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REVIEW

Wnt signaling: a common theme in animal development

Ken M. Cadigan and Roel Nusse[1]

Howard Hughes Medical Institute, Department of Developmental Biology, Beckman Center, Stanford University Medical Center, Stanford, California 94305-5323 USA

Wnt proteins are now recognized as one of the major families of developmentally important signaling molecules, with mutations in *Wnt* genes displaying remarkable phenotypes in the mouse, *Caenorhabditis elegans*, and *Drosophila*. Among functions provided by Wnt proteins are such intriguing processes as embryonic induction, the generation of cell polarity, and the specification of cell fate. Until recently, our knowledge of the molecular mechanism of Wnt signaling was very limited, but over the past year, several major gaps have been filled. These include the identification of cell-surface receptors and a novel mechanism of relaying the signal to the cell nucleus. In addition, several components of Wnt signaling are implicated in the genesis of human cancer. These insights have come from different corners of the animal kingdom and have converged on a common pathway. At this junction in this rapidly evolving field, we review our current understanding of Wnt function and signaling mechanisms, doing so in a comparative approach. We have put emphasis on the latest findings, highlighting novelty and underscoring questions that remain. For additional literature, we refer to several previous reviews (McMahon 1992; Nusse and Varmus 1992; Klingensmith and Nusse 1994; Miller and Moon 1996; Moon et al. 1997). We have limited the number of references, particularly in the tables. Fully referenced forms of these tables can be found on the Wnt homepage (http://www-leland.stanford.edu/~rnusse/wntwindow.html).

The Wnt Family

Wnt genes are defined by sequence homology to the original members *Wnt*-1 in the mouse (first called *int*-1; Nusse and Varmus 1982; Van Ooyen and Nusse 1984) and *wingless* (*wg*) in *Drosophila* (Cabrera et al. 1987; Rijsewijk et al. 1987). They encode secreted glycoproteins, usually 350–400 amino acids in length. Homologous genes have been found in increasing numbers in organisms ranging from mammals to the nematode *C. elegans*. The degree of sequence identity in Wnt proteins is minimally 18%, including a conserved pattern of 23–24 cysteine residues, in addition to other invariant amino acids. By phylogenetic analysis, several of these genes have been assigned as orthologs of each other across species, mostly within vertebrates (Sidow 1992). Vertebrate genomes also contain several combinations of highly similar *Wnt* genes, usually referred to as A-B pairs. Table 1 lists the known *Wnt* genes in the most relevant species as groups of orthologs.

wg in *Drosophila* (Sharma and Chopra 1976; Baker 1987; Rijsewijk et al. 1987) is the best understood *Wnt* family member and has been a paradigm in elucidating *Wnt* gene function in other organisms. The first insight into understanding the mechanism of Wnt signal transduction came from the existence of several fly genes with mutant phenotypes consistent with defects in Wg signaling (Nüsslein-Volhard and Wieschaus 1980; Wieschaus and Riggleman 1987; Perrimon et al. 1989). *porcupine* (*porc*), *dishevelled* (*dsh*), *armadillo* (*arm;* the *Drosophila* homolog of β-catenin) and *pangolin* (*pan*,D*Tcf*) mutant embryos have segment polarity defects similar to *wg*, while *zeste-white 3* (*zw3*) mutants have the opposite phenotype. These genes have been ordered in a genetic pathway (Fig. 1; Klingensmith et al. 1994; Noordermeer et al. 1994; Peifer et al. 1994b; Siegfried et al. 1994; Brunner et al. 1997; Van de Wetering et al. 1997), and recent studies have focused on understanding the biochemical relationship between the protein products. This effort has been greatly augmented by the study of the vertebrate counterparts of these genes, giving us our current understanding of *Wnt* signal transduction summarized in this review.

More *Wnt* phenotypes

Many of the known *Wnt* loss-of-function mutations have been generated in the mouse, and some highly intriguing phenotypes are seen. For example, inactivation of *Wnt*-7A results in animals with ventralized limbs (Parr and McMahon 1995). This phenotype concurs with the dorsal epidermal expression pattern of *Wnt*-7A and with the consequences of ectopic expression of the gene in chicken limb buds, which dorsalizes the limbs (Yang and Niswander 1995). Similarly interesting is the phenotype of *Wnt*-4 mutations: the absence of kidneys (Stark et al. 1994). This role of *Wnt*-4 in kidney development is underscored by ectopic expression studies, showing that

[1]Corresponding author.
E-MAIL rnusse@cmgm.stanford.edu; FAX (650) 723-1399.

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Table 1. Wnt *genes in various organisms*

Gene	Mouse	Human	*Xenopus*	Chicken	Zebrafish	*Drosophila*	*C. elegans*[a,b]
Wnt-1	●	●	●		●	*wg*	*Ce-Wnt*-1
Wnt-2	●	●	●		●		*Ce-Wnt*-2
Wnt-2B		●	●				*lin*-44
Wnt-3	●	●	●		●		*mom*-2
Wnt-3A	●		●				*egl*-20
Wnt-4	●	●	●	●	●		
Wnt-5A	●	●	●	●	●	*DWnt*-3/5	
Wnt-5B	●						
Wnt-6	●		●				
Wnt-7A	●	●	●	●	●	*DWnt*-2	
Wnt-7B	●	●	●				
Wnt-7C			●				
Wnt-8A	●		●	●	●		
Wnt-8B[c]	●	●	●		●		
Wnt-8C				●[d]			
Wnt-9[e]							
Wnt-10A	●		●		●		
Wnt-10B	●	●					
Wnt-11	●	●	●	●			
(*Wnt*-12, *Wnt*-13)[f]							
						DWnt-4[g]	

●Identification of the gene.
[a]The *C. elegans Wnt* genes are not assigned as orthologs of vertebrate genes.
[b]C. Kenyon (pers. comm.).
[c]Mouse *Wnt*-8B unpublished, isolated by John Mason (pers. comm.).
[d]Chicken *Wnt*-8C might be considered the true ortholog of mouse and *Xenopus Wnt*-8A, as these genes are very similar. In addition, there are no other chicken *Wnt*-8 genes yet, nor have separate orthologs of *CWnt*-8C been cloned from the mouse and the human.
[e]A partial sequence of *Wnt*-9 has been isolated from hagfisch and thresher shark only.
[f]There have been reports on *Wnt* genes called *Wnt*-12 and *Wnt*-13, but they are either identical to one another (*Wnt*-12 is the same as *Wnt*-10B) or similar (*Wnt*-13 should be called *Wnt*-2B). More information on the nomenclature and classification of *Wnt* genes can be found on the *Wnt* gene homepage (http://www-leland.stanford.edu/~rnusse/wntwindow.html).
[g]*DWnt*-4 is too divergent to be assigned as an ortholog.

this gene may function in the mesenchymal–epithelial transitions occurring during the formation of this organ (Herzlinger et al. 1994; Stark et al. 1994). (See Table 2 for a comprehensive list of *Wnt* mutations and phenotypes.)

Wnt *mutations in* C. elegans

The exciting recent findings on *Wnt* mutations in the nematode *C. elegans* have given the field another model system that rivals *Drosophila* in its power of genetic analysis. There are at least five *Wnt* genes in the worm, one of which (*mom-2*) is implicated in setting up the polarity of the embryo. In four-cell-stage embryos, the P2 cell, itself part of the germ-line lineage, polarizes the adjacent EMS cell which will then divide into a endodermal (E) and mesodermal (MS) precursor (for review, see Bowerman 1997; Figure 2). Genetic screens have identified a set of maternal genes called *mom* (for more mesoderm), where the E cell adopts a MS cell fate. One of these genes, *mom-2*, encodes a *Wnt* gene that is required in the P2 cell, suggesting that *mom*-2 is a major signal for the polarization of the EMS cell (Rocheleau et al. 1997; Thorpe et al. 1997). Other *mom* mutants include *mom*-1, encoding a homolog of *Drosophila porc* and *mom*-5, which belongs to the Frizzled (Fz) family of cell-surface proteins, recently implicated as Wnt receptors (Bhanot et al. 1996; Rocheleau et al. 1997). In addition, RNA interference experiments provide evidence for an Arm/β-catenin homolog functioning in this pathway (Rocheleau et al. 1997). The *pop*-1 gene (Lin et al. 1995), which has the opposite phenotype of the *mom* genes (transforming MS into an E cell fate) encodes a high mobility group (HMG) box transcription factor with homology to LEF-1 and the Tcf family, which interact with Arm/β-catenin to regulate Wnt targets in flies and vertebrates (this paper; for review, see Nusse 1997). The other identified *Wnt* mutation *lin*-44 (Herman et al. 1995) is also required for certain asymmetric cell divisions, in this case in the larval male tail, where *lin*-44 acts nonautonomously to polarize adjacent cells. These target cells require a Fz protein encoded by *lin*-17 for their asymmetric cell divisions to occur (Sawa et al. 1996). It appears therefore that the Wnt signaling pathway found in flies and vertebrates is similar in worms (see Fig. 3), though there may be important differences, which will be discussed.

