__Review Article__

Regulation ofWnt signaling by heterotrimeric G-proteins

Abstract

Wnt signal transduction pathways play crucial roles in animal development, and after birth, they are involved in maintaining cellular and tissue homeostasis. Deregulation of the Wnt-mediated pathways occurs in many human diseases, including a wide range of human cancers. Many years ago, due to the topological similarity between Frizzled receptors and G-protein-coupled receptors, it was proposed that heterotrimeric G-proteins might be involved in the regulation of Wnt/Frizzled-mediated signaling pathways. Currently, there is a wealth of evidence indicating that heterotrimeric G-proteins regulate both canonical and non-canonical Wnt signaling pathways. This review article will discuss some of this evidence and the significance of this interaction.

Keywords: Wnt signaling; G proteins; cancer

1. Introduction

The Wnt/Frizzled-mediated signal transduction pathways are involved in regulating a wide range of biological responses. Consequently, the deregulation of these signaling pathways leads to developmental abnormalities in multicellular animals and many chronic diseases, including neurological disorders and various types of cancers in adults [1-7]. The investigation of Wnt signaling began over 40 years ago [8-10]. These pathways have been categorized as canonical and non-canonical based on the involvement of the β-Catenin protein [1-7]. While the canonical Wnt signaling seems to be a single pathway dependent on a central and critical protein called β-Catenin, the non-canonical Wnt/Frizzled signaling comprises several pathways involved in diverse biological responses. These responses include planar cell polarity (PCP), small GTPase-mediated cytoskeleton reorganization, and intracellular calcium homeostasis [1-4]. Numerous excellent review articles about Wnt signaling pathways and their biological and clinical significance have been authored by pioneering scientists in the field, as well as other scientists worldwide. Interested readers can explore the Wnt homepage (*http://web.stanford.edu/group/nusselab/cgi-bin/wnt/*), which provides a list of intriguing review articles.

For decades, Wnt signaling pathways have captured the attention of various biological and clinical scientists across different fields, including developmental biology, cancer biology, neuroscience, and stem cell biology. Two crucial and interconnected questions arise: a) How precisely are the Wnt signaling pathways regulated in various tissues? And b) how can we overcome the deregulation of these pathways in disease conditions? It has become evident that addressing these questions is no easy task and has grown even more challenging over time due to the expanding number of components and regulators within these signaling pathways.

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In 1997, when I joined the University of Pennsylvania (USA) as a post-doctoral fellow, my involvement centered around the search for potential new regulators of Wnt signaling. One captivating feature that drew my attention was the structure of Frizzled proteins. By that time, several Frizzled proteins from different organisms had been cloned and identified [11-15]. The overarching structure and topology of Frizzled proteins closely resembled those of G protein-coupled receptors, sharing a common feature of seven hydrophobic transmembrane domains [15] (Figure 1). This prompted the question of whether heterotrimeric G protein signaling pathways control Wnt signaling or if Frizzled proteins belong to a subfamily of GPCRs (G protein-coupled receptors).

2. Frizzled Receptors

The first Frizzled was originally identified in *Drosophila* as a gene encoding a protein involved in determining tissue polarity or planar cell polarity [15,16]. This conclusion was drawn due to mutagenesis experiments that resulted in a lack of orientation of the wing hairs [15,16]. Frizzled receptors are restricted to multicellular animals because they have not been found in single-cell eukaryotes (like yeasts) [17,18]. Interestingly, Frizzled proteins have also not been found in plants [17,18]. The human genome encodes at least 10 different Frizzled proteins involved in diverse cellular activities, including cell proliferation, cell differentiation, cell polarity, and maintaining cellular and tissue homeostasis [3,19].

Frizzled proteins are identified as the main receptors for Wnt glycoproteins. They are integral membrane proteins with seven hydrophobic transmembrane domains [1-5]. Frizzled proteins consist of 500 to 700 amino acids with a molecular weight of 45 to 60 kDa [18,19]. The extracellular amino terminal of a Frizzled protein includes a signal sequence at the extreme end and a cysteine-rich domain (CRD) which is involved in physical interaction with the Wnt ligands [18,19]. The CRD motif consists of 120-125 amino acids with 10 conserved cysteine residues. Interrupted by a hydrophilic domain of 40 to 100 amino acids, the seven hydrophobic transmembrane domains are located in the middle of the protein. After the 7th transmembrane domain, there is a conserved 6 amino acid motif (KTXXXW) that is required for activation of the canonical Wnt signaling [18,19]. The carboxy-terminal is located intracellularly and is the most diverse section among different Frizzled proteins. Some Frizzled receptors also have a PDZ-binding domain (S/T-X-V) at the extreme carboxy-terminal, which is believed to be required for interaction with the PDZ domain of Dvl proteins (the mammalian homologs of Dishevelled in *Drosophila*) [18,19]. The Frizzled proteins are involved in both canonical and non-canonical Wnt pathways and receive assistance from co-receptors (LRP5/6 and ROR2/RYK) to interact with the Wnt glycoproteins [1-4]. Additionally, there are reports that the interaction of Wnt causes dimerization of Frizzled receptors [18,19].

