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Review

G-protein signaling: back to the future

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Abstract. Heterotrimeric G-proteins are intracellular partners of G-protein-coupled receptors (GPCRs). GPCRs act on inactive Gα·GDP/G$\beta\gamma$ heterotrimers to promote GDP release and GTP binding, resulting in liberation of Gα from G$\beta\gamma$. Gα·GTP and G$\beta\gamma$ target effectors including adenylyl cyclases, phospholipases and ion channels. Signaling is terminated by intrinsic GTPase activity of Gα and heterotrimer reformation – a cycle accelerated by 'regulators of G-protein signaling' (RGS proteins). Recent studies have identified several unconventional G-protein signaling pathways that diverge from this standard model. Whereas phospholipase C (PLC) β is activated by Gα_q and G$\beta\gamma$, novel PLC isoforms are regulated by both heterotrimeric and Ras-superfamily G-proteins. An *Arabidopsis* protein has been discovered containing both GPCR and RGS domains within the same protein. Most surprisingly, a receptor-independent Gα nucleotide cycle that regulates cell division has been delineated in both *Caenorhabditis elegans* and *Drosophila melanogaster*. Here, we revisit classical heterotrimeric G-protein signaling and explore these new, non-canonical G-protein signaling pathways.

Key words. Asymmetric cell division; GoLoco motif; G-protein; phospholipase C; RGS proteins.

The standard model of heterotrimeric G-protein signaling

Cellular signaling is accomplished by a myriad of proteins, peptides, lipids, ions and small molecules. Signals are commonly transmitted by the actions of hormones released from the same cell (autocrine), a neighboring cell (paracrine) or distant cells (endocrine). For example, neurotransmitter release at the synaptic cleft can propogate signals to neurons, muscle cells and neuroendocrine cells, and can also participate in autocrine feedback signals to the neuron releasing the neurotransmitter. Kinases, phosphatases, proteases and nucleotide binding proteins all contribute to the intracellular propagation of signaling. Many of these proteins alternate between an 'on' and an 'off' state to regulate the duration and intensity of the signal. Guanine nucleotide binding proteins or 'G-proteins' are among the most ubiquitous of these cellular switches and alternate between a GDP-bound off state and a GTP-bound on state.

The standard model of G-protein-coupled receptor (GPCR) signaling is outlined in figure 1. Heterotrimeric G-proteins are the intracellular partners of seven transmembrane-domain (7TM) GPCRs. Membrane-bound heterotrimers composed of Gα, Gβ and Gγ subunits are closely associated with the intracellular faces of GPCRs. GDP-bound Gα subunits bind tightly to the obligate heterodimer of G$\beta\gamma$. This association aids Gα localization to the plasma membrane (e.g. [1]; reviewed in [2]) and is essential for functional coupling to GPCRs [3]. In addition, G$\beta\gamma$ binding to GDP-bound Gα slows the spontaneous rate of GDP release, thus acting as a guanine-nucleotide dissociation inhibitor (GDI) [4, 5]. Agonist-bound GPCRs act as guanine nucleotide exchange factors (GEFs), promoting the release of bound GDP by Gα.

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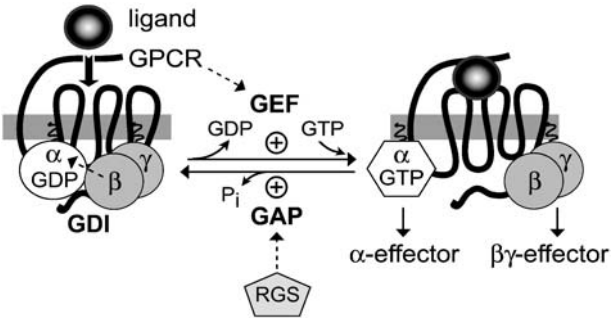

Figure 1. Standard model of the GDP/GTP cycle governing activation of heterotrimeric GPCR signaling pathways. In the absence of ligand, the Gα subunit is GDP bound and closely associated with the G$\beta\gamma$ heterodimer. This Gα·GDP/G$\beta\gamma$ heterotrimer interacts with the cytosolic loops of a seven-transmembrane-domain G-protein-coupled receptor (GPCR). G$\beta\gamma$ facilitates the coupling of Gα to receptor and also acts as a guanine nucleotide dissociation inhibitor (GDI) for Gα·GDP, slowing the spontaneous exchange of GDP for GTP. Ligand-bound GPCRs act as guanine nucleotide exchange factors (GEFs) by inducing a conformational change in the Gα subunit, allowing it to exchange GTP for GDP. G$\beta\gamma$ dissociates from Gα·GTP, and both Gα·GTP and G$\beta\gamma$ are competent to signal to their respective effectors. The cycle returns to the basal state when Gα hydrolyzes the gamma-phosphate moiety of GTP, a reaction that is augmented by GTPase-accelerating proteins (GAPs) such as the Regulator of G-protein Signaling (RGS) proteins.

