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# WNT-FRIZZLED SIGNALING VIA CYCLIC GMP

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## 1. ABSTRACT

Wnt-Frizzled signaling is an essential aspect of early development, regulating cell fate, polarity, differentiation, and migration. In addition to the well-known Wnt/beta-catenin pathway characterized for Frizzled-1, there are other pathways regulated by Wnts that are not mediated by Frizzled-1 and do not lead to stabilization of beta-catenin and activation of the Lef/Tcf-sensitive transcription of genes. The first of these non-canonical pathways to be identified is the Wnt/Ca++ pathway in which Frizzled-2 activation leads to release of beta/gamma subunit complexes from heterotrimeric G-proteins (presumably Go and Gt) to activate phospholipase C and other effectors to stimulate a mobilization of intracellular Ca++. More recently a second, related pathway of Wnt-Frizzled signaling has been discovered that regulates the intracellular levels of cyclic GMP. Frizzled-2, established as a member of the family of 7TMS receptors that couple by heterotrimeric G-proteins to effectors, can signal via the G-protein Gt2, transducin, a G-protein prominent in phototransduction in the eye, to cyclic GMP phosphodiesterase. The discovery of the expression of Gt2 in embryonic cells was co-incident with the demonstration that inhibitors of cyclic GMP phosphodiesterase potently blocked various features of Frizzled-2 signaling in mouse embryonic F9 cells and in zebrafish embryos. The signal linkage map from Wnt to changes in intracellular cyclic GMP and development is the focus of this review. The molecular features of how changes in intracellular cyclic GMP concentrations control development remain to be elucidated.

## 2. ATYPICAL, NON-CANONICAL PATHWAYS OF WNT-FRIZZLED SIGNALING

It is now well-known that Wnt-Frizzled signaling extends beyond the pathway by which Frizzled-1 can increase the stabilization of beta-catenin and activate the Lef/Tcf-sensitive transcription of genes (1). Several Wnts that do not bind to Frizzled-1 and have little effect on beta-catenin stabilization exert influences on other aspects of development(2). The first pathway, the Wnt/Ca++ pathway, recognized as "atypical" or "non-canonical" was an important addition to our understanding of Wnt-Frizzled signaling (3), heralding in what would be other atypical pathways regulated by Wnt signaling (4). The demonstration that Frizzled-2 activation by Wnt-5a increased the mobilization of Ca++ and activated calcium-sensitive downstream effectors provided the proof-of-concept that the canonical pathway could not possibly explain the full richness of Wnt signaling in development (5). The details of this seminal discovery and its significance to the area of Wnt signaling are treated elsewhere in this volume (see M. Kuhl).

Central to search for novel pathways of Wnt-Frizzled signaling was the molecular cloning of Frizzleds (6). The elucidation of the larger family of mammalian Frizzled clearly demonstrated that these cell-surface proteins are members of the superfamily of receptors with seven transmembrane segments (7TMS) in their primary sequence (7). Since the bulk of these 7TMS receptors couple to downstream signaling elements via members of the family of heterotrimeric G-proteins, a hypothesis that Frizzled receptors are G-protein-coupled receptors was proposed and tested(8). During analysis of Frizzled-2 action on the intracellular concentration of Ca++ it became obvious that a second, non-canonical pathway of Frizzled signaling must be operating. The evolution of this idea and the strategies employed to elucidate the Wnt-Frizzled regulation of cyclic GMP phosphodiesterase, a well-known effector of GPCRs, are described below.

## 3. FRIZZLED-2 AS A G-PROTEIN-COUPLED RECEPTOR

More than 5% of the human genome encodes cell-surface receptors that display seven, hydrophobic, transmembrane segments in the hydropathy plots of their