3. G protein-coupled receptors (GPCRs)

The most diverse family of proteins is the trimeric G protein-coupled receptors (GPCRs), which are estimated to encompass more than 1000 different proteins encoded by over 2% of human protein-encoding genes [20-23]. The sheer number of these receptors indicates that GPCRs are likely involved in an extensive range of biological activities, and indeed, this is the case.

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Alongside our fundamental senses (sight, hearing, smell, taste, and touch), GPCRs play a role in regulating metabolic reactions, neurotransmission, immune responses, heartbeats, and numerous other physiological functions [20-23]. The ligands for GPCRs are also remarkably diverse, including photons, odors, ions, biogenic amines, neurotransmitters, peptides, hormones, and glycoproteins [20-23].

Similar to Frizzled proteins, nearly all GPCRs possess seven hydrophobic transmembrane domains that bridge the extracellular and intracellular domains [24]. According to classical models, when a GPCR is activated, often through interaction with its cognate ligands, the receptor undergoes a conformational change that prompts the Gα subunit (in the trimeric G-protein complex) – which is initially GDP-bound – to adopt a new conformation for substituting GDP with GTP [20-24]. In live active cells, the concentration of GTP is estimated to be around 10 times higher than that of GDP [25]. Nevertheless, the Gα subunits in their initial trimeric state exhibit a much higher affinity for GDP [25,26]. This trait is a captivating feature shared by all GTP-binding proteins, including members of the Ras superfamily. Gα-GTP physically dissociates from Gβγ, and both subunits can subsequently regulate their effectors [25-27].After transmitting the signal, many GPCRs become inactivated or desensitized through membrane endocytosis, typically resulting in two outcomes [28-30]. The first involves receptor recycling and its relocalization to the cell membrane, while the second entails receptor proteolysis [28-30]. Endocytosis of GPCRs necessitates G protein receptor kinases (GRKs) that phosphorylate these receptors at their C-terminus. The β-arrestin protein recognizes the phosphorylated GPCRs, leading to the formation of Clathrin-coated endocytic vesicles [28-30].

Structural studies of GPCRs are hampered by technical difficulties in the overexpression, purification, and crystallization of these membrane proteins. To date, only a handful of GPCRs have had their structures resolved, including bovine Rhodopsin, adenosine A2 receptor, and adrenergic receptors [20-23]. Structural analysis of Rhodopsin has suggested that retinal isomerization triggers three intramolecular activation pathways via receptor transmembrane domains 2, 3, 5, 6, and 7 [20,22]. A similar mode of activation has been proposed for other GPCRs [22]. Itis hypothesized that different ligands might induce distinct active conformations of the receptor, thereby initiating specific responses [21,22]. An important aspect to consider is domain coupling, where a change in one segment of a GPCR could impact neighboring domains and their associated functions [21]. Such investigations have provided new insights into how ligands activate GPCRs. Another model suggests an equilibrium between the active and inactive states of a GPCR, with the presence of the ligand tilting the balance toward the active state [23].

There are four subfamilies of Gα proteins (Gαs, Gαi, Gαq, and Gα12/13), each of which comprises several members [26,27]. For instance, the Gαq subfamily includes Gαq itself, Gα11, Gα14, and Gα15/16. Additionally, mammalian cells encode 5 beta and 12 gamma subunits [26,27]. As a result, numerous combinations of α, β, and γ subunits are expected. Coupled with the substantial diversity of G protein-coupled receptors (GPCRs), it becomes evident that GPCR/G protein-mediated signaling pathways are not only involved in nearly all biological functions of eukaryotic cells but are also among the most intricate signaling pathways.

3.1Are Frizzled proteins a subfamily of GPCRs?

The amino acid sequences of Frizzled proteins are not very similar to those of GPCRs, and the partial similarity is limited to the hydrophobic transmembrane domains [18,19]. However, the

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topological similarity between Frizzled and GPCR receptors (both having seven hydrophobic transmembrane domains) (Figure 1) serves as evidence supporting the notion that Frizzleds belong to a subfamily of GPCRs, characterized by specific features such as a cysteine-rich domain (CRD) at the amino-terminal and (or) a PDZ-interacting domain at the extreme carboxy-terminal [18,19].