Nucleotide-free Gα then binds GTP, which is present at a significant molar excess over GDP in cells. The binding of GTP results in conformational changes within the three flexible switch regions of Gα [6], resulting in the dissociation of G$\beta\gamma$. Both GTP-bound Gα and free G$\beta\gamma$ are capable of initiating signals by interacting with downstream effector proteins. The intrinsic guanosine triphosphatase (GTPase) activity of the Gα subunit causes the hydrolysis of GTP to GDP, returning the Gα subunit to its inactive state. Reassociation of G$\beta\gamma$ with Gα·GDP terminates all effector interactions [7, 8]. Thus, the standard model of GPCR signaling assumes that the Gα subunit's lifetime in the GTP-bound state controls the duration of signaling of both Gα·GTP and free G$\beta\gamma$ subunits.

G-protein subunits

The Gα subunit

There are 16 Gα genes in the human genome which encode 23 known Gα proteins. These proteins can be divided into four major classes based on sequence similarity: $G\alpha_{(s\,/\,olf)}$, $G\alpha_{(i1\,/\,i2\,/\,i3\,/\,o\,/\,t\text{-}rod\,/\,t\text{-}cone\,/\,gust\,/\,z)}$, $G\alpha_{(q\,/\,11\,/\,14\,/\,16)}$ and $G\alpha_{(12\,/\,13)}$ [9]. Gα subunits range in size from 39 to 45 kilodaltons (kDa) [10], and are N-terminally modified by the covalent attachment of the fatty acids myristate and/or palmitate. N-myristoylation of $G\alpha_i$ family members is the result of co-translational addition of the saturated 14-carbon fatty acid to the glycine at the new N-terminus of the protein following removal of the initiator methionine [11]. All Gα subunits except the photoreception-specific Gα ('transducin' or $G\alpha_t$) contain a 16-carbon palmitate reversibly attached through a thioester bond to a cysteine near the N-terminus [11]. Lipid modification of Gα subunits is important for membrane localization. Palmitoylation results in the stable attachment of Gα subunits to the membrane [12]. Myristoylation contributes to membrane localization, although expression of myristoylated but not palmitoylated $G\alpha_{i/o}$ results in the localization of a substantial portion of the Gα subunits to the cytosolic fraction [13–15]. Myristoylation and/or palmitoylation of Gα subunits affects targeting to specific cell membrane regions and regulates interactions with other proteins such as adenylyl cyclase, G$\beta\gamma$, and GPCRs [16–19].

The G$\beta\gamma$ dimer

There are 5 known human Gβ [20, 21] and 12 human Gγ subunit genes [9, 22, 23], resulting in a large number of potential combinations of G$\beta\gamma$ dimers. All Gγ subunits are C-terminally prenylated post-synthetically: Gγ1, Gγ8 and Gγ11 with a 15-carbon farnesyl moiety, and the rest with a 20-carbon geranylgeranyl group [11]. This lipid modification of the Gγ polypeptide is important for the resultant membrane localization of the G$\beta\gamma$ dimer. Most G$\beta\gamma$ combinations can form functional heterodimers [24]; however, there are exceptions; e.g. $G\beta_2$ can pair with $G\gamma_2$ but not $G\gamma_1$ [25].

Evidence supporting the role of specific G$\beta\gamma$ combinations in receptor coupling and effector activation is sparse but growing [24]. Most in vitro assays show little difference in receptor coupling profile or effector activation. However, there are some in vivo examples of the importance of specific G$\beta\gamma$ pairs for specific signaling pathways. $G\beta_1\gamma_1$ interacts more robustly with rhodopsin and phosducin than other G$\beta\gamma$ combinations [26]. Inhibition of α1H low-voltage-activated T-type ($Ca_v3.2$) calcium channels is mediated selectively by $G\beta_2\gamma_2$ [27]. $G\gamma_3$ was shown to be important for coupling the somatostatin receptor to voltage-sensitive L-type calcium channels, while $G\gamma_4$ was found to be required for coupling the muscarinic receptor to the same channels [28].