primary sequence. These 7TMS receptors constitute the superfamily of G-protein-coupled receptors, composed of five families segregated based upon homology and the nature of the ligand binding domain of each (7). As a class, GPCRs display many characteristics including the following: exofacially-disposed N-terminal sequences that are N-glycosylated; C-terminal "tails" that are disposed into the cytoplasm where they interact with G-proteins and are subject to phosphorylation by protein kinases; a GTP-dependent shift in agonist-specific affinity of the receptor; and, downstream signaling that is dependent upon the presence of their cognate G-proteins. The Frizzled-2 receptor has been studied both by *in silico* analysis, by expression in cells to which Wnt-containing media can be employed to activate downstream signaling, as well as through donation its cytoplasmic domains to a chimeric receptor that includes the exofacial and 7TMS regions of a prototypic GPCR, the beta2-adrenergic receptor (9). The Frizzled-2 receptor displays an N-glycosylated, N-terminus disposed to the exofacial side of the lipid bilayer and 7TMS much like all GPCRs. The N-terminus displays cysteine-rich domains and organization very similar to the gonadotropin receptors for follicle-stimulating hormone (FSH) and leutinizing hormone (LH) that, like the Wnt ligand, are secreted glycoproteins and also coupled to G-proteins. The C-terminus of members of the Frizzleds are disposed to the cytoplasm and display canonical sites for phosphorylation by protein kinase C and likely other kinases known to phosphorylate and to regulate GPCRs. Other cytoplasmic domains of Frizzleds display canonical sites for phosphorylation by protein kinase A and casein kinase II. Through the creation of the Frizzled chimera for which the cytoplasmic domains of Frizzleds are substituted for those of the beta2-adrenergic receptor (8), it has been possible to test if these Frizzled cytoplasmic domains can perform a GTP-dependent shift in agonist affinity, a cardinal property of GPCRs. The Frizzled chimera display a bona fide GTP-dependent, agonist-specific shift in receptor affinity(10), like other GPCRs. The ability to display a GTP-dependent shift in affinity is dependent upon the presence of the heterotrimeric G-proteins linked to Frizzled downstream signaling, suppression of Go and Gq, for example, blocks Frizzled-1 signaling and the GTP-dependent shift of the Rfz1 chimera (11). The very fact that Frizzled cytoplasmic domains can be spliced onto the exofacial and 7TMS domains of the beta2-adrenergic receptor also provides compelling evidence to support the hypothesis that Frizzled-2 is a *bona fide* GPCR. A more-detailed analysis of the Frizzleds as GPCRs is found elsewhere in this journal (see C. Malbon).

## 4. DISCOVERY OF Gt ALPHA SUBUNIT EXPRESSION IN EMBYRONIC CELLS

One of the most interesting and unexpected discoveries made during a routine screen of G-protein family members by antisense oligodeoxynucleotide suppression (12) was the discovery of a role for Gtalpha subunit in Frizzled-2 signaling. The screen included antisense oligodeoxynucleotides to Gt1 alpha and Gt2 alpha subunits that are typically believed to be expressed only in the eye (13), more specifically in the rods (Gt1) and in the cones (Gt2), and functioning in the visual pathway. In vertebrate vision, the GPCR rhodopsin (actually a photopigment sensitive to light, not a true "receptor") is activated by photon capture by its 11-cis-retinal moiety covalently attached in the 7TMS, activates a Gt-protein, which in turn activates a cyclic GMP phosphodiesterase, leading to a reduction in intracellular cyclic GMP and the closure of a cyclic nucleotide-gated channel in the membrane (13). Gt1 and Gt2 were included as G-protein targets of suppression, simply as a "control" and it was surprising that antisense suppression of a G-protein believed to be exclusively expression in the visual system should have any effect on Frizzled-2 signaling (14). Interestingly, suppression of only Gt2 blocked Frizzled-2 signaling.

Follow-up experiments employing reverse transcription polymerase chain reaction (RT-PCR) amplification to test for the presence of Gt2 mRNA in mouse F9 teratocarcinoma embryonic cells, one of the model systems used to probe the role of G-proteins in Frizzled signaling, revealed expression of Gt2 mRNA (14). To extend the analysis, primers for the RT-PCR amplification were selected to encompass significant portions of the Gt2 sequence. The products of the amplification were sequenced, revealing identity with the corresponding Gt2 sequences. At the protein level, the presence of Gt2 was probed using whole-cell extracts of F9 cells and immunoblotting with anti-Gt2 alpha antibodies. A ~40kDa species with an $M_r$ equivalent to that of authentic Gt2 alpha isolated from mammalian eye was identified in the blots from the F9 cells. Staining of Gt2 alpha using an antipeptide antibody could be blocked by addition of the competing peptide in the blotting protocol (14). Thus, at the level of mRNA and at the protein level, Gt2 is expressed in mammalian embryonic cells.

## 5. INHIBITION OF FRIZZLED-2 SIGNALING BY INHIBITORS OF CYCLIC GMP PHOSPHODIESTERASE

During the course of the screens of G-protein alpha subunits by antisense suppression, the Frizzled-2 stimulated formation of primitive endoderm in F9 cells was being analyzed using a battery of well-characterized enzyme inhibitors that target well-known signaling pathways. The results of the screens revealed several interesting new insights, but most remarkable observation was the ability of a methylxanthine (methylisobutylxanthine, MIX) to block the Frizzled-2 signaling. MIX is most often thought of as an inhibitor of cyclic AMP phosphodiesterases, Frizzled-2 signaling to stimulate formation of primitive endoderm had already been shown, however, to be unaffected by elevation of intracellular cyclic AMP levels by either exogenously added cyclic nucleotides or by treatment of cells with cholera toxin (15). Interestingly, MIX is known to be a methylxanthine that can inhibit phosphodiesterases that metabolize either cyclic AMP or cyclic GMP. To test further the nature of the MIX effects, other phosphodiesterase inhibitors with selectivity for cyclic GMP-specific phosphodiesterases were screened as