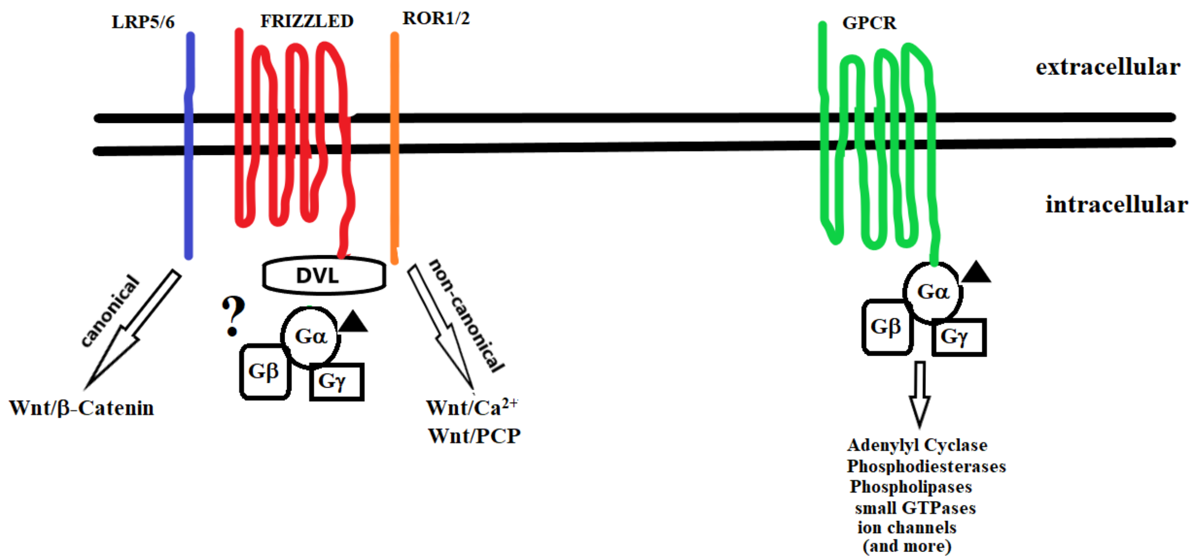

Figure 1.A very simple demonstration of Frizzled and GPCR signaling pathways at the upstream level. The topology of both receptors is very similar and therefore it has been proposed that activation of Frizzled signaling may pass through heterotrimeric G-proteins including Gα, Gβ, and Gγ subunits (**?** mark). Frizzled receptors get help from co-receptors to transmit signals which are known as canonical (Wnt/β-Catenin) and non-canonical (Wnt/Ca^{2+} and Wnt/PCP) Wnt signaling pathways. Single transmembrane proteins, LRP5/6 and ROR1/2 function as co-receptors for the canonical and non-canonical Wnt pathways respectively. The small filled triangle attached to the Gα subunits, represents GDP (in the resting state) or GTP (in the active state). The effectors of GPCR/G protein signaling are very diverse and only some are listed in the figure. LRP5/6, Low-density lipoprotein *receptor*-related protein5/6; ROR1/2, receptor tyrosine kinase–like orphan receptor-1 and 2; PCP, planar cell polarity; GPCR, G protein-coupled receptors.

For both canonical and non-canonical Wnt signaling pathways, there are coreceptors (LRP5/6 and ROR1/2, respectively) that assist Frizzleds in gathering the necessary physiological signals [1-5]. In certain reports, Frizzleds and Smoothened are categorized as class F GPCRs [24]. To

qualify as a subfamily of GPCRs, these receptors are expected to directly bind and activate heterotrimeric G-proteins. While Smoothened has been shown to directly interact with trimeric G-proteins from the Gi and G12/13 subfamilies [24], the direct interaction of Frizzleds with trimeric G-proteins is still being investigated [18,24]. Both we and others possess evidence supporting a direct interaction between certain members of Frizzled receptors and the Gα subunits of heterotrimeric G-proteins. I will discuss these results in the subsequent sections of this article.

The findings from investigations concerning the interaction between Wnt/Frizzled and G-protein signaling pathways hold several significant implications. GPCR/G protein signaling may contribute to specifying signals through different Wnt and Frizzled proteins. Furthermore, G protein signaling pathways may aid in the discovery of additional components and regulators of Wnt signaling. These findings might also propose new clinical targets for preventing and (or) treating chronic diseases, such as human cancers, that are in some way linked to the deregulation of Wnt signaling pathways. It has been estimated that more than 35% of the therapeutic drugs currently used in clinics target either GPCRs or G-proteins [31,32].

