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Review

Wnt-frizzled signaling to G-protein-coupled effectors

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Abstract. Wnt proteins signal via cell surface receptors termed Frizzleds. Frizzleds display many properties characteristic of members of the superfamily of G-protein-coupled receptors, including heptihelical hydropathy plots; an exofacial N-terminal region that is glycosylated; a cytoplasmic C-terminal region that includes canonical motifs for phosphorylation by protein kinase A, protein kinase C and casein kinase II; cytoplasmic domains that couple to heterotrimeric G proteins, as evidenced by a GTP-shift in receptor affinity; receptor-mediated responses sensitive to depletion of specific G protein subunits and receptor-mediated responses sensitive to bacterial toxins that target G proteins. Evidence from a variety of developmental systems demonstrates Wnt-Frizzled (Fz) signaling via pathways other than the Wnt/β-catenin pathway linked to transcription controlled by Lef/Tcf. Prominent among these additional pathways is a Wnt-Fz pathway regulating intracellular [Ca^{++}] and cyclic GMP levels. The essential role of heterotrimeric G proteins in Wnt-Fz signaling is highlighted.

Key words. Frizzled; Wnt; cell signaling; Ca^{++}; cyclic GMP; PDE; development; G proteins; G-protein-coupled receptors.

Signaling by Wnt-Fz

Wnts comprise a class of mammalian genes encoding 350–380-amino-acid, secreted signaling ligands, which play diverse and essential roles in generation of cell polarity, embryonic induction, specification of cell fate and in diseases such as cancer [1–3]. At the molecular level, Wnt proteins operate largely via receptor-mediated signaling pathways, and these receptors appear to be members of the *frizzled* (Fz) family of genes [4, 5]. A canonical Wnt8-Fz-1 pathway has evolved based on genetic evidence in which Fz-1 activation by Wnt is coupled to activation of the novel phosphoprotein Dishevelled (Dvl), which acts in complex with the multivalent scaffold protein Axin, and the product of the adenomatous polyposis coli (APC) gene, to suppress glycogen synthase kinase 3-β (GSK3β) activity [1, 2, 6]. In the absence of Wnt, GSK3 acts unopposed to phosphorylate both the scaffold Axin as well as the β-catenin, which destabilizes β-catenin, directing it to degradation by the proteosome [7]. In the presence of Wnt, Fz-1 action inactivates GSK3β, stabilizes β-catenin, and leads to accumulation and translocation of β-catenin to the nucleus where it combines with members of the Tcf family of DNA-binding proteins, enabling transcription [8–10] (fig. 1) [11, 12]. β-Catenin has been shown to compete with the transcriptional repressor Groucho, transforming the transcription factor Tcf from a repressor to an activator [11, 13].

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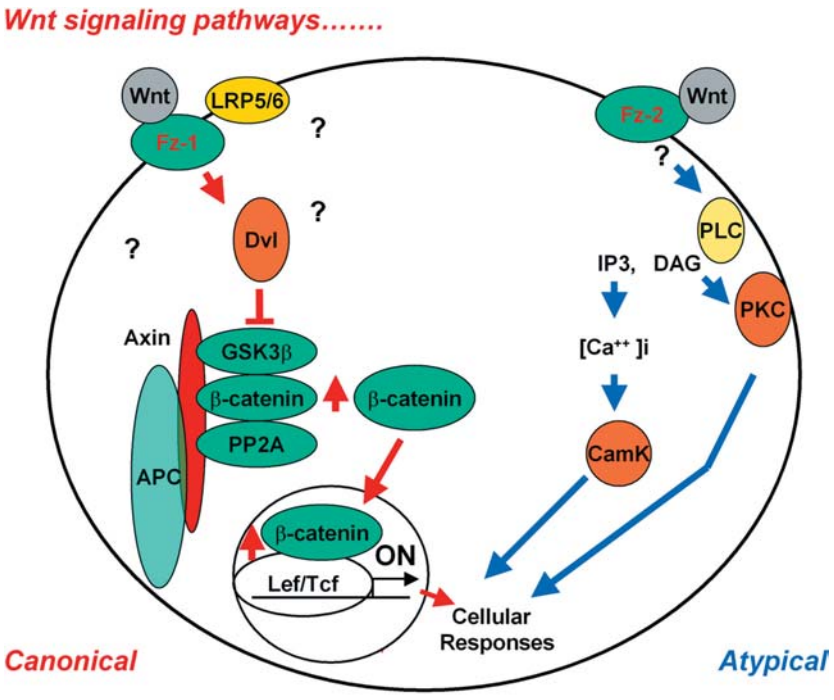