Recent studies by Robishaw and co-workers have defined specific roles for $G\gamma_7$ in coupling heterotrimeric G-proteins to receptors [29–32]. Endogenous $G\gamma_7$ expression was suppressed in HEK-293 cells using a ribozyme approach, resulting in a coincident decrease in expression of $G\beta_1$ but not $G\beta_2$ through $G\beta_5$ subunits. PGE1, muscarinic and purinergic GPCR signals were unaffected by $G\gamma_7$ knockdown; however, isoproterenol-induced adenylyl cyclase activity was abrogated suggesting a specific role for $G\gamma_7$ in β-adrenergic receptor signaling. $G\gamma_7$-knockout mice are fertile and of normal weight, but exhibit an

increased startle response. Notable are the striking changes in the striatum, with $G\alpha_{olf}$ expression reduced 82%, whereas $G\alpha_o$, $G\alpha_q$, $G\alpha_s$ and $G\alpha_{13}$ are expressed at normal levels. $G\gamma_7$-null mice also show reduced levels of D1 dopamine receptor-induced adenylyl cyclase activity in the striatum, complementing the reduced D1 receptor function found previously in $G\gamma_7$-knockdown cells.

G-protein structure

The Gα subunit

The Gα subunit (fig. 2A) is composed of two domains: a nucleotide binding domain with high structural homology to Ras-superfamily GTPases, and an all-alpha-helical domain that, in combination with the Ras-like domain, helps to form a deep pocket for binding guanine nucleotide (fig. 2B; reviewed in [33]). Gα subunits contain three flexible regions designated switch-I, -II and -III that change conformation in response to GTP binding and hydrolysis [34–38]. The GTP-bound conformation of Gα, which can be mimicked by the nonhydrolyzable GTP analogue, GTPγS, results in decreased affinity for G$\beta\gamma$, subunit dissociation and increased affinity for Gα effectors. The planar ion aluminum tetrafluoride (AlF_4^-) mimics the conformation of the terminal γ-phosphate of GTP during the transition state of GTP hydrolysis [39], and is useful for studying the in vitro interactions of Gα subunits with various regulators and effectors [40, 41].

Structural studies of Gα·GDP·AlF_4^- alone [34, 38] and in combination with RGS4 [37] have provided a better understanding of how nucleotide hydrolysis occurs. Indeed, mutations to a critical arginine (e.g. R178 in $G\alpha_{i1}$; fig. 2B) or glutamine (e.g. Q204 in $G\alpha_{i1}$), each involved in stabilizing the γ-phosphate leaving group during hydrolysis [34, 38], are commonly used to make Gα subunits GTPase-deficient and thus constitutively active (e.g. [42]). The Ras-like domain, a variation on the nucleotide-binding fold [43], adopts a conformation also seen in EF-Tu, Ras and Rap1A [44–46]. The helical domain, an insertion between the α1 helix and β2 strand of the core Ras-like domain, folds into a six-alpha-helix bundle (fig. 2A). Interactions of residues which span the domain interface are thought to be involved in receptor-mediated nucleotide exchange and subsequent G-protein dissociation [47]. Gα subunits also contain an extended N-terminal region of 26–36 residues. The first 23 residues are disordered in the structure of $G\alpha_t$ in both the GDP and the GTPγS bound state [35, 48]. Structures of the heterotrimer show that this region forms an α-helix that interacts with Gβ [36, 49]. Recent evidence from Hamm and colleagues suggests that the N-terminal myristate of the Gα subunit imparts conformational rigidity to the amino terminus of the Gα subunit and implies that the N-terminus of Gα may be highly ordered in vivo [50].