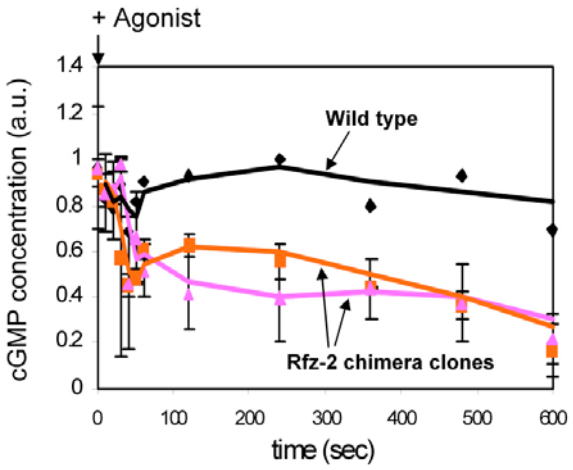


**Figure 1.** Activation of the Frizzled-2 chimera stimulates decreased intracellular levels of cyclic GMP. Mouse F9 cells (wild type) and clones expressing the beta2-adrenergic receptor/rat Frizzled-2 receptor chimera (Rfz-2 chimera) were treated at time = 0 with beta-adrenergic agonist (+Agonist, 0.01 mM isoproterenol) and the level of intracellular cyclic GMP determined at various intervals over a 10-min time-course. The results presented are in arbitrary units (a.u.) as the mean values of assays derived from three separate experiments for each of the two individual clones.

possible inhibitors of the Frizzled-2 signaling. Two additional inhibitors of Frizzled-2 signaling were identified as a result of the inhibitor screens, namely dipyridamole and zaprinast. Both dipyridamole and zaprinast were found to be potent inhibitors of the Frizzled-2 signaling to primitive endoderm formation (14). Since one prominent target of both of these inhibitors in the cyclic GMP phosphodiesterase PDE6, an effector for Gt alphas (13), the hypothesis that Frizzled-2 is a GPCR coupled via Gt2 to a cyclic GMP phosphodiesterase was created for direct testing.

## 6. FRIZZLED-2 REGULATES INTRACELLULAR CONCENTRATIONS OF CYCLIC GMP

The provocative role of the "visual" G-protein Gt2 in Frizzled-2 signaling and the demonstration that cyclic GMP phosphodiesterase-selective inhibitors block Frizzled –2 action called the question, does Frizzled-2 signal via cyclic GMP in development? The analysis of intracellular cyclic GMP levels in mouse F9 cells demonstrates that activation of Frizzled-2 itself or via use of the Frizzled-2 chimera stimulates a rapid and profound reduction in intracellular cyclic GMP levels (figure 1). These experiments were the first to demonstrate the role of a cyclic nucleotide (cyclic GMP) in the cell signaling of Frizzleds (14). Analysis of the decline in cyclic GMP in response to Frizzled-2 chimera activation was mimicked in F9 cells expressing the native rat Frizzled-2 in response to stimulation by Wnt-5a, but not Wnt-8. Treating cells with either dipyridamole or zaprinast blocks the ability of Frizzled-2 to stimulate the decline in intracellular levels of cyclic GMP and, when used at higher levels, leads to an increase in the ambient level of cyclic GMP that approaches 1-fold. Expression of the constitutively active mutant version of Gt2 alpha ( but not that of Gs or Go) lead to a decline in the intracellular concentration of cyclic GMP, mimicking the activation of Frizzled-2 (14). These data provide a direct linkage from Frizzled-2 via Gt2 alpha to the regulation of intracellular cyclic GMP levels.

## 7. INHIBITION OF CYCLIC GMP PHOSPHODIESTERASES BLOCKS DEVELOPMENT IN ZEBRAFISH

The mouse F9 cells are totipotent and provide an excellent, accessible model system for study of the early mouse development. The observations derived from the use of these cells were tested further, however, by examining the effects of the phosphodiesterase inhibitors on Frizzled action in zebrafish embryos. Much of what we have learned about Wnt/Ca++ signaling and Frizzled-2 action has been derived from studies in zebrafish embryos (16-18). The cyclic GMP phosphodiesterase-selective inhibitors dipyridamole and zaprinast were shown to potently inhibit the increases in Ca++ mobilization of zebrafish embryos in response to Frizzled-2 activation (14). This provides new insights that the newest paradigm in non-canonical Wnt pathways merges at some point with the first non-canonical pathway described, the Wnt/Ca++ pathway. The observations suggest that cyclic GMP levels or the activation of a cyclic GMP-sensitive effector is upstream of the Wnt/Ca++ response in these embryos. Cyclic GMP and Ca++ are well-known intracellular second messengers that often display interactions, such as those observed in phototransduction. Delineating the precise molecular details of the Ca++/cyclic GMP interaction in Frizzled-2 signaling remains an essential task.