Figure 1. Wnt-Fz signaling pathways, the 'canonical' and 'atypical' pathways. In the absence of Wnt, the Fz-1 and its coreceptor LRP5/6 do not suppress the activity of the downstream protein kinase, GSK3*β*. Upon stimulation, the novel phosphoprotein Dvl exerts an inhibitory effect on GSK3*β*, leading to a reduction in the phosphorylation of *β*-catenin and the scaffold protein Axin. In the absence of phosphorylation, *β*-catenin is not targeted to the proteosome for degradation, but rather accumulates in the cytosol and eventually in the nucleus, where it combines with the Lef/Tcf transcriptional complex to stimulate gene expression. Other Wnts, acting via Fz-2, do not lead to activation of Dvl and regulation of GSK3*β*.This pathway, which controls mobilization of intracellular calcium, was termed atypical, although it makes use of several key elements in signaling common to GPCRs.

Fz-2 receptor and the 'non canonical' pathway

The Fz-2 heptihelical receptor displays a 'noncanonical' pathway that links to changes in the mobilization of intracellular Ca^{++}, and not to the canonical Fz-1-mediated, *β*-catenin pathway (fig. 1). Recent evidence suggests that some Wnts function through this pathway, a pathway that is distinct from the one described above, which requires Dvl activation [3, 14–16]. Xwnt5a, for example, unlike Xwnt1, does not induce duplication of the axis, but rather induces morphogenetic defects [14, 15]. These effects noted are unique to Xwnt5a, as Xwnt1, -3a, -8 and -8b are functionally equivalent in the induction of axis, whereas Xwnt5a, -4 and 11 are functionally distinct in this atypical Wnt-Fz pathway which activates protein kinase C [17], calcium mobilization, Ca^{++}/calmodulin-dependent protein kinase II (CamKII) [18, 19] and other more recently discovered downstream effectors [20]. Biochemical studies of Wnt-Fz signaling have been hampered by the formidable task of purifying biologically active Wnt ligands, which has been frustratingly difficult and only recently achieved [21]. Biochemical description of the Wnt/*β*-catenin, canonical pathway have emerged, but comparatively little information was available until recently on the molecular features of how Fz2 receptors signal.

Frizzled receptors: are they G protein linked?

One of the fundamental question in Wnt signaling is whether Frizzleds signal in some manner via heterotrimeric G proteins (G proteins). *frizzled* gene products display a predicted protein sequence with many of the landmarks of G-protein-coupled receptors (GPCRs), including a heptihelical hydropathy plot predicting seven hydrophobic segments of sufficient length to be transmembrane-spanning domains [4, 22, 23], a complex N-terminal region that is exofacial and N-glycosylated and not unlike that for the gonadotropin hormone receptors that are GPCRs [23], a cytoplasmic C-terminal 'tail' that is replete with sites suitable for possible phosphorylation by protein kinase A, protein kinase C and casein kinase II [4], and finally, significant homology to other known GPCRs [20]. Although there are > 18 reported Frizzleds in mammals, insufficient data exist on the downstream signaling character of each to enable construction of a classification scheme with the same level of detail as that comparing Fz-1 and Fz-2. Analysis of the primary sequences of rat Fz-1 versus rat Fz-2, however, reveals high overall homology, but nine significant non-identities exist for residues located in the proposed cytoplasmic domains that link to het-

erotrimeric G proteins and other downstream signaling components.

In zebrafish embryos, for example, signaling via rat Fz-2 (Rfz2), but not rat Fz-1 (Rfz1), leads to activation of calcium transients [24], which by many criteria mimics a well-known response of other GPCRs that leads to activation of the G protein effector phospholipase C-β (PLCβ), generation of water-soluble inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (DAG) accumulation, and ultimately to activation of protein kinase C [24, 25]. In frog embryos, another model of early vertebrate development, activation of Rfz2, but not Rfz1, leads to activation of protein kinase C and to recruitment of green fluorescent protein (GFP)-tagged protein kinase C from the cytoplasm to the cell membrane [17] (fig. 2). Pertussis toxin catalyzed ADP ribosylation of members of the Gαi, Gαo and Gαt families of G protein subunits, inactivating these subunits and blocking signaling from the G protein to its effectors. The activation of PLCβ, protein kinase C and the recruitment of protein kinase C to the cell membrane can be blocked by pretreatment of the cells with pertussis toxin [24]. Finally, it has been shown that actions of some Wnts are regulated by a member of the newly discovered family of *Regulators of G Protein Signaling*, or RGSs [26].