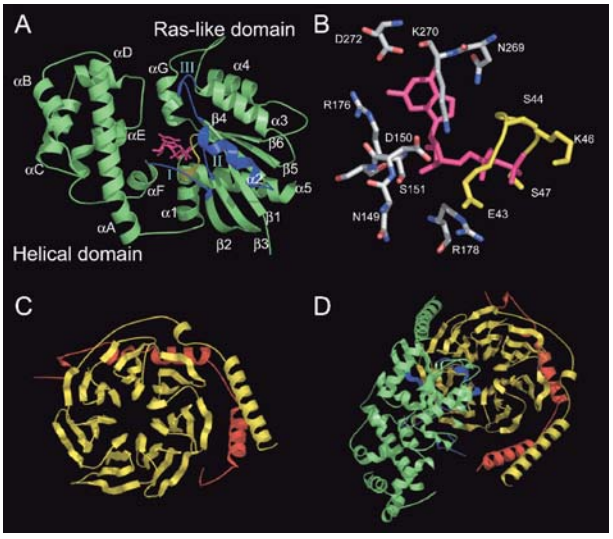

Figure 2. Structural features of heterotrimeric G-protein subunits. (*A*) The crystal structure of $G\alpha_t$·GDP·AlF_4^- (Protein Data Bank identifier: 1TAD) illustrates the Ras-like domain, the all-alpha-helical domain and the bound nucleotide at the interdomain interface. Switch regions I, II and III are shown in blue, GDP in magenta and the phosphate binding loop (P-loop) in yellow. Alpha-helices and beta-sheets are labeled according to traditional designations. (*B*) Close-up view of the guanine nucleotide binding pocket of a chimeric $G\alpha_{i/i1}$ subunit (structural coordinates from PBD ID: 1GOT). Residues that contact the guanine base, ribose sugar, and α and β phosphates are labeled. P-loop residues are shown in yellow and GDP in magenta. (*C*) The structure of the $G\beta_1\gamma_1$ dimer (PDB ID: 1TBG) shows that Gβ (yellow) forms a seven-bladed propeller consisting of seven WD40 repeats. Gγ (red) forms two alpha helices that bind to the single alpha-helix of Gβ and to several of the WD40 blades. (*D*) The crystal structure of the heterotrimer (PDB ID: 1GOT) shows that the switch regions of Gα (blue) form part of the interface for interaction with G$\beta\gamma$.

The G$\beta\gamma$ dimer

The G$\beta\gamma$ subunit is a functional heterodimer (fig. 2C, D) that forms a stable structural unit. All Gβ subunits contain seven WD-40 repeats, a tryptophan-aspartic acid sequence that repeats about every 40 amino acids and forms small antiparallel β strands [51]. Crystal structures of the G$\beta\gamma$ dimer (fig. 2C) and G$\alpha\beta\gamma$ trimer (fig. 2D) revealed that the seven WD-40 repeats of the Gβ subunit folds into a seven-bladed β-propeller or torus-like structure, while the N-terminus forms an α-helix [36, 49, 52]. Gγ folds into two α-helices; the N-terminal helix forms a coiled-coil with the α-helix of Gβ, while the C-terminal helix makes extensive contacts with the base of the Gβ torus [36, 49, 52]. Unlike the conformationally flexible Gα subunit, the G$\beta\gamma$ dimer does not change conformation when it dissociates from the G-protein heterotrimer [52]. In addition, G$\beta\gamma$ association with Gα prevents G$\beta\gamma$ from activating its effectors. These two findings suggest that the binding sites on G$\beta\gamma$ for Gα and G$\beta\gamma$ effectors are at least partially shared. In support of this hypothesis,

mutation of several residues on Gβ that contact Gα can abrogate G$\beta\gamma$-mediated phospholipase C-β_2 and adenylyl cyclase activation [7, 8]. Several groups have identified other regions of Gβ that are important for effector activation, indicating that the Gα binding site on G$\beta\gamma$ is not the only effector contact region [53, 54].

G-protein signaling pathways

Gα effectors

All four classes of Gα subunits now have well-established cellular targets. The first recognized Gα effector was adenylyl cyclase (AC), first described by Sutherland and Rall [55, 56]. Nearly 20 years after the identification of AC as an important component of intracellular signaling, a GTP binding protein that stimulated AC was isolated; it has since been termed Gα_s [57]. Shortly thereafter, Gα_i, which inhibits AC and thus opposes the action of Gα_s, was identified [58–61]. In recent years, it has become clear that the membrane-bound ACs exhibit a diverse expression pattern and respond positively or negatively to distinct sets of regulatory inputs including G$\beta\gamma$ and divalent cations [62, 63].

Gα protein signaling is also critically involved in sensory transduction. GPCRs can act as tastant and odorant receptors, coupling internally to G-proteins such as Gα_{gust} and Gα_{olf}, respectively [64, 65]. Similarly, vision is dependent on GPCR-mediated phototransduction, a unique signaling cascade that utlilizes Gα_t to regulate a cyclic GMP-gated Na^+/Ca^{2+} channel through its effector cGMP phosphodiesterase [66].