4. Regulation of Wnt signaling by GPCR/G protein signal transduction pathways

In 1997, when I embarked on my first post-doctorate training at the University of Pennsylvania (USA), no published results were available concerning the interaction between Wnt/Frizzled signaling and heterotrimeric G-protein pathways. The topological resemblance between Frizzled receptors and GPCRs compelled me to initiate an exploration into the potential interaction between these two signaling pathways. To commence, I hypothesized that if heterotrimeric G-proteins were capable of regulating Wnt signaling, general activators of G-protein pathways (such as nonhydrolyzable GTP analogs and aluminum fluoride, $ALF4^{-}$) might influence the activity of Wnt signaling. At the time, Wnt signaling was not as comprehensively understood as it is today. It's intriguing to note that despite the initial discovery of Frizzled-encoding genes as contributors to tissue polarity in *Drosophila* [16], the Wnt/Frizzled-mediated signaling pathway was initially recognized as what we now term the "canonical Wnt signaling" or "the Wnt/β-Catenin pathway" [1-6]. According to an established model, activation of the canonical Wnt signaling pathway led to GSK-3β inhibition and the subsequent accumulation of cellular β-Catenin [1-5]. In light of this, I opted for assays to assess GSK-3β kinase activity (using Tau protein as a substrate) and cellular levels of β-Catenin. Utilizing *Xenopus* oocytes as a cellular system, I discovered that general activators of G-protein signaling (GTP-γS and aluminum fluoride) diminished GSK-3β kinase activity and stabilized cellular β-Catenin protein levels. Given that these activators, particularly GTP-γS, could also activate members of the Ras superfamily, I employed active mutants of various Gα subunits (GαsQL, GαiQL, GαqQL, and GαzQL) available in the lab. Intriguingly, expression of all these active Gα subunits led to GSK-3β inhibition and the accumulation of cellular β-Catenin, with GαqQL exhibiting a more pronounced effect. The wild-type Gαq displayed a similar behavior, albeit with a weaker effect compared to its active mutant [33]. Corresponding outcomes were obtained when using the mammalian HEK293T cell line as the cellular system [34]. Based on these findings, we postulated that heterotrimeric G-proteins likely regulate Wnt signaling, and we suggested that among various classes of Gα proteins, Gαq plays a more significant role [33, 34].

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Gαq is recognized as an activator of the beta isoform of phospholipase C (PLCβ) [33, 34]. Activated PLCβ converts PIP2 (Phosphatidylinositol 4, 5-bisphosphate) into two critical second messengers: IP3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). IP3 interaction with its receptors on the endoplasmic reticulum prompts calcium release into the cytosol. Meanwhile, DAG directly activates protein kinase C (PKC) [33, 34]. Therefore, it was postulated that Gαq might inhibit GSK-3β by activating PKC. This notion was consistent with results from Dr. Trevor Dale's laboratory in the UK, revealing that the *Drosophila* homolog of Wnt proteins (Wingless) could deactivate GSK-3β through a pathway dependent on protein kinase C, PKC [35]. Notably, Dr. Dale's group did not mention the potential involvement of G-proteins in the regulation of Wingless signaling in their publication [35]. While G proteins are not the exclusive regulators of PKC, the interaction between Wingless and PKC served as a cue for further exploration into the relationship between Wnt signaling and G proteins.

Another insight into the role of trimeric G proteins in regulating Wnt signaling emerged from Dr. Randal Moon's laboratory in late 1997 [36]. Their work demonstrated that the expression of Wnt5a in *Zebrafish* embryos triggered the activation of phosphoinositide signaling and the release of calcium from intracellular stores in a manner sensitive to pertussis toxin [36]. It was already established that certain members of the Gi class of G proteins can activate phosphoinositide signaling, with this class being pertussis toxin-sensitive [37].

The discovery of Axin, a negative regulator of Wnt signaling, in 1997 revealed the presence of an RGS (regulator of G protein signaling) domain within the protein's structure [38]. The RGS domain is commonly found in a family of proteins that expedite the GTPase activity of alpha subunits of heterotrimeric G proteins [39, 40]. While the RGS domain of Axin is known to interact with the APC (Adenomatous Polyposis Coli) protein, it remains uncertain whether this domain can genuinely activate the GTPase activity of a specific Gα subunit, even though interactions between Axin and certain G alpha subunits like Gαs and Gα12 have been suggested [41-46]. The discovery of Axin's RGS domain provided another clue indicating G proteins' potential role in Wnt signaling regulation. Additionally, through *Xenopus* embryo axis-duplication assays, it was revealed that the expression of RGS4 could impede Wnt signaling [47]. These early findings paved the way for more comprehensive investigations into the potential interactions between Wnt/Frizzled and heterotrimeric G-protein signaling pathways.

Research indicated that inducing primitive endoderm production in mouse F9 teratocarcinoma stem cells relied on canonical Wnt signaling [48, 49]. In 1999, it was reported that primitive endoderm formation in F9 cells expressing Frizzled 7 was inhibited by pertussis toxin (a Gi signaling inhibitor) or transfection with antisense oligonucleotides targeting Gαq or Gαi. Inhibitors of protein kinase C (PKC) also blocked this assay [48]. These outcomes suggested the involvement of Gαq and/or Gαi in regulating canonical Wnt signaling. Subsequently, this research group engineered a genetic construct encoding a chimeric seven-transmembrane protein receptor, merging the ligand-binding and transmembrane domains of the β2-adrenergic receptor (a GPCR) with the intracellular domains of Rat Frizzled-1 [49]. This chimeric receptor facilitated rapid activation and deactivation of Frizzled protein through a GPCR agonist, isoproterenol. Treating F9 teratocarcinoma cells expressing this chimeric receptor with isoproterenol led to β-Catenin stabilization and activation of its transcriptional activity [49]. Once more, these responses were inhibited by either pertussis toxin or antisense oligonucleotides against Gαq and

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Gαi [49], further supporting the role of these G proteins in regulating canonical Wnt signaling. Using a similar approach, it was demonstrated that Rat Frizzled-2 expression could activate cGMP phosphodiesterase in a pertussis toxin-sensitive manner [50], with suggestions of the involvement of a transducin-like Gα protein [50].