Are Wnt *genes involved in embryonic axis specification in vertebrates?*

In *Xenopus*, injection of various *Wnt* genes as RNAs into

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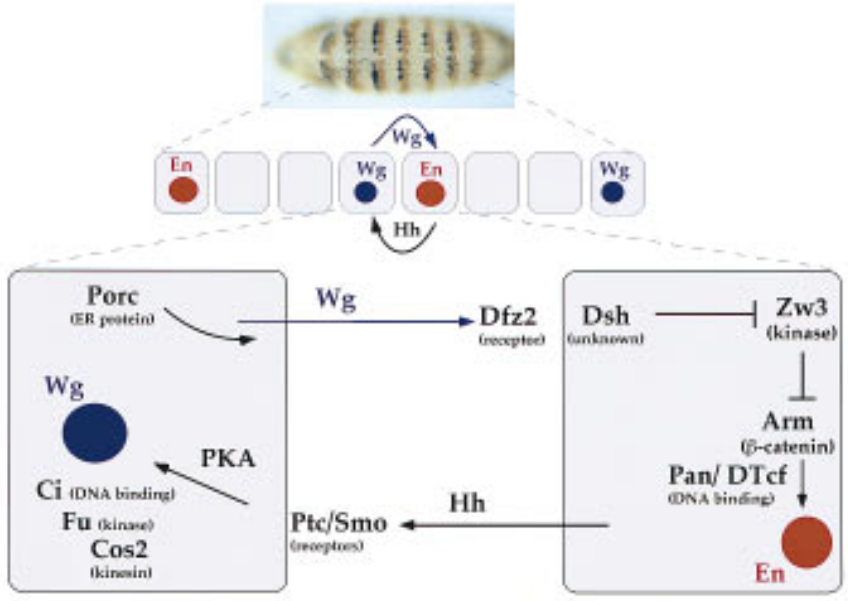


Figure 1. Intercellular signaling during *Drosophila* embryogenesis. (*Top*) A *Drosophila* embryo stained for expression of Wg (blue) and En (brown). Below is a representation of two parasegments (the parasegment boundary is between the Wg-and the En-expressing cells). Wg signals to maintain En expression; the En cells activate Wg expression by secreting the Hedgehog (Hh) protein. The Wg protein is secreted with the assistance of Porc, an ER transmembrane protein. Wg can act through the Dfrizzled-2 (Dfz2) receptor, although there is no genetic evidence that Dfz2 is required. Within the target cell, the PDZ-containing protein Dsh is required to transduce the signal leading to the inactivation of the protein kinase Zw3. In cells that do not receive Wg, Zw3 acts to destabilize the Arm protein. Together with DTcf (also known as *pan*) Arm can activate transcription of target genes, including *en*. The Hh protein, made by the En cells, binds to Patched (Ptc), which together with the Smoothened (Smo) protein forms a receptor complex. Within the target cell, the Hh signal is transduced by a complex between Cubitus interruptus (Ci), Fused (Fu), and Costal-2 (Cos2) to control Wg expression. Protein kinase A (PKA) probably acts in parallel to this pathway.

early ventral blastomeres leads to induction of dorsal mesoderm and a duplicated body axis (McMahon and Moon 1989; Moon 1993). Such *Wnt* genes can also rescue primary axis formation in developmentally compromised embryos. These observations are intriguing and have provided the field with useful assays for *Wnt* genes. Nonetheless, there are no data implicating an endogenous *Wnt* in induction of the primary axis, as no known *Wnt* is expressed in the right place at the right time. *XWnt*-8, for example, has potent axis-inducing effects (Smith and Harland 1991; Sokol et al. 1991) but is expressed too late, after the onset of zygotic transcription and in the wrong area [ventral marginal cells (Christian et al. 1991; Christian and Moon 1993)]. In addition, dominant-negative forms of Wnt (Hoppler et al. 1996), Fz (Leyns et al. 1997; Wang et al. 1997a), or Dsh (Sokol 1996) block secondary axis formation if coinjected with Wnt proteins, but they fail to block primary axis formation. At present, it seems unlikely therefore that a Fz–Wnt interaction is required for normal axis formation in frogs (Moon et al. 1997).

Table 2. Wnt *gene phenotypes in various organisms*

Gene	Organism	Phenotype
Wnt-1 (*swaying*)	mouse	deletion portion midbrain, cerebellum
Wnt-2	mouse	placental defects
Wnt-3A (*vestigial tail*)	mouse	tail, tailbud, caudal somites
Wnt-4	mouse	kidney defect
Wnt-7A	mouse	dorsal–ventral polarity limbs
wg	*Drosophila*	segment polarity; many others
DWnt-2	*Drosophila*	testis; adult muscles[a]
egl-20	*C. elegans*	Q-cell migration[b]
lin-44	*C. elegans*	T-cell polarity tail
mom-2	*C. elegans*	loss of endoderm, excess mesoderm in embryo

[a]K. Kozopas and R. Nusse (unpubl.).
[b]C. Kenyon (pers. comm.).

There is, however, compelling evidence that downstream members of the Wnt signaling pathway are essential for inducing the endogenous axis. Depletion of maternal β-catenin prevents the induction of the primary axis (Heasman et al. 1994). β-Catenin accumulates in the nuclei of dorsal blastomeres, consistent with activation of a Wnt pathway (Schneider et al. 1996; Larabell et al. 1997). This accumulation is blocked by overexpression of the zw3 homolog GSK-3 (Larabell et al. 1997). Likewise, overexpression of GSK-3 inhibits primary axis formation (Dominguez et al. 1995; He et al. 1995; Pierce and Kimelman 1996), as does a dominant-negative form of XTcf-3 that cannot bind β-catenin (Molenaar et al. 1996). Taken together, a picture emerges in which a non-Wnt mechanism inhibits GSK-3, stabilizing β-catenin and promoting a complex with XTcf-3 in dorsal nuclei (Fig. 3).

In the mouse, a naturally occurring recessive mutation, *fused*, has a duplicated axis phenotype similar to that seen after Wnt misexpression in *Xenopus* (Zeng et

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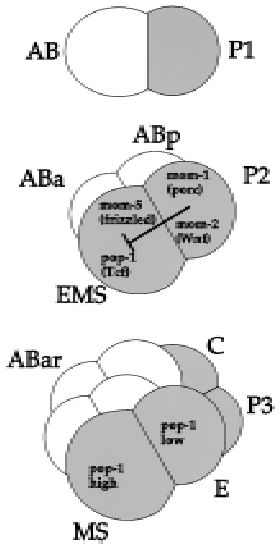


Figure 2. Intercellular signaling during *C. elegans* embryogenesis. The first division of the zygote gives rise to an anterior AB and a posterior P1 cell. The P1 cell divides into an anterior E/MS and a posterior P2 cell. A signal from P2 polarizes the E/MS blastomere, such that its anterior daughter (MS) will give rise to mesoderm and the posterior daughter (E) makes endoderm. In the absence of this signal, both daughters adopt the MS cell fate. The signal requires a Wnt (*mom*-2) and a *porc* homolog (*mom*-1), both required in P2. The *mom*-2 signal is probably received by the Fz homolog *mom*-5, resulting in down-regulation of the Tcf-related *pop*-1 protein in the E cell nucleus (compared to MS nuclei). The ABar blastomere, whose mitotic spindle orientation is disrupted in *mom*-1, *mom*-2, and *mom*-5 mutants, is also shown.

al. 1997). The cloned product of *fused*, a protein called Axin, can inhibit the formation of the primary axis in *Xenopus* when injected into dorsal blastomeres (Zeng et al. 1997). A ventrally injected dominant-negative version of the Axin protein results in frog embryos with defects similar to mouse *fused* mutants. In *Xenopus*, it appears that Axin inhibits β-catenin by activating GSK-3 or by acting on an unidentified protein between GSK-3 and β-catenin. The gastrulation phenotype of mice mutant for β-catenin (Haegel et al. 1995) is also consistent with an antagonistic relationship between Axin and β-catenin. Axin may act directly in the *Wnt* pathway, or it may be the target of the putative non-Wnt signal discussed above (Fig. 3).

Early misexpression of *Wnt*-8 (using the chicken gene called *Wnt*-8C (Hume and Dodd 1993) in mouse embryos can also induce a secondary axis (Pöpperl et al. 1997). As in frogs, endogenous mouse *Wnt*-8A lacks the correct expression pattern to be a strong candidate for the primary axis-promoting signal (Bouillet et al. 1996), although mouse *Wnt*-8A, like chicken *Wnt*-8C (Hume and Dodd 1993), is expressed in intriguing sites, including the primitive streak. The generation of null mutations in more mouse *Wnt* genes, in particular *Wnt*-8A, may reveal what role, if any, Wnt genes play in axis formation in vertebrates.

Wnt proteins

Working with Wnt proteins as biological agents has proven to be problematic. There are numerous unpublished tales of failed attempts to produce secreted Wnt proteins in cell culture. In general, overexpression of the genes in cultured cells results in accumulation of misfolded protein in the endoplasmic reticulum (ER; Kitajewski et al. 1992). Secreted forms of Wnt proteins can be found in the extracellular matrix or the cell surface (Bradley and Brown 1990; Papkoff and Schryver 1990; Burrus and McMahon 1995; Schryver et al. 1996), but efforts to solubilize this material have not been successful. Addition of suramin or heparin to cells can lead to a significant increase of Wnt protein in the medium (Bradley and Brown 1990; Papkoff and Schryver 1990), but this protein has not been shown to be biologically active (Papkoff 1989; Burrus and McMahon 1995).

While under any circumstance most Wnt protein is cell bound, several systems have more recently been developed that produce soluble forms. The *Drosophila* Wg (Van Leeuwen et al. 1994) and DWnt-3 (Fradkin et al. 1995) proteins and the mouse Wnt-1 protein (Bradley and Brown 1995) have been recovered from the medium of cultured cells. The amounts secreted are minor, but using in vitro assays for activity, these soluble forms have been shown to be biologically active. Wg protein can be tested for the stabilization of the Arm protein (Van Leeuwen et al. 1994; Fig. 3), and Wnt-1 protein can induce morphological transformation of target cells (Jue et al. 1992; Bradley and Brown 1995). Furthermore, using a hematopoietic stem cell proliferation assay, several Wnt proteins have been shown to be active in solution, and one of these, Wnt-5A, has been partially purified while retaining activity (Austin et al. 1997). These assays for soluble Wnt proteins are critical for defining Wnt protein interactions with other proteins, in particular cell-surface receptors. Moreover, they may lead to the purification to homogeneity of active protein and ultimately to the determination of Wnt protein structure.

Based on interallelic complementation between different *wg* alleles, it has been suggested that the Wg protein consists of different functional domains. These domains apparently have different functions in the patterning of the embryonic cuticle, and they have been suggested to interact with different receptors (Bejsovec and Wieschaus 1995; Hays et al. 1997). Evidence for different domains in Wnt proteins has also emerged from analyzing the phenotype of chimeric Wnt proteins in frog embryos (Du et al. 1995).