G-protein subunits of the Gα_q class (Gα_q, Gα_{11}, Gα_{14} and Gα_{16}) activate phosphoinositide-specific phospholipase C (PI-PLC) isozymes [67]. PI-PLCs hydrolyze the phosphoester bond of the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], generating the ubiquitous second messengers inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG) [67]. Regulation of PLC isozymes by heterotrimeric G-proteins is discussed in a subsequent section.

G$\alpha_{12/13}$ proteins can regulate the small G-protein RhoA via effectors that possess Dbl-homology (DH) and pleckstrin-homology (PH) domain cassettes characteristic of Rho-family guanine nucleotide exchange factors [68]. Activated Gα_{12} and Gα_{13} subunits can stimulate PDZ-RhoGEF activity [69, 70]; in common with activated Gα_q, G$\alpha_{12/13}$ subunits are also capable of stimulating the activity of leukemia-associated RhoGEF (LARG) [71, 72]. Furthermore, Gα_{13} (but not Gα_{12}) can stimulate p115RhoGEF activity [73, 74]. Each of these three RhoGEFs activate RhoA by promoting exchange of GDP for GTP which, in the case of constitutive overactivation, can result in cell transformation [75–78].

G$\beta\gamma$ effectors

The G$\beta\gamma$ dimer was once thought only to facilitate coupling of G$\alpha\beta\gamma$ heterotrimers to GPCRs and act as a Gα inhibitor given its guanine nucleotide dissociation inhibitor (GDI) activity. However, it is now known that, following dissociation of Gα·GTP, G$\beta\gamma$ is free to activate a large number of its own effectors [21, 24]. The first G$\beta\gamma$ effectors identified were the G-protein-regulated inward-rectifier K^+ channels (GIRK or $K_{ir}3$ channels) [79]. Since then, G$\beta\gamma$ has been found to bind directly to both the N- and C-termini of GIRK1-4 [80–85]. GIRK channels are synergistically activated by PtdIns(4,5)P_2, intracellular Na^+ and G$\beta\gamma$ [86, 87]. Neuronal N- and P/Q-type Ca^{2+} channels are also regulated by both Gα and G$\beta\gamma$ subunits [88–90]. A number of findings suggest that the interaction between G$\beta\gamma$ and Ca^{2+} channels is direct. For example, overexpression of G$\beta\gamma$ in various cell lines inhibits Ca^{2+} channel activity [91], while overexpression of G$\beta\gamma$ scavengers, such as the C-terminus of G-protein-coupled receptor kinase-2 (GRK2), suppresses this effect [88]. Furthermore, mutation of residues within the putative G$\beta\gamma$ binding site on the α_1 pore-forming subunit of Ca^{2+} channels eliminates channel inhibition caused by GPCR activation [91–93].

G$\beta\gamma$ subunits can also regulate kinases and small G-proteins. Activation of certain GPCRs results in G$\beta\gamma$-mediated stimulation of ERK1/2, JNK and p38 mitogen-activated protein kinases (MAPKs); a response that is inhibited by agents that sequester G$\beta\gamma$ dimers [94–97]. Phosphoinositide-3′ kinase-γ (PI3Kγ), a key signaling enzyme found downstream of GPCRs in leukocytes, is directly activated by G$\beta\gamma$ subunits [98–101]. G$\beta\gamma$ has been shown to both positively and negatively regulate various AC isoforms [102–104], activate PLC-β and PLC-ε [67, 105, 106], and localize GRK2 and GRK3 to the plasma membrane (reviewed in [107, 108]). A recent exciting finding has been the purification of a Ptdins(3,4,5)P_3-dependent Rac nucleotide exchange factor (P-Rex1) from neutrophil extracts [109]. The P-Rex1 protein serves as a coincidence detector for PI3K and G$\beta\gamma$ signaling to facilitate Rac activation [109]. Ptdins(3,4,5)P_3 produced from receptor-mediated PI3Kγ activation synergizes with receptor-mobilized free G$\beta\gamma$ to regulate Rac activation via the tandem DH/PH domains of P-Rex1 [109]. Although P-Rex1 has a PH domain (along with DH, tandem DEP, tandem PDZ and inositol phosphatase domains), its G$\beta\gamma$ interaction site has yet to be delineated.