Studying Frizzleds in mammalian cells

Expression of Rfz1 and -2 in mammalian F9 stem cells has yielded additional evidence in support of these receptors as GPCRs. Expression of Rfz2 followed by the coculture of cells secreting Wnt5a, but not Wnt8, induced formation of primitive endoderm (PE), as measured by the expression of two prominent PE marker proteins, tissue plasminogen activator (tPA) and cytokeratin endo A, the TROMA antigen [27]. The use of conditioned media from clones secreting a Wnt ligand to activate the Fz-2 receptor is labor intensive, subject to the generic criticism of all such studies making use of conditioned media (i.e. a complex mixture), but can, in a limited manner, be used to test the biology of a response using the native receptor. Consistent with the expression of the PE markers in response to activation of Rfz2 was the loss of the expression of the embryonic marker antigen SSEA1 in the F9 cells expressing the Rfz2 when cocultured with cells expressing Wnt5a. Parallel experiments performed with conditioned media from cells expressing either Wnt5a, Wnt8 or the empty-vector control confirmed the fidelity of Wnt/Fz signaling, i.e. only Wnt5a-containing conditioned media activates Rfz2 and only Wnt8 activates Rfz1, as observed in frog and zebrafish studies [28]. Pertussis toxin treatment abolished Rfz2 signaling in the F9 cells, blocking Wnt5a from promoting PE formation in Rfz2-bearing cells [27]. The expression of two G proteins, Gαo and Gαt2, were shown to be obligate for Rfz-2 signaling to PE formation. The ability of pertussis toxin and antisense oligodeoxynucleotide (ODN)-mediated suppression of Gα subunits to block Rfz2-mediated responses supports the central hypothesis, i.e. that Fz-2 signals via heterotrimeric G proteins [20].

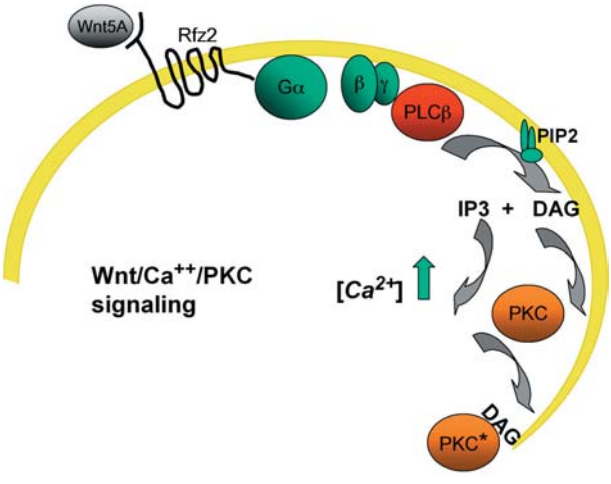

Figure 2. Delineation of the Wnt/Ca^{++}/protein kinase C signaling by Fz-2. Activation of Fz-2 by Wnt5A leads to the activation of heterotrimeric G proteins that release G-β/γ subunits that can activate phospholipase Cβ. The activation of PLC leads to hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP2) and the release of the water-soluble inositol phosphate IP3, which acts to mobilize intracellular calcium stores, and to the creation of diacylglycerol, which leads to migration of and activation of protein kinase C in the cells. The asterisk represents an 'activated' molecule.

Evidence for a role of visual elements in Frizzled signaling

Two important features of Rfz2 signaling uncovered recently implicate elements of the well-known visual pathway, namely pertussis toxin sensitivity and a requirement for Gαt2. The possible involvement of Gαt2 in Frizzled signaling was an exciting possibility, since the expression of Gαt in vertebrates previously was thought to be confined almost exclusively to the visual pathway. Recent data demonstrate, however, the expression of Gαt2 in these stem cells as well as Chinese hamster ovary cells in culture [29]. Another tantalizing piece of data was derived from screens of Fz-2 signaling with selective enzyme inhibitors. Both zaprinast and dipyridamole, well-known inhibitors of cyclic GMP-selective phosphodiesterase (PDE), were found to block signaling from Rfz2 [29]. Sensitivity to pertussis toxin, to suppression of Gαt and to inhibitors of cyclic GMP PDE focused our attention on analogies of the Fz-2 signaling to the well-known visual pathway.

