 1996, Huber et al 1996). This rather unexpected finding has been corroborated by the identification of the LEF-1 homologue *pan/dTCF* as a segment polarity gene in *Drosophila*, which acts downstream of *arm* in Wg signaling (Brunner et al 1997, van de Wetering et al 1997). In *Xenopus*, injection of RNA encoding a dominant-negative form of the LEF-1 homolog *XTcf-3* blocks axis duplication by β-catenin and leads to ventralization, indicating that *XTcf-3* acts downstream of β-catenin and is essential for formation of the endogenous axis (Molenaar et al 1996).

All these findings imply a function of Arm/β-catenin in the nucleus, which is supported by in situ analysis of Arm/β-catenin protein localization in *Drosophila* and *Xenopus* embryos and in cultured cells (Funayama et al 1995, Behrens et al 1996, Huber et al 1996, Molenaar et al 1996, Orsulic & Peifer 1996, Schneider et al 1996, Larabell et al 1997, Miller & Moon 1997).

In conclusion, Wg/Wnt signaling stabilizes uncomplexed Arm/β-catenin in the cytoplasm, which can then translocate to the nucleus to associate with transcription factors of the HMG-box protein family to directly regulate transcription of Wg/Wnt target genes.

However, HMG-box proteins do not always act synergistically with Wnts: The *pop-1* gene encodes a HMG-box protein participating in a Wnt cascade that regulates the asymmetric division of the EMS cell in *C. elegans*. Mutation of this gene leads to the opposite phenotype of those phenotypes produced by mutations in *mom-2* (Wnt) or *wrm-1* (β-catenin) (Lin et al 1995, Rocheleau et al 1997, Thorpe et al 1997) (Figure 1). Constitutive repression of a target gene promoter in the absence of nuclear β-catenin has also been demonstrated for XTcf-3 (Brannon et al 1997). Thus, HMG-box proteins appear to have

the intrinsic ability to either activate or repress transcription of target genes, a decision that may be regulated by interaction with Arm/β-catenin.

For further information the reader is referred to several recent reviews (Barth et al 1997, Cavallo et al 1997, Nusse 1997, Willert & Nusse 1998).

TARGET GENES OF WNT SIGNALING

In most models, the main function of Wnt signaling is the regulation of cell fate decisions by altering the transcriptional program of target cells in an instructive fashion. Consistent with these models, mutation of Wnt genes or inappropriate expression of Wnts usually leads to cell fate changes, which are reflected by altered gene expression. Not surprisingly, many genes directly or indirectly regulated by Wnts are transcription factors or secreted signaling molecules that are likely to be key players in a hierarchy of regulatory genes. Among the best studied examples of such genes are members of the homeobox family of genes, e.g. *engrailed* (*en*) and *ultrabithorax* (*ubx*). For two of these genes, *ubx* and *siamois*, the latter a gene expressed in the Nieuwkoop center in the *Xenopus* embryo, there is evidence for direct transcriptional activation by binding of a complex consisting of Arm/β-catenin and HMG-box proteins to specific sites in the target gene promoter (Brannon et al 1997, Riese et al 1997). Both studies found that transcriptional activation by Wnt signaling alone is not sufficient to explain the normal expression pattern of the target gene. Additional inputs from other sources of patterning information appear to be integrated at the level of the target gene promoter, giving rise to the final transcriptional pattern. Such a combinatorial mechanism of gene activation may explain how Wnt signaling can regulate a large number of different target genes and participate in apparently unrelated processes depending on the cellular context in which signaling takes place. One example for tissue-specific differences in target gene regulation is the effect of Wg on expression of *achaete* (*ac*), a proneural gene. In the wing imaginal disc, Wg activates *ac* expression (Couso et al 1994), whereas in the eye imaginal disc, Wg acts as a repressor of *ac* (Cadigan & Nusse 1996). Cooperation with other signaling pathways at the level of target gene promoters may also explain why ubiquitous expression of Wg does not lead to ubiquitous expression of Wg target genes (Noordermeer et al 1992, Sampedro et al 1993, Baylies et al 1995).

Do Wnts Act as Morphogens?

One of the most interesting questions in pattern formation has been whether secreted signaling molecules such as Wnts can form gradients and activate target genes in a concentration-dependent manner. In the case of Wg, ample evidence indicates that this is indeed the case (Zecca et al 1996, Neumann & Cohen 1997). In the wing imaginal disc, expression of the Wg target genes

neuralized (*neur*), *Distalless* (*Dll*), and *vestigial* (*vg*) depends on the distance from Wg-expressing cells and is abolished or strongly reduced in clones of *dsh* or *arm* mutant cells, even at a distance of more than 10 cell diameters away from the stripe of Wg at the wing margin. Furthermore, ectopic expression of Wg, but not of Dsh or constitutively active Arm, leads to non-autonomous activation of Wg target genes (Zecca et al 1996, Neumann & Cohen 1997). These results indicate that Wg can move over a considerable distance and argue against the existence of a signal relay mechanism to explain the long-range action of Wg. In the *Drosophila* wing imaginal disc, the stability and distribution of the Wg protein appears to be regulated by the concentration of one of its putative receptors, *Dfz2*. Because *Dfz2* expression itself is subject to transcriptional downregulation by *wg*, the *wg* gene controls the shape of its own morphogen gradient in a negative feed-back loop (Cadigan et al 1998).

PERSPECTIVE

Despite the impressive amount of novel data that have substantially improved our understanding of Wnt signaling, many gaps remain to be filled before we will get a glimpse of the complete picture. Open questions include: (*a*) What determines the specificity of interactions between Wnts and Frizzled receptors? (*b*) How do Frizzleds activate downstream signaling components? (*c*) What is the function of Dsh? (*d*) What is the relationship between Wnt signaling and tissue polarity signaling? (*e*) Is there a role for G proteins and PKC in Wnt signaling? (*f*) How do Wnts affect cell polarity and cell adhesion? (*g*) What is the role of Wnt signaling in cancer?

Given the multitude of experimental systems being used to study Wnt signaling, answers to remaining questions will undoubtedly come from many different directions, at an accelerated pace.

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