In general, the mechanism of G$\beta\gamma$ interaction with its effectors is not entirely clear. Many, but not all, G$\beta\gamma$ effectors contain PH domains; however, not all PH domain-containing proteins interact with G$\beta\gamma$, making the prediction of all PH-domain G$\beta\gamma$ interaction sites challenging. The molecular determinants of G$\beta_1\gamma_2$ interaction with the GRK2 PH domain has recently been elu-

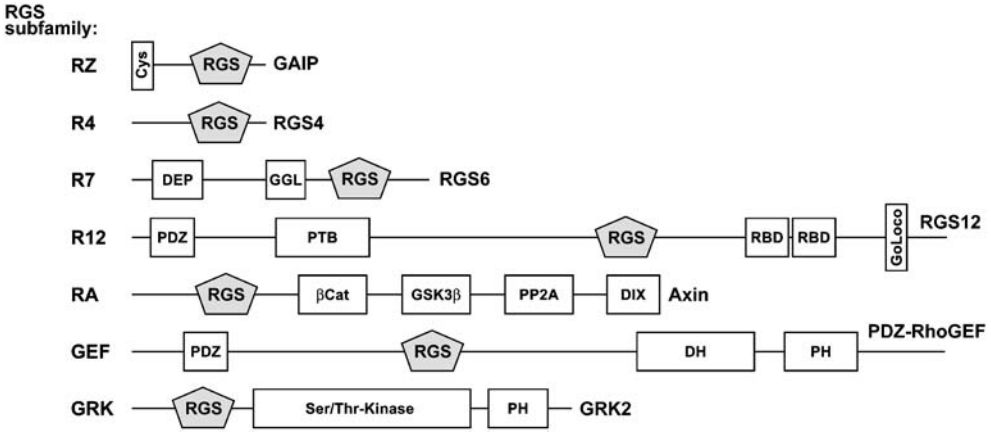

Figure 3. Schematic of the varied multi-domain architectures of RGS family proteins. RGS subfamily nomenclature follows that first established by Wilkie and Ross [318]. Abbreviations used are Cys (cysteine-rich region), RGS (Regulator of G-protein Signaling domain), DEP (Dishevelled/EGL-10/Pleckstrin homology domain), GGL (Gγ-like domain), PDZ (PSD-95/Dlg/ZO-1 homology domain), PTB (phosphotyrosine binding domain), RBD (Ras binding domain), GoLoco (G$\alpha_{i/o}$-Loco interacting motif), βCat (β-catenin binding domain), GSK3β (glycogen synthase kinase-3β binding domain), PP2A (phosphatase PP2A binding domain), DIX (domain present in Dishevelled and Axin), DH (Dbl homology domain), PH (Pleckstrin homology domain), Ser/Thr-kinase (serine-threonine kinase domain).

cidated by Tesmer and colleagues [110], and this may provide a basis for the discovery of novel PH-domain containing G$\beta\gamma$ effectors.

The G-protein signaling field is becoming increasingly populated with findings of cross-talk and integration between previously 'distinct' signaling pathways, and thus many new targets of Gα and G$\beta\gamma$ regulation are being described. In such situations, a clear distinction between 'direct' and 'indirect' effectors should be made. The test for the former should ideally include demonstrations of (i) direct interaction between homogenous purified components, but also (ii) physiologically relevant (i.e. endogenous) interaction of proposed signaling partners.

Regulation of heterotrimeric G-protein signaling

RGS domain-containing proteins

It was originally thought that the duration of heterotrimeric G-protein signaling could be modulated by only two factors: the intrinsic GTP hydrolysis rate of the Gα subunit and acceleration of that rate by certain Gα effectors such as PLC-β [111]. In 1996, several groups discovered a new family of GTPase-accelerating proteins (GAPs) for Gα proteins: the 'regulators of G-protein signaling' or RGS proteins (fig. 3) [112–114]. Each RGS protein contains a hallmark ~120 amino-acid 'RGS domain' – a nine-alpha-helix bundle which contacts the Gα switch regions, stabilizing the transition state for GTP hydrolysis [37, 41]. Many RGS proteins catalyze rapid GTP hydrolysis by isolated Gα subunits in vitro and attenuate agonist/GPCR-stimulated cellular responses in vivo [115]. Because of their GAP activity, RGS proteins are now considered key desensitizers of heterotrimeric G-protein-signaling pathways.