Biochemistry of the vertebrate visual pathway: parallels to Fz-2 signaling

The visual pathway has been studied extensively and involves a central triad of signaling molecules, rhodopsin, either Gαt1 (expressed in rods) or Gαt2 (expressed in cones), and a cyclic GMP-selective PDE (fig. 3A). Rhodopsin, the photopigment of the vertebrate eye, is a GPCR with seven mostly α-helical transmembrane segments [30] and hydropathy plots much like that of Fz-2. The 11-cis-retinal chromophore captures a photon and undergoes a transition to the all-trans state, serving as an embedded agonist ligand in photoexcited rhodopsin. The change in the conformation of the photolyzed rhodopsin is transduced to the cytoplasmic domains that form a binding site for its cognate heterotrimeric G protein Gαt1/2. The activation of Gαt catalyzed by photolyzed rhodopsin, in turn, activates a cyclic GMP PDE, PDE6, by relieving the suppression of the PDE by its inhibitory PDE6γ subunit. The PDEα subunits are catalytic, responsible for the hydrolysis of cyclic GMP and its decline in intracellular levels, which, in turn, leads to changes in the activity of cyclic GMP-regulated effectors, e.g. cyclic GMP-gated channels controlling membrane conductance (fig. 3A).

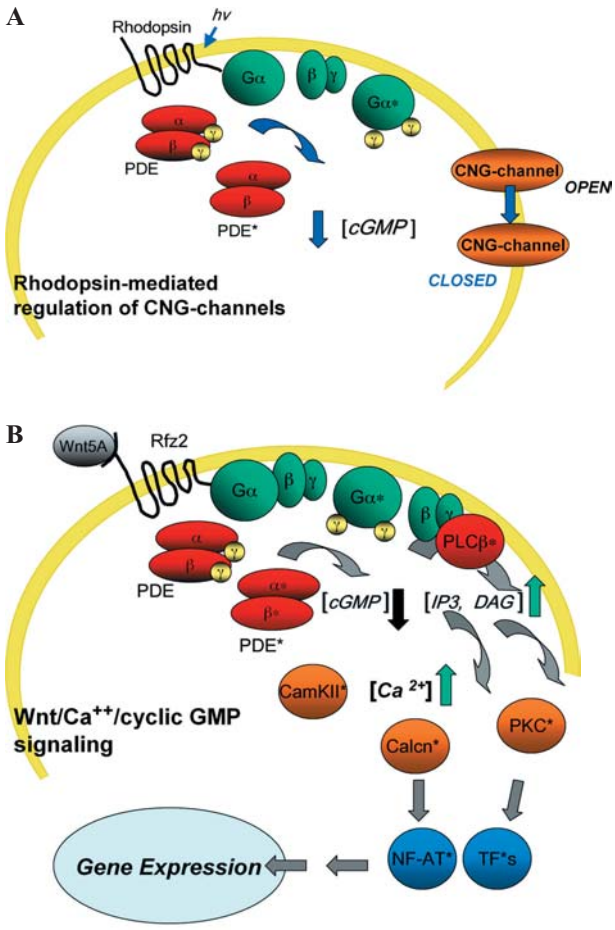

Figure 3. Fz-2 regulation of cell signaling and gene expression via the activation of PLC and cyclic GMP phosphodiesterase. (*A*) Light (*hv*) leads to photoexcitation of the photopigment rhodopsin, which in turn activates the heterotrimeric G protein Gαt (also known as transducin). The activated Gαt abstracts the inhibitory subunits of PDE6, releasing the catalytic subunits (α and β) to degrade cyclic GMP, reducing the intracellular concentration of this cyclic nucleotide. The cyclic nucleotide-gated (CNG) channel closes at the lower levels of cyclic GMP, provoking a change in membrane conductance. (*B*) Activation of Fz-2, likewise activates heterotrimeric G proteins, Gαt and other G-proteins. Activation of Gαt leads to activation of the PDEs and a decline in intracellular levels of cyclic GMP. The G$\beta\gamma$ subunit complexes activate PLCβ, leading to increased accumulation of IP3 and a corresponding increase in intracellular calcium, and activation of calcium/calmodulin-sensitive protein kinase II and the phosphoprotein phosphatase calcineurin. Diacylglycerol (DAG) leads to activation of protein kinase C (PKC) and phosphorylation of various transcription factors (TFs). Downstream of these steps is the activation of transcription by nuclear factor of activated T cells (NF-AT, via dephosphorylation) and other transcription factors (by phosphorylation?) that regulate gene expression.