The effects of the phosphodiesterase inhibitors were evaluated for their influence on zebrafish gastrulation, since the Wnt/Ca++ pathway have been shown to function in the movement of cells at the future dorsal side, extension, and narrowing of the embryo along the anterior-posterior axis. Zebrafish embryos treated with either dipyridamole or zaprinast during gastrulation lacked extension along the anterior-posterior axis (14). *In situ* staining of the somite marker *MyoD* revealed decreased dorsal convergence in the embryos treated with inhibitors of cyclic GMP phosphodiesterase activity. More 75% of the dipyridamole-treated embryos displayed epiboly defects. Many of these defects are similar to those identified by genetic mutations in the Wnt/Frizzled pathways (14). Thus, cyclic GMP appears to be a key intracellular second messenger involved in Frizzled-2 signaling.

## 8. CYCLIC GMP AND DEVELOPMENT: CONCLUDING REMARKS

The recent results from many laboratories provides the a working model of the Wnt/Ca++ and Wnt/cyclic GMP pathways (figure 2). This signaling pathway is the product of many lines of research derived

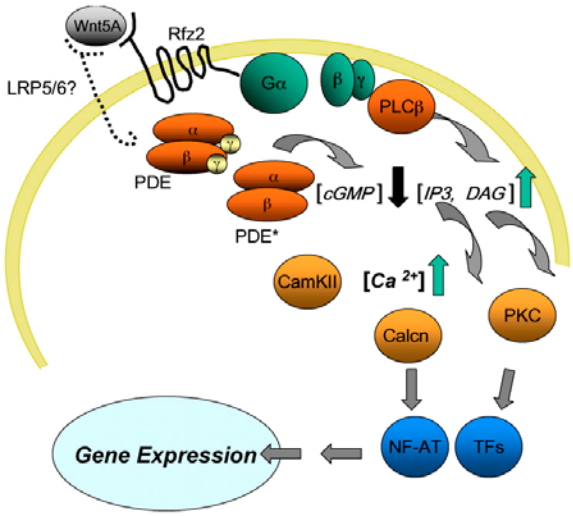


**Figure 2.** Schematic model of Frizzled-2 signaling via heterotrimeric G-proteins. The current data available support the following scheme for Wnt-Frizzled-2 activation of phospholipase C (PLCbeta) and cyclic GMP phosphodiesterase (PDE). Wnt-5a binds rat Frizzled-2 (Rfz2), a process that may require the LRP co-receptors LRP5/6. Activation of Frizzled-2 leads to activation of heterotrimeric G-proteins (including Go and Gt2), releasing Gbeta/gamma subunit complexes that activate phospholipase C. Phospholipase Cbeta metabolized phosphatidylinositol bisphosphate to yield water soluble inositol phosphates (such as IP3) as well as diacylglycerol (DAG). The IP3 stimulates mobilization of intracellular Ca++ and activation of Ca++/calmodulin-sensitive protein kinase II (CamKII) and the protein phosphatase calcineurin (Calcn), while the DAG activates protein kinase C (PKC). Various transcription factors (TFs) are sensitive to activation of protein kinase C. Activation of calcineurin leads to activation of the transcription factor NF-AT and presumably other TFs. Activation of Gt2 also leads to activation of cyclic GMP phosphodiesterase, stimulating a sharp decline in the intracellular concentration of cyclic GMP. Downstream targets that are known to sense changes in intracellular cyclic GMP concentrations include cyclic nucleotide-gated ion channels, guanylyl cyclases, protein kinase G, and other phosphodiesterases. The identity of the downstream targets of cyclic GMP involved in Frizzled-2 signaling in development remains to be elucidated.

from work in *Xenopus*, zebrafish, and mouse teratocarcinoma cells. The central feature of the Frizzled-2 is its role as a GPCR, relying upon the action of Go and Gt2 to mediate its effects to the levels of intracellular Ca++ and cyclic GMP. One obvious question to be addressed is what are the downstream, cellular sensors for the changes in cyclic GMP that follow the activation of Frizzled-2. The literature identifies several prominent candidates, including cyclic nucleotide-gated channels, protein kinase G, guanylylcyclases, and other phosphodiesterases.

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