The mechanism of Wnt secretion; the role of porc

There are several lines of evidence suggesting that Wnt proteins require specific accessory functions for optimal secretion. The association between overproduced Wnt and Bip proteins (Kitajewski et al. 1992) in the ER indicates that most Wnt protein is misfolded under those conditions. This could be attributable either to a general mishandling of overproduced cysteine-rich proteins or to a limiting concentration of a specific binding partner.

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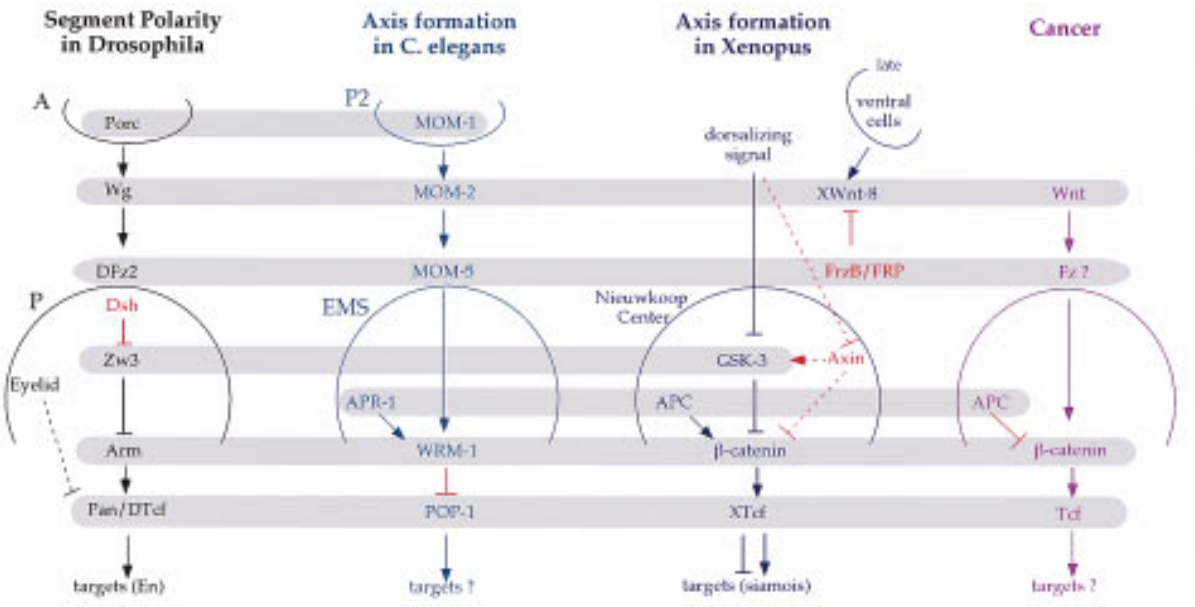


Figure 3. Comparison of Wnt pathways in embryogenesis and carcinogenesis. Related genes are highlighted across the different systems. Potential differences in the pathways are shown in red. Broken lines indicate alternative pathways. During segment polarity in *Drosophila*, anterior (A) cells signal to posterior (P) cells using Wg and the genes shown here and in Fig. 1, resulting in the activation of Arm. There is genetic evidence for an antagonism of Wg signaling by the gene *eyelid*, possibly at the level of DTcf. No role for *Drosophila* adenomatus polyposis coli (APC) in Wg signaling has yet been found. During *C. elegans* embryogenesis, the activity of the Wnt protein MOM-2 in the P2 cell polarizes the E/MS cell and down-regulates nuclear levels of the Tcf-related POP-1 protein. In the target EMS cell, the Fz-related protein MOM-5, the APC homolog APR-1, and the Arm/β-catenin-related WRM-1 protein are required for POP-1 down-regulation. APR-1 is shown acting in parallel to MOM-5 to activate WRM-1, but a direct role in the pathway has not been rule out. Targets of POP-1 have not yet been identified. The *Xenopus* primary axis is specified by a dorsalizing signal that does not appear to be a Wnt or require Dsh, but involves down-regulation of GSK-3, activating β-catenin. Axin could be a direct Wnt signaling component, inhibiting the pathway, possibly by activating GSK-3 or inactivating β-catenin. Axin could be inhibited by the dorsalizing signal or act in parallel. APC can activate the pathway upstream of β-catenin, but its relationship to the other proteins is not clear. XTcf-3 represses expression of the *siamois* gene, but upon binding with β-catenin, activates *siamois*, inducing the formation of the Spemann's organizer. After the onset of zygotic transcription, cells from the Spemann's organizer secrete soluble forms of Fz, called FRP or FrzB, which can counteract the activity of the ventralizing Xwnt-8 signal. In colorectal tumors and some melanomas, mutations in either APC (truncating the protein) or β-catenin (stabilizing it) lead to increased activity of β-catenin/hTcf-4 transcription complexes, which may play a causal role in promoting carcinogenesis. Wnt expression can lead to breast cancer in mice. (See text for more discussion and references.)

The identification of such a putative counterpart may have to await the purification of Wnt in an active form. Initial steps in purifying active Wg protein in our laboratory using sizing chromatography show that the secreted form is considerably larger than monomeric Wg. This may imply that Wg is secreted as a multimer of itself or in a complex with another molecule. Although either explanation is possible, Wg is not linked by disulfide bridges to possible other components, because under nonreducing denaturing gel electrophoresis, Wg runs as a monomer (C. Harryman Samos and R. Nusse, unpubl.).

A genetic clue that Wg secretion requires a specific accessory function is the phenotype of the segment polarity gene *porc*. Embryos mutant for *porc* have the same phenotype as *wg* mutants, and *porc* is required for Wg signaling in larval tissues as well (Cadigan and Nusse 1996; Kadowaki et al. 1996). Like *wg*, *porc* mutant clones behave noncell autonomously, indicating a role in producing the Wg signal (Kadowaki et al. 1996). In contrast to the diffuse staining of Wg protein seen in wild-type embryos, Wg in *porc* mutants is confined to the producing cells (van den Heuvel et al. 1993). The *porc* gene encodes a protein with eight transmembrane domains and is located perinuclearly in transfected cells (Kadowaki et al. 1996). Overexpression of Porc and Wg simultaneously changes the Wg glycosylation pattern but does not lead to increased Wg secretion (Kadowaki et al. 1996). These observations all suggest that Porc has a function within the secretory pathway to facilitate Wg synthesis or processing.

In worm embryos, *mom*-1 encodes a Porc-like protein (Rocheleau et al. 1997). Because it is required in the same cell (the P2 blastomere) as the Wnt gene *mom*-2 (Thorpe et al. 1997), it may have a similar relationship with *mom*-2 as *porc* does with *wg*. In addition, *mom*-3 is also required only in the P2 cell (Thorpe et al. 1997). It has not yet been cloned but may be an additional factor required for Wnt processing or secretion.

Although Porc or MOM-1 is, respectively, required for Wg and MOM-2 secretion, it is not known whether they

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are required for other members of the Wnt family. The role of Porc in the secretion or function of other Wnt proteins has not yet been looked at, but the data from *C. elegans* suggest that at least one *Wnt* besides *mom*-2 may require *mom*-1 and *mom*-3 for normal function. These genes have a highly penetrant defect in vulva formation that is not seen in *mom*-2 mutants that appear to be null (Thorpe et al. 1997). This suggests that *mom*-1 and *mom*-3 are required for the production of another worm Wnt protein in the vulva.

Wnt proteins as morphogens

Secreted Wnt proteins can in principle pattern cells over long distances. How far they actually travel from producing cells is difficult to determine because of the poor antigenicity of most Wnt proteins, but for Wg, where good antibodies are available, the protein can be found several cell diameters from the site of synthesis (Van den Heuvel et al. 1989; González et al. 1991; Neumann and Cohen 1997a). Consistent with this, *wg* mutants have patterning defects over a greater area than encompassed by its RNA expression domain. It has been suggested that Wg acts as a morphogen in several tissues (Struhl and Basler 1993; Hoppler and Bienz 1995; Lawrence et al. 1996), that is, it can alter gene expression in a concentration-dependent manner, eliciting different responses at various distances from the Wg-secreting cells. These studies have not adequately ruled out the possibility of a relay mechanism where Wg acts on these cells indirectly, perhaps by activating the expression of another secreted factor, which then patterns cells at a distance.

Two recent papers appear to have settled this debate, at least in the developing wing blade, where Wg has both short- and long-range targets (Zecca et al. 1996; Neumann and Cohen 1997b). A relay mechanism was ruled out by engineering patches of cells to express normal Wg, a membrane tethered form of Wg, or a constitutively activated Arm protein (Zecca et al. 1996; see section on Arm below). Although Wg could activate target genes at a distance from the site of synthesis, the membrane-bound form only works on immediately adjoining cells and the activated Arm could only act cell autonomously, that is, within the cells expressing the construct. The expression pattern of target genes in wings containing Wg-expressing clones and experiments where Wg was partially inactivated were all consistent with the morphogen model, where the shorter-range targets require more Wg activity than the longer-range ones for activation. Wg also activates gene expression noncell autonomously in leg and eye discs (Zecca et al. 1996; Lecuit and Cohen 1997), so Wg may, in general, act as a morphogen.