Liu et al. (2005) employed Wnt-mediated rapid dissociation of GSK-3β/Axin and stabilization of cellular β-Catenin as indicators to study canonical Wnt signaling (51). Using mouse fibroblasts, they exhibited that antisense oligonucleotides against Gαq and Gαo could impede the canonical Wnt pathway. Consistent outcomes were achieved by using GTPγ-S (a general G-protein signaling activator) in the absence of exogenous Wnt ligands. Notably, they also observed a physical interaction between Frizzled and Gαo, which could be disrupted by the addition of Wnt-3a [51]. The role of Gαo in transducing Wg signaling and the planar cell polarity (PCP) pathway was also highlighted through genetic investigations of *Drosophila* [52]. Overexpressing wild-type Gαo or an active Gαo mutant (Gαo-GTP) activated both pathways [52]. Intriguingly, the effect of wild-type Gαo depended on Frizzled, while Gαo-GTP could activate both pathways in the absence of the receptor, implying direct involvement of heterotrimeric G-proteins in regulating Wnt/Frizzled signal transduction [52].

In previous studies, we revealed that Gαq signaling positively modulates the canonical Wnt pathway, potentially through inhibiting GSK-3β enzymatic activity [33, 34]. Consequently, we proposed that the inhibition of GSK-3β might occur via PKC activation, a recognized effector of Gαq signaling [33, 34]. Numerous research teams obtained congruent findings. For instance, it was reported that Gαq-mediated inositol polyphosphate generation (notably inositol pentakisphosphate, IP5) could activate CK2 (casein kinase 2). CK2, in turn, inhibits GSK-3β, leading to the accumulation of β-Catenin and potentially culminating in β-Catenin-dependent gene transcription [53]. Utilizing F9 teratocarcinoma cells stably expressing Rat Frizzled-1, it was also demonstrated that Wnt3a treatment activated inositol polyphosphate kinases, producing IP5, possibly through Gαq-signaling-mediated activation of phospholipase Cβ1/3 [53].

Colon epithelial cell homeostasis heavily relies on the canonical Wnt/β-Catenin pathway, and as such, many colon cancer cells and tissues exhibit signs of pathway deregulation [54-60]. Analysis of GPCR expression in these cells and tissues has unveiled intriguing findings, showcasing elevated expression of specific GPCR types that preferentially couple with trimeric Gq proteins [61-64]. Gq-coupled GPCRs like protease-activated receptors 1/2 (PAR1/2), LPA2 (lysophosphatidic acid receptor 2), and metabotropic glutamate receptors exhibit higher expression levels in colon cancer cells and tissues compared to normal colon epithelium [61-64]. These outcomes raise the possibility that these GPCRs might contribute to the upregulation of Wnt/β-Catenin in colon cancer cells and tissues. Preliminary unpublished results from our research indicate that treating HT-29 colon cancer cells with certain GPCR agonists like Thrombin, Trypsin, and carbachol (activating PAR1, PAR2, and m3AcR respectively) significantly increases cytoplasmic β-Catenin protein levels and boosts β-Catenin-mediated gene transcription.

It has also been reported that heightened expression and activity of the prostaglandin E2 receptor (EP2) upregulate β-Catenin expression and function [46]. EP2, a GPCR that primarily couples to Gs,is a class of trimeric G-proteins involved in adenylate cyclase activation, cAMP increase, and

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protein kinase A (PKA) activation [26, 27, 46]. PKA can phosphorylate β-Catenin at residue S675, potentially hindering its ubiquitination and degradation [65, 66]. Another proposed mechanism suggests the interaction between Gαs and Axin RGS domain might disengage Axin from the β-Catenin destruction complex, leading to cytoplasmic β-Catenin stabilization [46]. As previously mentioned, under normal conditions, the Axin RGS domain interacts with the scaffold protein APC [41-46]. Furthermore, evidence indicates that activated Gα12/13 enhances β-Catenin signaling by interacting with E-Cadherin, facilitating the release of β-Catenin from the cell membrane into the cytoplasm [67]. It appears that Gα proteins from various classes (s, i, q, and 12/13) can regulate Wnt signaling through diverse mechanisms. It is plausible that specific combinations of heterotrimeric G proteins govern both canonical and non-canonical Wnt pathways in different cells and tissues, potentially accounting for the contextual specificity of Wnt signaling pathways.