RGS proteins are no longer considered exclusively as desensitizing agents, but also as scaffolds that coordinate multiple components of GPCR signaling to overcome diffusional limitations and facilitate rapid, receptor-specific signal onset and termination. For example, studies of GPCR signaling to G-protein-regulated inward rectifier potassium (GIRK) channels have found that RGS1, -2, -3, -4, -5, -7 and -8 accelerate both the activation and deactivation kinetics of agonist-dependent GIRK currents without necessarily altering either current amplitudes or steady-state dose-response relationships [116–121]. Modulatory effects of RGS proteins on GPCR signaling are not easily predicted solely on the basis of RGS domain-mediated Gα GAP activity. There is an emerging view that RGS domain-containing proteins have multifaceted functions in signal transduction.

As shown in figure 3, several RGS family members contain multiple signaling and scaffolding domains. The R7 subfamily of RGS proteins, consisting of RGS6, -7, -9 and -11, have an additional domain that interacts with Gβ_5 subunits: the G-protein γ subunit-like (GGL) domain [122–125]. R7 subfamily members contain not only the GGL domain, but also a DEP (Dishevelled/EGL-10/Pleckstrin) homology domain, likely important for membrane targeting [126, 127]. RGS12 contains several protein-protein interaction domains including a PDZ (PSD95/Dlg/ZO-1) domain, which binds GPCR C-termini in vitro and a phosphotyrosine binding (PTB) domain that facilitates phosphotyrosine-dependent recruitment of RGS12 to the α1B-subunit of the N-type calcium channel [128, 129]. In addition, RGS12 and its R12 subfamily

member, RGS14, contain tandem Ras-binding domains (RBDs) [130]; in the case of RGS14, these RBDs bind the Rap subfamily of small G-proteins [131]. RGS12 and RGS14 also share a single GoLoco motif that binds $G\alpha_i$ subunits [132–134]. Members of the RhoGEF subfamily of RGS proteins couple GPCR activation to RhoA via $G\alpha$ binding to their N-terminal RGS domains and the consequent activation of the RhoA-directed GEF activity embodied in their tandem DH/PH domains [70, 71, 73, 74, 135].

GRK2 is involved in desensitization and downregulation of GPCR activation via phosphorylation of the intracellular loops and carboxy-terminus of activated GPCRs; GRK2 has also been shown to act as an effector antagonist for $G\alpha_q$ via its conserved RGS domain [136, 137]. The recent structural determination of GRK2 in complex with $G\beta_1\gamma_2$ underscores the fact that GRKs are multifaceted signaling regulators given the ability of GRK2 to directly attenuate $G\alpha_q$, $G\beta_1\gamma_2$ and GPCR signaling [110]. Mounting evidence suggest distinct modalities of regulation by GRKs may apply for different GPCRs. For instance, GRK2 regulates metabotropic glutamate receptor signaling by a kinase-independent mechanism, presumably by sequestration of both $G\alpha_q$ and $G\beta\gamma$ subunits [138, 139]. This is in stark contrast to the traditional model of regulation (such as that of the β-adrenergic receptor), in which GRK2 phosphorylates the receptor to facilitate arrestin binding, thus preventing further G-protein activation while also facilitating downstream signaling cascades [140].

Further insight into the regulatory mechanisms governing GRK2-mediated desensitization has recently been described [141]. The Raf kinase inhibitor protein (RKIP) is a physiological inhibitor of both GRK2 and Raf-1 [141, 142]: RKIP can sterically inhibit protein-protein interactions transacted by both kinases. Lorenz and co-workers observed that GPCR activation causes protein kinase-C (PKC)-mediated phosphorylation of RKIP on Ser153 [141]. Ser153 phosphorylation is sufficient to change RKIP specificity from Raf-1 to GRK2. Thus, a two-pronged facilitation of GPCR signaling can occur: PKC activity removes tonic inhibition of Raf-1 by RKIP and, simultaneously, GRK2-mediated phosphorylation and internalization of the GPCR is prevented. Hence, this is a novel mechanism of positive feedback for GPCR signaling or, potentially, cross-talk between GPCRs coupled to different effector pathways (e.g. PLC/PKC versus MAPK). Provocatively, the site of RKIP/GRK2 interaction maps to the N-terminal 185 amino acids of GRK2. This region encompasses the RGS domain of GRK2. Thus, it is tempting to speculate that RKIP binds to the GRK2 RGS domain. Defining the molecular determinants of this protein complex could shed additional light on $G\alpha$-independent protein-protein interactions mediated by RGS domains, as first identified in the case of the Axin RGS domain binding to the adenomatous polyposis coli (APC) tumor suppressor protein [143].