Whether other Wnt proteins act in vivo as long-range patterning molecules is less clear. One of the best-characterized *Wnt* phenotypes in the mouse is the absence of a large part of the midbrain in *Wnt*-1 mutant animals (McMahon and Bradley 1990; Thomas and Capecchi 1990). Although *Wnt*-1 is initially expressed in the midbrain, expression becomes restricted to a narrow band at the midbrain–hindbrain junction (Wilkinson et al. 1987). Possibly, *Wnt*-1 controls patterning of the CNS beyond its expression domain. The mouse *engrailed-1* (*en-1*) gene is normally expressed in a similar pattern as *Wnt*-1 and its expression decays in a *Wnt*-1 mutant, suggesting that it is a target of *Wnt*-1 signaling (McMahon et al. 1992). When *en*-1 is placed under the control of the *Wnt*-1 promoter, this transgene can significantly rescue the *Wnt*-1 midbrain defect (Danielian and McMahon 1996). This suggests that if there is a nonautonomous action of *Wnt*-1 in the brain, it occurs through a relay mechanism. Likewise, in *Xenopus*, the Wnt signaling pathway appears to induce axis formation in a sequential way, inducing the formation of the Nieuwkoop organizer (Fig. 3), which then secretes factors that induce dorsal mesoderm and notochord (He et al. 1995; Lemaire et al. 1995; Wylie et al. 1996). Clearly, the ability of Wnt proteins to act as morphogens must be examined on a case-by-case basis.

Fz proteins act as receptors for Wnt proteins

For a long time, a significant gap in understanding the mechanism of Wnt signaling was the lack of receptors. The difficulties in generating sufficient quantities of soluble and pure Wnt protein have precluded the identification of specific cell-surface receptors using conventional methods, such as cDNA expression cloning. Recently, however, a series of genetic, cell biological, and biochemical experiments have provided good evidence that members of the Fz family of cell-surface proteins function as receptors for Wnt proteins. *fz* genes encode seven transmembrane receptor-like proteins with an amino-terminal extension rich in cysteine residues that is predicted to be positioned outside of the cell (Figs. 4 and 5; Vinson et al. 1989).

In *Drosophila*, mutations in the first discovered *fz* gene display a tissue or planar polarity defect. In normal wings, the epithelial cells comprising the wing blade are all aligned similarly, so that the wing hairs, one of which is secreted by each cell, all point in a distal direction (Adler 1992). Flies mutant for null alleles of *fz* are viable, but the alignment of epithelial cells is disrupted, resulting in wing hairs pointing in several directions (Vinson and Adler 1987). *fz* mutants also have disruptions in the direction of bristles on the notum and legs (Adler 1992), and in the orientation of the ommatidia comprising the insect compound eye (Zheng et al. 1995). This phenotype is also associated with several other mutations (Wong and Adler 1993; Strutt et al. 1997), including *dsh* (Theisen et al. 1994; Krasnow et al. 1995), which is required for Wg signaling. This raises the possibility that Fz-like molecules might be involved in Wnt reception.

A *fz*-related gene in *Drosophila*, *Dfz2*, is a good candidate for being a specific receptor for Wg. In assays using soluble Wg protein, various cell lines transfected with *Dfz2* bind Wg on their cell surface (Bhanot et al. 1996). Moreover, stable transfection of *Dfz2* into cells that are nonresponsive to Wg (and do not normally express *Dfz2*) confers upon these cells the ability to accumulate Arm protein in a Wg-dependent manner. However, Wg pro-

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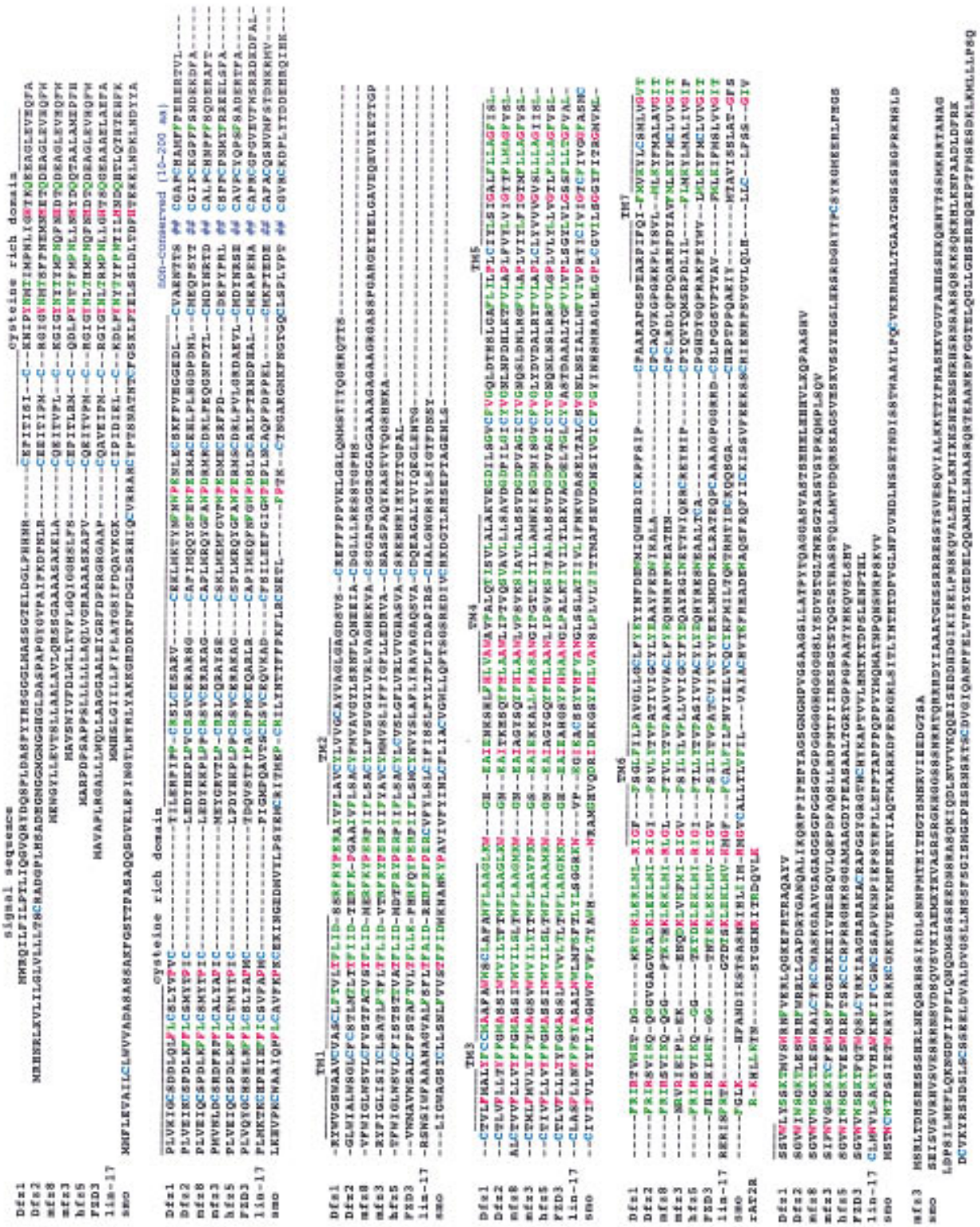


Figure 4. (*See facing page for legend.*)

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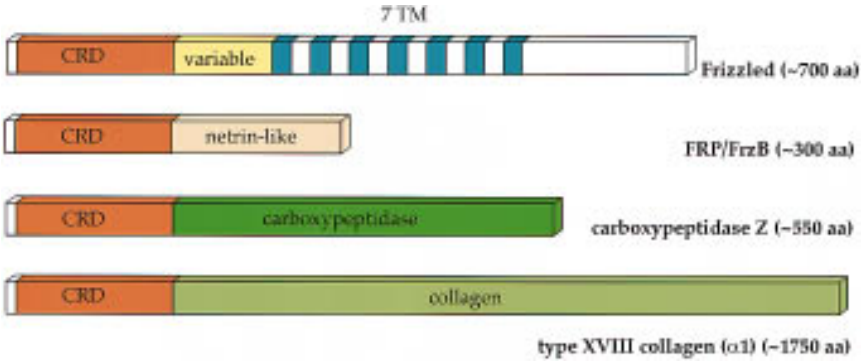


Figure 5. Schematic structures of proteins containing related Fz cysteine-rich domains (CRDs). In addition to the CRDs, the Fz proteins contain seven transmembrane (TM) domains; the FRP/FrzB molecules have some homology to netrins, and the protease carboxypeptidase has an enzymatic domain. A special subtype of collagens also has a CRD domain.

tein does not uniquely bind to cells expressing *Dfz2;* a variety of other Fz family members (Wang et al. 1996; Y.K. Wang et al. 1997), including the original Fz, also enable cells to interact with Wg (Table 3; Bhanot et al. 1996). Because binding affinity cannot be measured in these assays, there is no information on the relative strengths of these interactions. There is at present no mutant in the *Dfz2* gene, so it is possible that the gene is not required for Wg signaling in vivo and that Wg uses another receptor. Still, the demonstration that Dfz2 can bind and transduce the Wg signal in cell culture makes it an attractive candidate for a Wg receptor.

Genetic evidence for fz–Wnt *interactions in the worm*

The genetic evidence that Wnt proteins require Fz proteins for signaling, although lacking in flies, is accumulating in *C. elegans* (Table 3), though the story is a little complicated. Mutations in the *lin*-17 gene, which encodes a Fz protein (Sawa et al. 1996), affect the same cells that are influenced by the *Wnt* gene *lin*-44 (Herman et al. 1995), but with significant differences. Although *lin*-44 mutants have reversals of polarity in certain cells undergoing asymmetric divisions (Herman et al. 1995), these same cells in *lin*-17 mutants undergo symmetrical divisions; that is, polarity is completely lost (Sawa et al. 1996). One model to explain this is that there is a second signal, possibly another *Wnt*, which also works through the *lin*-17 receptor. Thus, the *lin*-17 phenotype is predicted to be the sum of defects in two signals.

In the EMS cell fate decision controlled by the *mom* genes, the *mom*-5 gene encodes a Fz member (Rocheleau et al. 1997), genetically interacting with the Wnt protein MOM-2. However, the penetrance of a null allele of *mom*-5 is very low (<10%), whereas the penetrance of the strong *mom*-2 *Wnt* mutations is >70%. This discrepancy could be explained by the presence of another *fz* gene. However, *mom*-2;*mom*-5 double mutants have a penetrance of only 8%, suggesting that *mom*-5 may in part play a negative role in *mom*-2 signaling. On the other hand, both *mom*-2 and *mom*-5 have a second defect (in the orientation of the mitotic spindle of the ABar blastomere at the eight-cell stage; Fig. 2) in which both genes have identical phenotypes with 100% penetrance (Rocheleau et al. 1997; Thorpe et al. 1997). Despite the complications, the story from the worm so far suggests a close relationship between Wnt and Fz proteins.