Gαt, the G protein of the visual pathway

The rod (*GNAT1* gene) and the cones (*GNAT2* gene) express differing, but homologous, forms of Gαt. Gαt is a member of the Gαi/Gαo family [31, 32], which are all substrates for ADP ribosylation and thereby inactivation by pertussis toxin. Although both Gαts are heterotrimeric, their β subunit composition also varies from rods to cones. Upon activation by rhodopsin, the GDP of the heterotrimer is exchanged for GTP, and the Gαt-GTP diffuses and binds to a membrane-associated photoreceptor-specific cyclic GMP PDE. The discovery of a role of Gαt2 in Fz-2 signaling was quite by accident, revealed in a screen with oligodeoxynucleotides (ODNs) antisense to a broad spectrum of G protein α and β subunits, including Gαt subunits. ODNs antisense to Gαt subunits were employed initially in a 'control' group. Antisense, but not sense or missense, ODNs to Gαt2, however, resulted in a blockade of Fz-2 signaling [27].

Cyclic GMP PDE6, a Gαt-driven effector

This provocative hypothesis that Gαt mediates Fz-2 signaling in a nonvisual pathway was tested. Retinal PDE6, the known effector for Gαt, is composed of four subunits, two catalytic (α and β in rods; α' and α' in cones) and two regulatory (γ) subunits [33–35]. The γ subunits for rods and cones are nonidentical. An additional subunit (δ) observed only in the rods can bind the PDE complex and disrupt its membrane association. The catalytic activity of the PDE provides for high gain, and the system appears to be regulated by members of the RGS family [36, 37], much as Wnt signaling in some instances is regulated by RGS proteins [26, 37]. Screens employing well-known

chemical inhibitors of signaling molecules revealed PDE inhibitors such as IBMX, zaprinast and dipyridamole as potent agents effectively blocking both Rfz2 signaling and Rfz2-induced formation of PE [27, 29] (fig. 3B).

Fz-2 regulation of intracellular cyclic GMP levels: closing the loop

Direct measurements of cyclic GMP levels in mammalian F9 clones expressing Frizzleds revealed a sharp, transient decline in cyclic GMP levels in response to activation of the Fz-2, but not Fz-1 [29]. Clones expressing the Rfz2 chimera displayed a reduction in the intracellular concentration of cyclic GMP in response to stimulation with isoproterenol. The clones expressing Rfz2 displayed a similar sharp decline in the intracellular concentrations of cyclic GMP in response to conditioned media containing Wnt5a, a response that could be abolished by treating the conditioned media first with anti-Wnt5a antibodies [29]. Inhibitors of cyclic GMP-selective PDEs, such as dipyridamole or zaprinast, blocked Fz-2 signaling and induction of primitive endoderm. To extend these studies further, the effects of these selective PDE inhibitors were examined in zebrafish embryos, in which Fz-2 signaling controls calcium mobilization [24, 25, 38]. In these embryos, treatment with either dipyridamole or zaprinast during gastrulation had several effects: blocking Fz-2 signaling to calcium signaling, blocking normal convergent extension, epiboly defects and several lateral expansion of somites in > 75% of the developing embryos [29]. Taken together, these data provide a compelling case to support the hypothesis that Fz-2 signaling makes use of Gαt and cyclic GMP PDE, well-known signaling elements in the visual pathway (fig. 4).