4.1 G-proteins may regulate Wnt secretion

Wntless:*Drosophila*Wntless (or Evenness Interrupted) was discovered in 2006 [68, 69]. Disruption of Wntless in *Drosophila* produces a Wg null phenotype [68, 69]. The mammalian homolog of Wntless is GPR177, a putative G-protein-coupled receptor (GPCR) known to be involved in membrane trafficking and secretion of the Wnt glycoproteins [70]. Although Wntless/GPR177 is structurally similar to GPCRs, the possible ligands that activate it or its coupling to a trimeric G-protein have not yet been investigated, and therefore these proteins have sometimes been called orphan GPCRs [70]. The intracellular location of the Wntless amino-terminal adds one more transmembrane domain to this protein. This feature is almost unusual for GPCRs, in which the N-terminal is located extracellularly. Interestingly, like many GPCRs, Wntless can be endocytosed upon activation [71]. Homozygous deletion of Gpr177 alleles is embryonically lethal in mice due to the lack of primitive streak and mesoderm formation [70].

Porcupine: Porcupine is an eight-hydrophobic transmembrane domain-containing protein located in the endoplasmic reticulum [72-75]. Porcupine collaborates with the Wntless protein and is involved in the secretion of Wnt proteins [74, 75]. Additionally, Porcupine has o-acyl transferase activity and can add a palmitoyl group to many Wnt proteins [74, 75]. This lipid modification, which occurs at a conserved cysteine residue, not only aids in the secretion of Wnt proteins but also facilitates their interaction with Frizzled receptors [74, 75]. The topology of Porcupine is very similar to that of Wntless, and there is no evidence that Porcupine is a GPCR. Most GPCRs do not have enzymatic activity, and therefore the possible interaction between Porcupine and G-proteins remains to be further investigated.

4.2 The evidence supporting that G-proteins regulate Wnt signaling directly

Axin RGS Domain: As briefly mentioned above, the regulators of G-protein signaling (RGS) belong to a family of proteins known to enhance the GTPase activity of Gα proteins, a function similar to that of GTPase-activating proteins for the members of the RAS superfamily [39, 40, 76]. The RGS domain is a 120-amino-acid domain present in at least two groups of proteins. The first group consists of proteins named based on the function of their RGS domains, although they may also have other regulatory functions [76]. The second group comprises proteins carrying an RGS or RGS-like domain, but it is not clear whether they play a role in regulating G-protein

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signaling. The RGS domain of Axin, located in the amino-terminal region of the protein, is responsible for binding APC (adenomatous polyposis coli) [41-44]. Additionally, there is in vitro evidence that the RGS domain of Axin binds to the activated forms of Gα12 or Gαs; however, this interaction does not affect the GTPase activity of the Gα subunit [45, 46]. Since the activated Gα12 also binds to the RGS domain of p115RhoGEF (an exchange factor for Rho), it has been proposed that the binding of Axin to Gα12 inhibits Rho activation [45].

Daple: Daple is a recently identified protein that interacts with both Dishevelled and Frizzled proteins and functions as a guanine-nucleotide exchange factor (GEF) for the Gαi subunit of G-proteins [77, 78]. Daple is known as an activator of the Wnt5/Fz7-mediated signaling pathway [77]. Thus, Daple appears to be a point of interaction between G-proteins and non-canonical Wnt signaling. The presented model suggests that upon ligand stimulation (Wnt activation), Daple dissociates from Dvl and binds the Frizzled-Gαi complex, acting as a GEF for Gαi [77, 78]. Daple binds Gαi via a domain called GBA (Gαi binding and activating domain). An interesting question arises: To what extent is the activation of G-proteins by a Frizzled receptor similar to that of classical GPCRs? In classical G-protein signaling, the activated GPCR (or, in some cases, the immediate downstream effector) acts as a GEF for the Gα subunit [25, 26]. It is worth mentioning that activation of Gαi by Wnt5/Fz7/Daple signaling leads to a decrease in cellular cAMP levels and activation of the PI3-kinase/AKT pathway. These cellular activities are expected upon activation of Gi signaling by Gαi and Gβγ, respectively [77, 78]. Although much remains to be learned about Daple and its role in regulating both canonical and non-canonical Frizzled receptors, these early findings provide clear biochemical evidence for a direct interaction between Frizzled proteins and heterotrimeric G-proteins. It is also intriguing to consider whether Daple (or a similar mediator) is required for the activation of certain classical GPCRs.

Regarding the interaction with cancer, while it has been reported that Daple functions as a tumor suppressor in early colon tumorigenesis, additional results suggest that this protein is involved in tumor invasiveness at later stages [77, 78]. Elevated protein levels of Daple in tumor samples have been considered indicators of poor prognosis [78].

Lgr5: Lgr5 is a leucine-rich repeat-containing G-protein-coupled receptor known as a marker for some types of cancer stem cells, including colon cancer stem cells [79-82]. It has been shown that signals through Lgr5 potentiate the Wnt/β-Catenin pathway. Therefore, greater expression of this receptor in cancer tissues may represent higher activation of the canonicalWnt signaling in those tumors [81, 83]. Despite being known as an orphan receptor for years, further studies have revealed that the R-Spondin protein functions as a ligand for Lgr5 [84].