A third example of Wnt–Fz interactions in *C. elegans* is between *egl*-20 (a *Wnt* gene; C. Kenyon, pers. comm.) and *lin*-17; both genes are required in the migration of the neuronal Q cell (Harris et al. 1996).

Other Fz–Wnt interactions

Experiments in *Xenopus* also demonstrate that Fz molecules can transduce Wnt signals. Coinjection of a rat Fz with XWnt-8 leads to relocation of the Wnt protein from the ER to the cell surface, presumably because of binding to the receptor (Yang-Snyder et al. 1996). Fz-injected embryos also become more sensitive to the axis-duplicating activity of injected Wnt proteins. One Wnt member, XWnt-5A is normally unable to produce a secondary axis, but when coinjected with human fz5, this response is elicited (He et al. 1997). This result indicates that *Xenopus* normally does not express the cognate Fz receptor for XWnt-5A. However, in the absence of exogenous Fz, XWnt-5A has an effect: it can block the axis-inducing activity of XWnt-8 (Torres et al. 1996). It is not clear how XWnt-5A mediates this inhibitory effect, though a decrease in cell adhesion was implicated, nor is it known whether a Fz member is involved in this activity.

Somewhat ironically, a ligand for the original Fz protein in *Drosophila* is not known. In vitro, the Wg protein can bind to Fz (Bhanot et al. 1996), but *wg* does not appear to have a tissue polarity phenotype (though the pleiotropy of *wg* mutations makes this difficult to test rigorously). Clonal analysis of *fz* has revealed a puzzling

Figure 4. Sequence comparison between various members of the Fz protein family: *Drosophila* Fz; *Drosophila* Fz2 (DFz2); two mouse proteins (mfz8 and mfz3); two human proteins (hfz5 and FZD3); the *C. elegans* LIN-17 protein; and the *Drosophila* Smoothened (Smo) protein. Cysteine residues are in cyan throughout. Absolutely conserved residues are in magenta, and residues conserved in at least 6/8 protein are in green. The positions of the cysteine-rich domain, the nonconserved linker domain, and the seven transmembrane domains (TM) are indicated above the alignment. The type 2 angiotensin II receptor (rAT2R) has a short stretch of weak homology with the Fz proteins (Mukoyama et al. 1993), which is indicated.

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Table 3. *Interactions between Fz and Wnt proteins*

fz	Species	Wnt interaction	Type of interaction
fz	*Drosophila*	Wg	binding
fz2	*Drosophila*	Wg	binding
lin-17	*C. elegans*	*lin*-44	genetic
lin-17	*C. elegans*	*egl*-20	genetic
mom-5	*C. elegans*	*mom-2*	genetic
fz1	rat/*Xenopus*	XWnt-8	binding
fz4	mouse	Wg	binding
fz7	mouse	Wg	binding
fz8	mouse	Wg	binding
fz5	human	Wg	binding
		XWnt-5A	axis induction in *Xenopus*
FZD3	human	Wg	binding

and interesting phenomenon termed directional nonautonomy, where cells outside the clone also display the mutant phenotype (Vinson and Adler 1987). These phenotypically mutant cells outside the clone are usually distal to the fz^- cells. One explanation for this effect is that the Fz ligand, possibly a Wnt, moves over the field of cells in a proximal to distal direction, but cannot traverse the *fz* clone. In the eye, the data are consistent with the polarity signal emanating from the equator (Zheng et al. 1995). Identification of the tissue polarity ligand(s) may shed light on this intriguing problem.

Are Fz proteins the only Wnt receptors?

The above studies make a compelling case that Fz proteins are required for Wnt reception, but are they sufficient? There is a requirement for sulfated proteoglycans for Wnt signal transduction (see below), perhaps acting as a coreceptor, analogous to the relationship between proteoglycans and fibroblast growth factor (FGF) receptors (Schlessinger et al. 1995). The cell-surface receptor Notch has been proposed to play a role in Wg signaling based on somewhat complicated genetic interactions (Couso and Martinez Arias 1994), but complete removal of Notch activity in the wing and embryo does not reveal a defect in Wg signaling (Rulifson and Blair 1995; Cadigan and Nusse 1996). Finally, the Smoothened (Smo) protein, a distantly related member of the *fz* gene family (Alcedo et al. 1996; Van Den Heuvel and Ingham 1996) can associate with the multiple transmembrane protein Patched (Ptc), to constitute a functional Hedgehog (Hh) receptor (Fig. 1; Stone et al. 1996). In this complex, Hh binds to Ptc (Marigo et al. 1996; Stone et al. 1996), but the Smo protein is thought to transduce the signal. Whether Smo has a separate ligand is not known [Wg protein does not bind to Smo-transfected cells (Nusse et al. 1997)], nor is it clear whether Ptc-related molecules interact with other Fz proteins. Further biochemical characterization of the Fz proteins is needed to clarify this issue.

FRP, FrzB, and other secreted forms of Fz proteins

In addition to the integral membrane Fz proteins described above, *Xenopus* and other vertebrates produce several secreted proteins (called FrzB or FRP), which consist of a cysteine-rich domain (CRD) very similar to those in Fz molecules, followed by a stretch of charged residues containing a short stretch of homology to the netrins (Fig. 5; Shirozu et al. 1996; Finch et al. 1997; Leyns et al. 1997; Rattner et al. 1997; S.W. Wang et al. 1997). At least one of these molecules, FrzB or FRP, is specifically expressed in the Spemann's organizer in *Xenopus* embryos, where it can function as an antagonist of XWnt-8, a ventralizing factor (Leyns et al. 1997; S.W. Wang et al. 1997). Antagonism is probably mediated by direct binding of XWnt-8 to the CRD of the FRP proteins. Whether all FRPs function to down-regulate Wnt protein function is not clear; one can also imagine that they promote Wnt secretion or otherwise function in the distribution of these ligands.

In addition to the FRP and Fz proteins, the CRD motif is found in two other proteins (Fig. 5): carboxypeptidase Z (Song and Fricker 1997); and several isoforms of type XVIII collagen (Rehn and Pihlajaniemi 1995). The function of these domains is not clear, nor is it known whether these molecules can bind to Wnt proteins.

The requirement for proteoglycans in Wnt signaling

The binding of Wnt proteins to proteoglycans such as heparin has long been noted, and more recently, several lines of evidence suggest that this interaction has physiological relevance. Two *Drosophila* genes with embryonic mutant phenotypes very similar to *wg* have been shown recently to encode, respectively, homologs of UDP–glucose dehydrogenase (*sugarless;* Binari et al. 1997; Häcker et al. 1997; Haerry et al. 1997) and *N*-deacetylase/*N*-sulfotransferase (X. Lin and N. Perrimon, in prep.), which are required for heparin sulfate biosynthesis. The *sugarless* mutant phenotype was partially rescued by injection of embryos with heparin sulfate, and injection of heparinase into wild-type embryos created *wg*-like phenotypes. Null alleles of *sugarless* strongly reduced but did not completely block Wg signaling (Häcker et al. 1997; Binari et al. 1997). This is in contrast to genes such as *dsh* and *arm*, which are absolutely required for Wg signaling even when Wg is grossly overexpressed (Noordermeer et al. 1994; Manoukian et al. 1995). These mutants provide in vivo evidence for the importance of sulfated proteoglycans in Wg function, although their exact role is unclear.

Sulfated glycosaminoglycans are required for soluble Wg to optimally stabilize Arm in a *Drosophila* cell line, and the addition of exogenous heparin can enhance Wg signaling in this assay (Reichsman et al. 1996). These results suggest a role for these proteoglycans in either binding of Wg to cells or the transduction of the signal.

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Interestingly, heparin sulfates are not required for Wg binding to DFz2 (Bhanot et al. 1996), although a decrease in affinity cannot be ruled out. Removal of heparin sulfate (via heparinase) has been shown to block XWnt-8 activity in animal cap assays in *Xenopus* (Itoh and Sokol 1994), suggesting that proteoglycans are a general requirement for Wnt signaling.

The mechanism of signal transduction by Fz proteins

The Fz receptors include seven transmembrane domains, an amino-terminal extension acting as a ligand binding domain in DFz2 (Bhanot et al. 1996), and a cytoplasmic tail (Figs. 4 and 5). The fact that almost all previously identified seven transmembrane receptors utilize G proteins for signaling suggests that Fz molecules may as well. At present, however, there is only circumstantial evidence for a G protein in Wnt or Fz signaling. Dsh and another potential component of Wnt signaling, Axin, both contain domains that are found in G-protein regulators (see below), but thus far there is no genetic or biochemical evidence for a G protein in any Wnt pathway. In addition, there is no sequence homology between Fz proteins and the known G protein-coupled receptors, save for a short stretch of similarity with the type II angiotensin 2 receptor in the third cytoplasmic loop (Fig. 4; Mukoyama et al. 1993). The importance of this homology is not clear, although it may be significant to note that G proteins in general are known to interact with the third cytoplasmic loop of their cognate receptors (Bourne 1997). These issues can now be addressed through site-directed mutagenesis of Fz proteins, followed by in vitro or in vivo characterization.

It has also been recognized that the carboxyl terminus of many Fz proteins contains a motif (SXV) that can interact with PDZ domains (Fig. 4). Dsh has a PDZ domain and is the first known component of Wg signaling downstream of the receptor, but experiments in our laboratory have not revealed a direct interaction with any Fz proteins (Nusse et al. 1997). This may not be surprising in light of the report demonstrating that only some PDZ domains bind to the SXV motif (Songyang et al. 1997), and the Dsh PDZ falls into the nonbinding class (Doyle et al. 1996; Morais Cabral et al. 1996). The importance of the SXV tail for Fz function is also put into question by the result that replacing it with a GFP moiety does not affect signaling in the case of *lin*-17 in the worm (Sawa et al. 1996). In summary, how Fz proteins transduce Wnt signals to the inside of the cell remains an open question.