Future prospects for the role of G proteins in Frizzled action and development

The discovery of Frizzled signaling via G proteins and to both PLCβ and to cyclic GMP PDE was unexpected and provoked many derivative questions. In the visual pathway, the change in cyclic GMP stimulated by activation of rhodopsin, Gαt, and finally PDE6 is 'read' by a cyclic nucleotide-gated (CNG) ion channel that controls membrane conductance in the eye. What in the embryo is reading the change in intracellular cyclic GMP and convert-

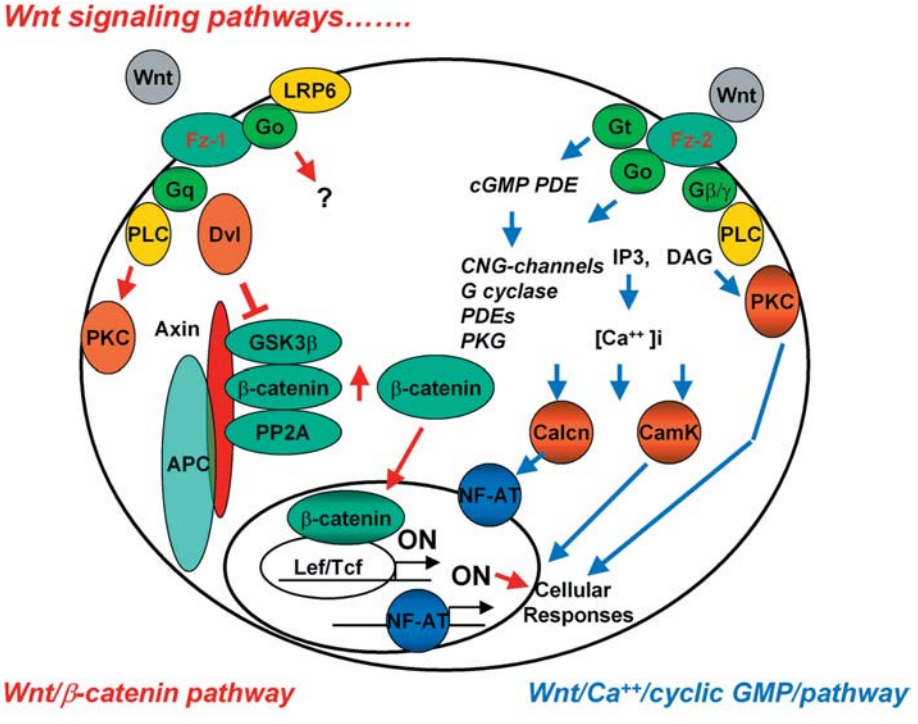

Figure 4. Wnt/Ca2+/cyclic GMP signaling via Fz-2. The binding of Wnt-5A activates the rat Fz-2, leading to activation of heterotrimeric G proteins (composed of Gα, β/γ subunits). Gαt2 then activates the phosphodiesterase (PDE > PDE6*), which stimulates a decline in intracellular [cyclic GMP]. The Gβ/γ subunits activate phospholipase Cβ (PLCβ), which hydrolyzes PIP2 to IP3 and diacylglycerol (DAG). IP3 catalyzes the release of stored, intracellular Ca^{2+}, activating Ca^{2+}/calmodulin-sensitive protein kinase II (CamKII) and the Ca^{2+}-sensitive protein phosphatase calcineurin (Calcn). The DAG activates protein kinase C (PKC) directly. Both PKC and Calcn directly and indirectly influence the activity of various transcription factors, using NF-AT as an example, to direct gene expression. The changes in cyclic GMP may regulate one or more known effectors, including cyclic nucleotide-gated channels, guanylycyclase, PDEs and/or protein kinase G. The Fz-1 pathway regulates the β-catenin-sensitive genes via the Lef/Tcf transcriptional complex and perhaps via other mechanisms.

ing this signal to another output? There are several possible effectors downstream of the cyclic GMP, including CNG-ion channels, guanylycyclase, protein kinase G and the PDEs themselves. Much further work will be required to sort out the complete set of downstream signals from Fz-2 and how these signals integrate with the other Frizzled-sensitive pathways [39, 40], such as the Wnt/β-catenin pathway, also shown recently to be coupled via heterotrimeric G proteins [41, 42]. The recent successful purification of biologically active Wnts will facilitate Wnt research [21]. Since Wnts can interact with multiple Frizzleds, however, the Frizzled chimeras remain invaluable tools with which to elucidate the downstream signaling pathway of a single *frizzled* gene product [39]. Used in tandem to address the Wnt-associated and Frizzled-specific pathways, these two approaches reveal much more information about Wnt-Frizzled signaling. Analysis of signaling and gene expression (perhaps by DNA microarray) will enable greater understanding how individual Wnts and individual Frizzleds stimulate and orchestrate these essential aspects of development (fig. 4).

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