Additional Data: Additional laboratory results provide biochemical evidence for a direct link between heterotrimeric G-proteins and Frizzled receptors. For example, one study demonstrated that the addition of Wnt3a to the membrane fraction isolated from rat brains or cultured cells leads to the replacement of GDP with GTP on Gαo/i proteins [85]. The same research group obtained similar observations using bacterially expressed Frizzled proteins [85]. Replacement of GDP with GTP on Gαo/i proteins due to the addition of Wnt proteins was found to be inhibited by the Wnt signaling antagonist sFRP (secreted Frizzled protein), as well as by pertussis toxin [85], a bacterial enzyme that ADP-ribosylates Gαo/i proteins, thereby inhibiting the interaction

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of Gαo/i with the cognate receptors [86]. This intriguing observation suggests that at least some members of the Frizzled family and GPCRs may function very similarly to induce the activation of Gα subunits. Additionally, a recent study revealed that an agonist for the Smoothened receptor (SAG1.3) also functions as a weaker agonist for Frizzled 6. Upon binding to this receptor, it induces a conformational change and activates G-proteins from the Gi class [87]. Sequence alignments and structural analysis have suggested that the binding domain for small ligands like SAG.1 is highly similar between the two receptors [87].

Many years ago, I conducted experiments using the *Baculovirus* expression system to investigate whether the Gαq class of Gα-proteins could be directly activated by some Frizzled proteins [88]. Gαq was chosen because my earlier results had indicated that the expression of this Gα protein in *Xenopus* oocytes could inhibit GSK-3β kinase activity and induce the cellular accumulation of β-Catenin. Several recombinant *Baculoviruses* encoding Frizzled proteins, Gαq, and phospholipase Cβ were constructed. A few of them have been indicated in Figure 2. SF9 insect cells were infected with the recombinant or non-recombinant (as control) viruses, and 48 hours post-infection, the membrane fractions were isolated from different sets of cells and subjected to a GTP [α-32P]-crosslinking assay. We had successfully employed this assay previously to study GTP-binding proteins [89]. If a Frizzled protein could directly activate a trimeric G-protein (like Gq), the cells expressing that Frizzled receptor were expected to show loading of the Gαq protein with GTP. Interestingly, I found that some members of Frizzled proteins, such as *Drosophila* Frizzled-1, could significantly enhance the GTP-binding of Gαq. This suggests that at least some Frizzled receptors may activate G-proteins directly (Figure 2).

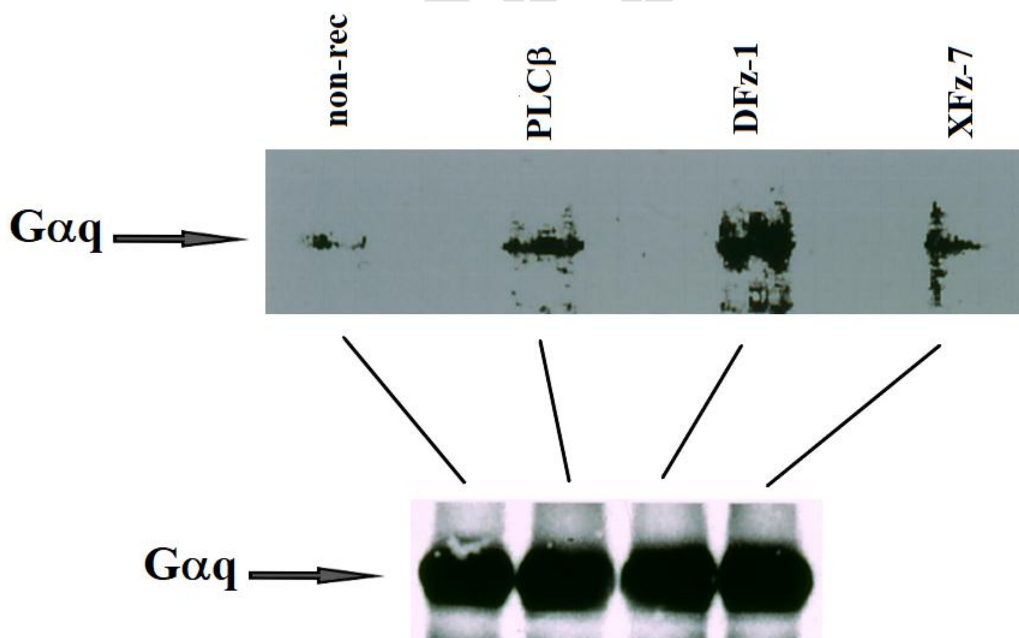

Figure 2.Frizzled receptors may activate G-proteins directly.

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SF9 insect cells were infected with non-recombinant (as control) or the recombinant *baculoviruses* encoding Phospholipase Cβ (PLCβ), *Drosophila* Frizzled-1 (DFz-1), or *Xenopus* Frizzled-7 (XFz-7). 48 hours post-infection the membrane fractions were isolated from different sets of cells and subjected to a GTP [α-^{32}P]-crosslinking assay [89].After crosslinking experiments, the samples were solubilized and used for immunoprecipitation of Gαq. The precipitated protein was divided into two equal parts and run on two similarly prepared SDS-PAGE. One gel (the top one) was dried and used for autoradiography and the second gel (the lower one) was used for western blotting to measure the amount of precipitated Gαq.