Signaling downstream of the receptor

dsh

In the genetic Wg signal transduction pathway, *wg* activates *dsh* (presumably through a Fz) which in turn inhibits *zw3*. *dsh* encodes a cytoplasmic protein (Klingensmith et al. 1994; Theisen et al. 1994) that has highly conserved counterparts in *Xenopus* and mouse, in particular in the amino terminus and in the central PDZ-containing domain (Fig. 6). In flies, *dsh* is required for Wg signaling in many tissues (Couso et al. 1994; Klingensmith et al. 1994; Theisen et al. 1994; Park et al. 1996a; Lecuit and Cohen 1997; Neumann and Cohen 1997b). Overexpression of Dsh can mimic Wnt signaling in *Drosophila* and *Xenopus* (Rothbacher et al. 1995; Sokol et al. 1995; Yanagawa et al. 1995; Axelrod et al. 1996; Cadigan and Nusse 1996; Park et al. 1996a). In mice, however, a knockout of a *dsh* gene did not display any of the dramatic developmental defects associated with Wnt proteins, though behavioral and neurological abnormalities were observed (Lijam et al. 1997). There are several other mouse *dsh* genes, all widely expressed (Klingensmith et al. 1996) and it seems likely that they act in a redundant manner. Thus far, no mutations in worm *dsh* genes have been reported.

It is not known how Dsh proteins work as Wnt signal-transducing components, but over the past few years, several motifs have been identified in these proteins. A picture is emerging of Dsh proteins being modular proteins that can interact with various other signaling components. Besides the PDZ domain discussed above, Dsh proteins contain two other domains found in proteins participating in G-protein-mediated signaling. The Axin protein, a negative regulator of Wnt signaling (Zeng et al. 1997) that contains a RGS (regulators of G-protein signaling) motif (Koelle 1997), shares a region of homology with Dsh proteins that we refer to as DIX (Fig. 6). Dsh proteins lack a RGS, but their carboxyl ends contain a so-called DEP domain (Ponting and Bork 1996), found in a variety of proteins, many of which participate in G-protein signaling.

The *Drosophila* Dsh is a phosphoprotein localized predominantly in the cytoplasm of the cell and not in the nucleus (Yanagawa et al. 1995). Wg stimulation in cells or embryos leads to hyperphosphorylation of Dsh (Yanagawa et al. 1995). It is not clear which protein kinase catalyzes this phosphorylation, although the Dsh protein can be found in a complex with casein kinase II (CKII, Willert et al. 1997) and is phosphorylated by CKII in vitro. Possibly, the hyperphosphorylated form of Dsh is the active form, and phosphorylated Dsh transduces the signal onto the next signaling component, directly or indirectly leading to the inhibition of Zw3. This view is somewhat oversimplified in light of the finding that under certain conditions, that is, overexpression of DFz2 in the absence of Wg, Dsh becomes hyperphosphorylated but does not activate the pathway (Willert et al. 1997). It may be that the phosphorylation pattern is not identical to that in Wg-stimulated cells, or it could be that hyperphosphorylation of Dsh is necessary but not sufficient for the signal to be transduced. Identification of more binding partners of Dsh will hopefully shed more light on its mechanism of action.

zw3/GSK-3

Flies mutant for *zw3* (Peifer et al. 1994b; Siegfried et al. 1994) and frog embryos expressing dominant-negative

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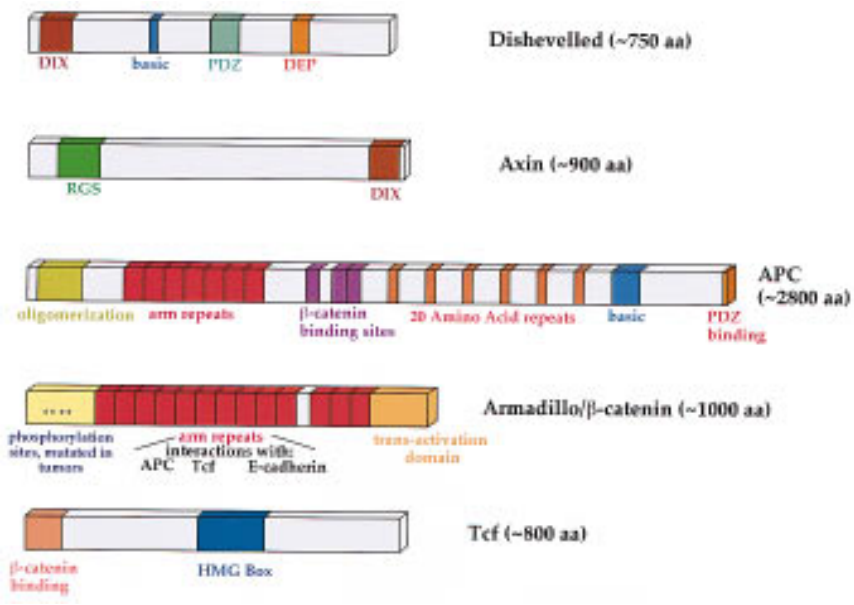


Figure 6. Schematic structure of intracellular Wnt signaling components. Dsh contains a domain also found in Axin (which we call the DIX domain), a conserved basic stretch, a PDZ (previously called GLGF or DHR) domain (Ponting et al. 1997), and a DEP (Dsh/egl-10/pleckstrin domain; Ponting and Bork 1996), the latter found in various proteins that interact with G proteins. Axin has an RGS motif (Koelle 1997) and a DIX domain. APC has seven Arm repeats, three β-catenin-binding sites, a set of internal repeats, and a basic domain. APC has a motif at the carboxyl terminus that can interact with PDZ domains (Matsumine et al. 1996). The Arm/β-catenin molecule has an amino terminus that regulates stability through several serine residues (asterisks). In addition to the internal Arm repeats, the protein has a transcriptional activator domain at the carboxyl terminus. The Tcf has a domain interacting with β-catenin and a HMG box-like DNA-binding domain.

versions of its vertebrate counterpart GSK-3 (Dominguez et al. 1995; He et al. 1995; Pierce and Kimelman 1996) both have phenotypes consistent with the constitutive activation of Wnt pathways through Arm/β-catenin. This has led to a model where Wnt acts to negatively regulate the Zw3/GSK-3 kinase, though the data equally support Zw3/GSK-3 acting in parallel as a repressor of Arm/β-catenin. The GSK-3 enzyme has been characterized extensively in mammalian cells and is unusual in that it is constitutively active in nonstimulated cells (Woodgett 1991). The enzyme activity can be down-regulated by the addition of insulin or epidermal growth factor (EGF) to serum-starved cells and is correlated with phosphorylation on residue Ser-9, probably via protein kinase rsk-90 or protein kinase B (Stambolic and Woodgett 1994; Cross et al. 1995). Thus, there is precedence for the idea that Wnt proteins inhibit Zw3/GSK-3 through covalent modification.

Cook et al. (1996) found that the addition of soluble Wg protein to the mammalian cell line C3H10T1/2 results in an approximate twofold down-regulation of GSK-3 activity in cell extracts. They showed that this effect was pharmacologically distinct from insulin and EGF-mediated inhibition of the kinase. A phorbol ester-sensitive protein kinase C (PKC) was shown to be required for the Wg effect. PKC is known to phosphorylate GSK-3 in vitro, lowering its activity (Goode et al. 1992). Identification of the in vitro phosphorylation sites of PKC on GSK-3 should allow the testing of the importance of these sites for in vivo regulation.

The fact that the reduction of GSK-3 activity upon stimulation with Wg is only 50% is cause for some concern (Cook et al. 1996). However, other inhibitory signals, such as insulin, inhibit to roughly the same degree. Is a twofold reduction of GSK-3 sufficient to transduce the Wnt signal, stabilizing β-catenin? This was not examined, but if Wg does cause the accumulation of β-catenin in these cells, would insulin do so as well? If the answer is no, this raises several interesting possibilities, such as different intracellular pools of enzyme, with only the Wnt-sensitive pool able to regulate β-catenin.

A complex among Zw3/GSK-3, Arm/β-catenin, and adenomatous polyposis coli

The Arm protein is similar to vertebrate β-catenin and plakoglobin, proteins binding to E-cadherin (McCrea et al. 1991) and linking adhesion complexes to the cytoskeleton. Arm/β-catenin proteins contain a set of 12 internal repeats, the structure of which was recently solved (Huber et al. 1997). Each repeat consists of 3 helices and the 12 repeats together form a superhelical, protease-resistant rod that contains a long, positively charged groove. This groove is suggested to be important in the binding of Arm/β-catenin to its various partners: cadherin, adenomatous polyposis coli (APC), and Tcf (see below; Fig. 6).

Although essential for cellular adhesion, *arm* mutants were first identified because of their *wg*-like phenotype in embryos (Wieschaus and Riggleman 1987). These mutations are carboxy-terminal truncations, leaving the internal repeats and cadherin-binding domains intact (Fig. 6). Alleles of *arm* disrupting the cadherin-binding domains show the expected cell adhesion defect (Orsulic and Peifer 1996). Antisense and overexpression experiments in *Xenopus* are also consistent with β-catenin playing a role in Wnt signaling in addition to its role in cell adhesion.

Wnt signaling regulates Arm/β-catenin levels post-transcriptionally in flies (Riggleman et al. 1990; Orsulic and Peifer 1996) and frogs (Larabell et al. 1997), leading to

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cytoplasmic and nuclear accumulation. This increase in Arm/β-catenin is attributable to increased stability in the presence of Wnt signaling (Hinck et al. 1994; Van Leeuwen et al. 1994). Consistent with its proposed place in the pathway, inactivation of zw3/GSK-3 also causes this accumulation (Peifer et al. 1994b; Stambolic et al. 1996; Yost et al. 1996; Larabell et al. 1997). The stabilized Arm protein is underphosphorylated compared to membrane-bound, cadherin-associated protein (Peifer et al. 1994a; Van Leeuwen et al. 1994). Thus, the simplest model would suggest that Zw3/GSK-3 directly phosphorylates Arm/β-catenin, destabilizing it, probably by promoting its entry into the ubiquitin–proteasome degradation pathway (Aberle et al. 1997). There are four potential GSK-3 phosphorylation sites in the amino-terminal portion of β-catenin that are conserved in Arm. Mutation of these sites led to a β-catenin that is more stable than wild-type β-catenin and considerably more potent in secondary axis formation in *Xenopus* (Yost et al. 1996). Deletions at the amino terminus of Arm (removing the four serine/threonine residues) result in a constitutively active Arm protein (Zecca et al. 1996; Pai et al. 1997). Both in flies and *Xenopus*, these mutant proteins are no longer sensitive to Zw3/GSK-3 regulation (Yost et al. 1996; Pai et al. 1997).

Although the above results make a compelling case for the importance of the amino-terminal phosphorylation sites in regulating Arm/β-catenin stability and signaling activity, the data that Zw3/GSK-3 is the direct kinase are less convincing. GSK-3 can phosphorylate β-catenin in vitro, and the activated form of β-catenin lacking the putative GSK-3 sites is phosphorylated less efficiently in vitro and in vivo (Yost et al. 1996). However, these assays were not quantitative, and the in vitro phosphorylation did not appear to be stoichiometric. In addition, other groups have not found Arm/β-catenin to be phosphorylated by Zw3/GSK-3 (Rubinfeld et al. 1996; Stambolic et al. 1996; Pai et al. 1997). Although there are many technical explanations for these discrepancies, it is also possible that GSK-3 is not the kinase that phosphorylates β-catenin in vivo.

If Zw3/GSK-3 does not directly interact with Arm/β-catenin, are there any known proteins that could form the bridge? An interesting candidate is the product of the adenomatous polyposis coli (*APC*) gene (for review, see Polakis 1997), mutations in which are correlated with colorectal cancer (see below). Tumor cell lines producing truncated forms of APC protein have high levels of cytosolic β-catenin because of increased stability (Rubinfeld et al. 1996).

Transfection of these cells with full-length APC or with fragments of the protein that are missing in the truncated forms reduces the β-catenin levels dramatically (Munemitsu et al. 1995). These tumor cell lines have been found to have complexes of GSK-3, β-catenin, and APC (Rubinfeld et al. 1996). The percentage of each protein in this complex is not clear, but GSK-3 is enriched in the β-catenin pool that also bound APC. GSK-3 was found to stoichiometrically phosphorylate an APC fragment, which stimulated its binding to β-catenin (Rubinfeld et al. 1996). These data suggest the possibility that GSK-3 destabilizes β-catenin through phosphorylation of APC, promoting APC binding to β-catenin and precipitating β-catenin degradation.

A positive role for APC in Wnt signaling

The data summarized above suggest that if APC plays a role in Wnt signaling, it would be a negative regulator of the pathway. However, some recent experiments are inconsistent with this view. *Xenopus* has an APC homolog (XAPC) that is found primarily in a complex with β-catenin (Vleminckx et al. 1997). Surprisingly, overexpression of XAPC in ventral blastomeres results in the induction of dorsal markers and a second notochord on the ventral side of embryos. This overexpression does not affect endogenous β-catenin levels, but β-catenin is necessary for the axis-inducing activity of XAPC. Fragments of human APC that have been shown to destabilize β-catenin in colon cancer cell lines also efficiently induced secondary axes (Vleminckx et al. 1997). These results suggest that APC has a positive signaling role in the Wnt pathway.

Results from *C. elegans* support the *Xenopus* findings. RNA interference studies, which are thought to specifically inhibit translation of the targeted message, with worm APC (*apr*-1)- and β-catenin (*wrm*-1)-related genes both produced embryos with *mom* phenotypes. The penetrance of the *wrm*-1 phenotype was 100%, but the *apr*-1 *mom* phenotype occurred only 26% of the time. When APR-1 interference was performed in a *mom*-2 (*Wnt* gene) or *mom*-5 (*fz*-related gene) background (which had 39% and 8% penetrance, respectively), 100% of the embryos lacked E cells (Rocheleau et al. 1997). This was taken as evidence that *mom*-2 and *apr*-1 act in parallel, converging at *wrm*-1, but these results can also be explained by another unidentified *Wnt* and *APC*-like gene acting redundantly with *mom*-2 and *apr*-1. In any case, once again APC is implicated positively in Wnt signaling.

Can the tumor cell culture results—where APC's primary role appears to be to stimulate β-catenin degradation—be reconciled with the frog and worm data? Perhaps APC, phosphorylated by Zw3/GSK3, binds to β-catenin and promotes its degradation. Upon Wnt stimulation, the nonphosphorylated form of APC still binds to β-catenin, promoting β-catenin signaling. In mammalian cells constitutively expressing Wnt-1, there is an increase in APC levels and in the stability of APC/β-catenin complexes, compared to untransfected cells (Papkoff et al. 1996). Further analysis of this effect in cell lines with inducible Wnt expression or after addition of soluble Wnt should help clarify the relationship between Wnt and APC.

An APC homolog in flies has also been identified (Hayashi et al. 1997). This protein can stimulate β-catenin turnover in colorectal tumor cell lines. However, embryos homozygous for a large deficiency removing the gene show no defect in Arm distribution (Hayashi et al. 1997). The analysis of additional mutations within this

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gene should be informative, in addition to testing for its phenotype in the absence of maternal contributions.

Binding of Arm/β-catenin to Tcf–LEF-1

At the same time that it was being appreciated that Arm and β-catenin accumulate in the nucleus after Wnt stimulation, several groups reported that β-catenin could bind to HMG box transcription factors of the Tcf–LEF-1 family. Coexpression of β-catenin and these proteins resulted in accumulation of β-catenin in the nucleus. Tcf and LEF-1 proteins were found originally as enhancer binding factors for T cell-specific genes (Clevers and Grosschedl 1996). Binding of Tcf proteins to DNA results in bending of the helix (Giese et al. 1992), but by themselves, these proteins are poor transcriptional activators. However, complexes between Tcf proteins and β-catenin act as potent transcriptional activators of reporter gene constructs containing the DNA element recognized by Tcf (Molenaar et al. 1996; Korinek et al. 1997; Morin et al. 1997). Overexpression of Lef-1 in *Xenopus* causes an axis duplication that is greatly enhanced by coinjection of β-catenin, whereas dominant-negative forms (that can bind DNA but not β-catenin) are able to block the formation of the primary and the Wnt-induced secondary axes (Behrens et al. 1996; Huber et al. 1996; Molenaar et al. 1996). The Tcf proteins have finally provided the link between Wnt signaling and transcriptional regulation.

Similar results—but underpinned by loss-of-function genetics—were obtained with a *Drosophila* homolog of Tcf, which is also named *pan* (Brunner et al. 1997; Van de Wetering et al. 1997). The DTcf protein binds to Arm (Brunner et al. 1997; Van de Wetering et al. 1997). Null mutations in DTcf cause a *wg*-like segment polarity phenotype, and a conditional allele can give defects in adults similar to *wg*. Genetically, DTcf mutations are downstream of Arm, consistent with the vertebrate results (Brunner et al. 1997; Van de Wetering et al. 1997).

In *C. elegans*, *pop*-1 encodes a protein with a HMG box, suggesting that it has a function similar to Tcf proteins (Lin et al. 1995). *pop*-1 is involved in the *Wnt*-dependent asymmetric cell division of the EMS blastomere referred to earlier. However, unlike *Drosophila*, where *wg* and *Dtcf/pan* have similar phenotypes, *pop*-1 has the opposite phenotype as the Wnt components of the *mom* class described above. Genetically, *pop*-1 is downstream of all the *mom* genes and the β-catenin-like gene *wrm*-1 (Rocheleau et al. 1997). The POP-1 protein is post-transcriptionally down-regulated by the Wnt pathway in the nuclei of the EMS daughter closest to the Wnt-producing P2 cell (Fig. 2; Rocheleau et al. 1997). The mechanism of this repression in not understood.

Is the *pop*-1 mutant phenotype evidence that Wnt signaling in worms is fundamentally different from flies and frogs? A few points in this regard need to be emphasized before reaching this conclusion. First, the conserved amino-terminal domain of the fly and vertebrate Tcf proteins (which binds Arm/β-catenin) is not found in POP-1 (Lin et al. 1995; Van de Wetering et al. 1997) so it may not even be the true Tcf worm counterpart. On the other hand, WRM-1 is distantly related to Arm and β-catenin, so perhaps WRM-1 and POP-1 can bind each other. This obviously needs to be tested directly. Second, the possible regulation of Tcf protein distribution by Wnt proteins has not yet been examined in flies or frogs, so it is not clear that Wnt-dependent down-regulation of POP-1 protein seen in the worm is unique. Finally, there are recent data on a target gene of the Wnt pathway in *Xenopus*, *siamois*, suggesting that the function of *pop*-1 in worm Wnt signaling may not be that different from the situation in frogs.

Several lines of evidence suggest that the homeobox gene *siamois* is a major target of β-catenin/XTcf-3 action in axis formation in frog embryos (Brannon and Kimelman 1996; Carnac et al. 1996; Fan and Sokol 1997), where it is expressed only on the dorsal side of gastrulating embryos. The siamois promoter contains several XTcf-3 binding sites, which are needed for β-catenin-mediated activation of *siamois* promoter reporter constructs (Brannon et al. 1997). Consistent with endogenous *siamois* expression, the wild-type reporter construct was expressed at low levels when injected ventrally. However, when the XTcf-3 binding sites were mutated, expression on the ventral side was almost as high as the parental construct's expression dorsally. This indicates that in addition to its activating role in conjunction with β-catenin, XTcf represses *siamois* expression in the absence of high levels of nuclear β-catenin (Brannon et al. 1997). Therefore, if the endogenous XTcf were mutated, a dorsalized embryo would be predicted—the opposite of the ventralized phenotype seen in β-catenin-depleted embryos. Thus, if *pop*-1 is a functional Tcf homolog in *C. elegans*, its phenotype may not be that unusual, depending on how important its repressing activity in the absence of Wnt signaling is.