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5. The biological and clinical significance of the regulation of Wnt/Frizzled signaling by heterotrimeric G-Proteins

The Wnt/Frizzled-mediated signaling pathways are generally divided into canonical and non-canonical pathways [1-5]. While canonical Wnt signaling appears to be a single pathway mainly dependent on the regulation of β-Catenin protein stability and its nuclear activation, non-canonical Wnt/Frizzled signaling includes several pathways involved in multiple biological functions. These functions encompass the regulation of cellular and tissue polarity, gene expression, phosphoinositide signaling, and the activation of crucial protein kinases [1-5].

The mammalian genome encodes 19 different Wnts and 10 different Frizzled proteins [3, 90]. Despite almost forty years of intensive research on Wnt signaling, the specificity of signals through various Wnt/Frizzled proteins is not yet fully understood [8]. The presence of co-receptors has added another layer of complexity to this story [1-3]. Identifying the specificity of signal transduction pathways is challenging due to the complex network resulting from crosstalk among various signal transduction pathways [91].

Despite the presence of around 800 genes encoding more than one thousand G-protein-coupled receptors (GPCRs), there are only four families of Gα proteins, comprising twenty-three members [26, 27, 92]. The human genome also encodes 5 beta and 12 gamma subunits [26, 27, 92]. These limited numbers of Gα, Gβ, and Gγ subunits can generate a significant number of trimeric combinations. Coupled with a multitude of ligands originating from different sources and diverse modes of GPCR activation, the signaling through G proteins and their receptors appears to be the most complex pathways in eukaryotic cells.

Although only about 10% of discovered GPCRs have been targeted for therapy, over 30% of drugs currently available in clinics target these receptors [31, 32]. The involvement of GPCR/G-protein signaling in regulating both canonical and non-canonical Wnt pathways raises the possibility of re-evaluating some known and clinically approved drugs for targeting cancer cells. On the other hand, the pharmaceutical industry likely possesses broader knowledge for discovering and designing new drugs to modulate G-protein-coupled receptors. Additionally, numerous biological compounds derived from bacteria, fungi, protoctists, plants, and animals

have been identified to modulate GPCR/G-protein signaling pathways [93]. Some of these compounds have been considered for clinical applications, and indeed, some have already received clinical approval [93].

The role of one or several members of almost all classes of Gα proteins (Gαs, Gαi, Gαq, and Gα12/13) in regulating Wnt signaling (both canonical and non-canonical) has been reported [33-53]. However, targeting Gα proteins may significantly influence cell viability and thus result in high levels of cytotoxicity and adverse effects. Targeting GPCRs seems to be a more favorable approach. Currently, our understanding of GPCR family members regulating Wnt signaling is limited. Comparing GPCR expression profiles between cancer cells with deregulated Wnt signaling and corresponding normal cells could provide valuable insights. Subsequently, exploring the interactions of differentially expressed GPCRs with canonical or non-canonical Wnt pathways could be a promising direction. A similar study has been conducted for different subgroups of acute myeloid leukemia (AML), although the interaction between GPCRs and Wnt/Frizzled signaling was not the focus of that study. Colorectal cancer cells, known for deregulated Wnt signaling, differentially express GPCRs primarily coupling to Gαq, a pathway primarily activating phospholipase C beta [61-64]. Among these GPCRs are protease-activated receptor 1 (PAR1), PAR2, m3-muscarinic acetylcholine receptor, LPA2 (lysophosphatidic acid receptor 2), and metabotropic glutamate receptors [61-64]. Given the deregulation of Wnt signaling in many colorectal cancer cells and tissues, it remains to be demonstrated whether these GPCRs are directly or indirectly involved in this deregulation.

6. Conclusions

Aside from the roles G-proteins play in Wnt signaling regulation and potentially other pathways, deregulation of GPCR/G-protein-mediated pathways itself has been observed in various human cancers, including melanoma, colon cancer, lung cancer, basal cell carcinoma, glioblastoma, and hepatocellular carcinoma [27, 31, 32, 94-99]. Due to the involvement of GPCR/G-protein signaling in nearly all cellular functions, it is plausible that these pathways affect various aspects of cancer cell biology, such as growth, proliferation, survival, invasion, stem cell properties, and immune response [27, 31, 32, 94-99]. Deregulation of GPCR/G-protein signaling pathways in human cancers can stem from gene mutations, gene overexpression, epigenetic silencing, changes in gene copy numbers, and potentially other unknown genetic, epigenetic, and biochemical alterations. Comprehensive review articles on this critical matter are available [27, 31, 32, 94-99]. Whether deregulation of G-protein signaling in malignancies affects Wnt/Frizzled pathways in actual cancer cells and tissues is an intriguing open question that needs to be investigated case by case.

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