In *Drosophila*, there is also evidence for derepression when the DTcf– binding site is mutated in the Wg response element of the Ultrabithorax (Ubx) promoter, but the effect is minor compared to that seen with *siamois* (Riese et al. 1997). Thus, at least for the few Wg targets so far examined, the activation activity of DTcf outweighs any derepression of target genes in DTcf mutants. More work is needed in all three model systems to determine the commonalities and differences in their Wnt pathways.

Wnt signaling components in cancer

The first *Wnt* gene discovered, mouse *Wnt*-1, was identified by virtue of its ability to form mouse mammary tumors when ectopically expressed due to proviral insertion (Nusse and Varmus 1982). Although the relation between Wnt proteins and mouse breast tumorigenesis has been extended, there is still no direct link between Wnt signaling and human breast cancer. However, APC and β-catenin implicate Wnt signaling in other forms of human cancer.

Mutations in the *APC* gene are found in familial and spontaneous colon carcinomas. As described above, tu-

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mor cell lines homozygous for *APC* mutations have abnormally high levels of β-catenin (Munemitsu et al. 1995). β-Catenin forms a complex with one of the human Tcf homologs (hTcf-4), which activates expression of reporter constructs containing hTcf-4-binding sites. Transfection of full-length *APC* into those cells inhibits expression of the reporter gene constructs (Korinek et al. 1997; Morin et al. 1997). Mutant forms of *APC*, which are unable to stimulate degradation of β-catenin, are incapable of blocking target gene expression. Thus, at least one effect of *APC* mutations is to activate a transcriptional complex that may contribute to the cell's oncogenic potential.

This theory is strengthened by the existence of several colon carcinoma cell lines with wild-type *APC* that nevertheless display strong expression of hTcf-4 reporter constructs (Korinek et al. 1997; Morin et al. 1997). These cell lines have mutations in the β-catenin gene similar to the activating mutations created in *Xenopus* and flies. Similar mutations are also present in several melanoma cell lines (Rubinfeld et al. 1997). These findings implicate stable β-catenin as the common feature of most colon carcinomas and many melanomas.

Does this mean that mutations in *APC* only contribute to tumorigenesis through stabilization of β-catenin? Results in cultured cells with expression of amino-terminal deleted versions of β-catenin (which constitutively activate Wnt signaling) show the formation of stable complexes of the mutant β-catenin and APC (Munemitsu et al. 1996; Barth et al. 1997). This raises the possibility that mutations in β-catenin may affect APC functions such as cell migration (Näthke et al. 1996) in addition to promoting Tcf-mediated transcriptional changes. It may be informative to stably transfect colon tumor cell lines with dominant-negative forms of hTcf-4 that cannot bind β-catenin (and presumably do not affect the APC protein) to see if their oncogenic characteristics can be reverted. When this is done with wild-type APC, the tumor cell growth rate is reduced sharply, because of increased apoptosis (Morin et al. 1997). If similar results are obtained with the mutated hTcf-4, this would strongly support hTcf-4 playing a causal role in colon cancer.

Do all Wnt functions work through Arm/β-catenin and Tcf proteins?

The *fz* and *dsh* genes function in the tissue polarity pathway in *Drosophila*, which regulates cell orientation in wings, legs, and eyes (Adler 1992; Theisen et al. 1994; Zheng et al. 1995). Because Fz can bind Wg (Bhanot et al. 1996), it is likely that a Wnt is the physiological polarity signal. However, this signaling pathway does not appear to be a standard Wnt pathway. Several other genes, *fuzzy*, *inturned*, and *rhoA* (Wong and Adler 1993; Park et al. 1996b; Strutt et al. 1997), have tissue polarity phenotypes and appear to act downstream of *fz* and *dsh*, but these genes have no apparent defects in Wg signaling. In addition, there is a *dsh* allele with a strong tissue polarity phenotype but no *wg*-like phenotypes (Theisen et al. 1994). The data suggest that the Wnt and planar polarity pathways branch at *dsh*, though it remains to be demonstrated that *zw3*, *arm*, and *DTcf* play no role in tissue polarity.

In *C. elegans*, it also appears that a branch occurs in a Wnt pathway. As described earlier, there is a signal from the P2 blastomere that polarizes the EMS cell. A *Wnt* gene (*mom*-2) and genes related to *porc* (*mom*-1), *fz* (*mom*-5), *APC* (APR-1), *arm* (WRM-1), and *Tcf* (*pop*-1) are required for this polarization (Rocheleau et al. 1997; Thorpe et al. 1997). A subset of these genes are also needed for the proper orientation of the mitotic spindle of the ABar cell (Fig. 2); *mom*-1, *mom*-2, and *mom*-5 are needed, but no requirement was seen for the others (Rocheleau et al. 1997), suggesting a branch in the pathway downstream of the MOM-5 receptor, perhaps at the as yet unidentified worm *dsh*. It will be interesting to see whether the ABar cell is a target for a signaling cascade similar to the fly planar polarity pathway.

wg *autoregulation*

In the embryonic epidermis, *wg* is required for the maintenance of its own transcription (Hooper 1994; Manoukian et al. 1995; Yoffe et al. 1995). This maintenance requires *porc*, but not *dsh* or *arm* (Manoukian et al. 1995). Unless one argues that *porc* mutants contain less stable Wg transcripts compared to *dsh* and *arm* mutants, embryonic *wg* autoregulation involves a different mechanism than most *wg* functions. In another study, using different double mutant combinations, it was found that *porc* and *dsh* were required for *wg* autoregulation but not *arm* (Hooper 1994). These studies clearly must be extended, hopefully by the identification of the Wg response elements in the Wg promoter.

In contrast to the embryo, *wg* negatively autoregulates its own expression at the dorsal/ventral boundary of the wing imaginal discs (Rulifson et al. 1996). This effect requires *dsh* but not *arm*. The Notch protein, which is the receptor in a pathway that is known to positively regulate Wg transcription at the dorsal/ventral boundary (Diaz-Benjumea and Cohen 1995; Rulifson and Blair 1995; Doherty et al. 1996; Rubinfeld et al. 1996) is required for Wg derepression (Rulifson et al. 1996). Although *Notch* could be acting in parallel with *dsh* in this process, it is interesting to note that Dsh protein has been shown to bind to and inhibit Notch activity when overexpressed in the wing (Axelrod et al. 1996). This makes for a model in which *wg* represses its own transcription by inhibiting *Notch* activity through *dsh*. Further studies of this side branch of the Wg pathway are needed, and it will be interesting to see whether this interaction is seen in other tissues.

Do Wnt proteins affect cell adhesion directly?

Before it was recognized that Arm/β-catenin forms a complex with Tcf proteins in the nucleus, a direct effect on cell adhesion was often suggested for Wnt proteins because of Arm/β-catenin's ability to bind cadherins.

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Cell lines transfected with Wnt proteins can have altered cell adhesion properties (Bradley et al. 1993; Hinck et al. 1994), but at least in one case, this was shown to be due to increased transcription of cadherin (Yanagawa et al. 1997). Overexpression experiments in flies with wild-type and a dominant-negative version of cadherin are consistent with the notion that regulation of cell adhesion is not the major readout of Wg signaling (Sanson et al. 1996). Overexpression of β-catenin mutant proteins that cannot bind cadherin can still induce a secondary axis in *Xenopus* embryos (Funayama et al. 1995).

In *Drosophila*, a thorough structure–function analysis of *arm* demonstrated that embryos mutant for an allele of *arm* that is wild type for adhesion function but appears null for Wg signaling (it can bind Tcf but cannot activate transcription) could be rescued by an *arm* transgene that is defective in adhesion function (Orsulic and Peifer 1996). Thus *arm*'s two functions can be completely separated. A more subtle role for nontranscriptional changes in cellular adhesion during Wnt signaling cannot be ruled out, but rigorously demonstrating the existence of such a role in a living organism will be difficult.

Future directions

The plethora of Wnt proteins playing important roles in many developmental systems and in human disease has attracted increasing numbers of researchers, dramatically accelerating progress toward understanding Wnt signaling. Still, at every level of the pathway, major questions remain. How do the Fz receptors work? What is Dsh doing to transduce the signal? What is the relationship between APC and Wnt signaling? How does Arm/β-catenin get into the nucleus? Identification of factors that interact with the identified components of the pathway will undoubtedly lead to new discoveries and insights in cell culture systems and organisms such as *Xenopus*. In addition, there is still plenty of genetics left to do in flies and worms.

Despite the pioneering role of *Drosophila* in elucidating the first outline of a Wnt signaling pathway, it is important to realize that despite the intensive effort already made, the genetics of *wg* signaling is still in its infancy. Although the *Drosophila* genome has been nearly saturated for zygotic mutants specifically affecting segmentation, most components of Wg signaling are expressed both maternally and zygotically. Only one chromosome, the X, has been searched extensively for such genes (Perrimon et al. 1989). Such screens are now being performed on the autosomes (Perrimon et al. 1996), which identified the proteoglycan synthesis mutants described earlier. Modifier screens in the embryo and eye turned up the *DTcf* mutants (Brunner et al. 1997), and another eye modifier screen showed a gene named *eyelid*, which encodes a Bright transcription family member (Treisman et al. 1997). This gene acts antagonistically towards *wg*, its phenotype suggesting that it acts in parallel to the pathway to restrict Wg target gene expression. The next few years will see many more interesting fly mutations affecting Wg signaling, and new mutations in the worm will surely be found. These genes will almost certainly have vertebrate counterparts acting in similar ways. Likewise, results obtained in vertebrates will influence the work done in model systems. Thus, the widespread occurrence of Wnt signaling in animals guarantees that the rapid increase in the understanding of the pathway will continue.

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Ken M. Cadigan and Roel Nusse

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