Novel G-protein signaling regulators

PLC-ε: a multifunctional nexus for heterotrimeric and monomeric G-protein signaling pathways

Stimulation of phosphoinositide-hydrolyzing PLC isozymes by extracellular stimuli such as neurotransmitters, hormones, chemokines, inflammatory mediators and odorants is one of the major signal transduction pathways used by cell surface receptors to mediate downstream signaling events [144]. At least five classes of PLC isozymes underlie these signals: PLC-β, PLC-γ, PLC-δ, PLC-ε and PLC-ζ (fig. 4) [67, 145]. Until recently, PLC-β was the isozyme most commonly found to be activated by GPCRs and heterotrimeric G-proteins. GPCRs activate PLC-β enzymes either via release of α-subunits of the G_q family of G-proteins [146–149] or by $G\beta\gamma$ dimers from activated G_i family members [105, 150, 151]. In contrast, PLC-γ, PLC-δ and PLC-ζ isoforms differ largely in their regulatory mechanisms. PLC-γ enzymes are regulated by receptor and non-receptor tyrosine kinases [152–154]. PLC-δ isoforms may be regulated by Ca^{2+} [155] and/or the high-molecular-weight G-protein (G_h) [67, 156]; however, the mechanisms by which PLC-δ enzymes couple to and are regulated by membrane receptors is less clear [67]. PLC-ζ, the most recently identified PLC isozyme, is reportedly responsible for sperm-mediated Ca^{2+} oscillations that occur during fertilization [145].

A novel class of PI-PLC was first revealed with the identification of the protein PLC210 in a screen for *Caenorhabditis elegans* Ras (LET-60) effectors [157]. Cloning of the full coding sequence of PLC210, the prototypical member of the PLC-ε family, identified functional domains not previously described in other PLCs. PI-PLCs generally contain a PH domain, an EF-hand domain, X and Y catalytic domains, and a C2 domain (notably PLC-ζ lacks a PH domain) (fig. 4). However, PLC210 and mammalian PLC-ε uniquely possess an N-terminal CDC25-homology domain and two C-terminal Ras-associating (RA) domains [157–160]. It is now known that upstream regulators of PLC-ε include Ras subfamily [158, 160] and Rho subfamily [161] GTPases, as well as subunits of the heterotrimeric G-protein family [106, 159]. Activation of PLC-ε by GPCRs coupled to $G\alpha$ subunits of the $G_{i/o}$, $G_{12/13}$ and G_s families has also been demonstrated, revealing that PLC-ε is yet another PLC isozyme regulated by GPCRs [162–164]. In addition to generating the second messengers $Ins(1,4,5)P_3$ and diacylglycerol, PLC-ε has also been shown to trigger other downstream signals independent of its phosphoinositide-hydrolyzing activity. PLC-ε, via

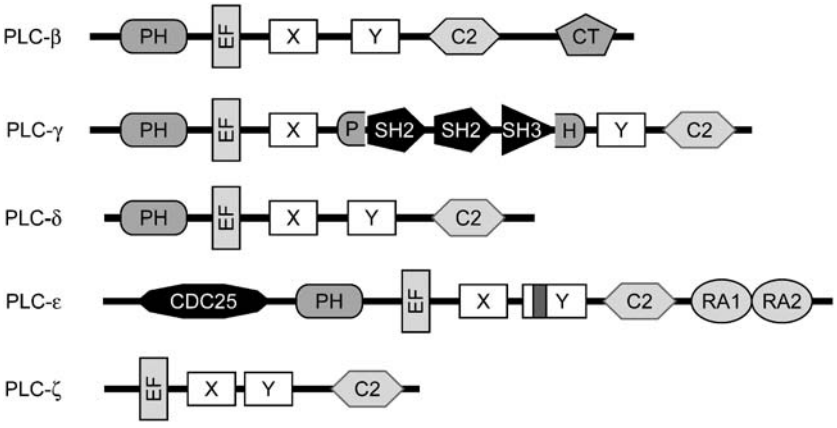

Figure 4. Domain architecture of mammalian PLC family members. Hallmarks of phosholipase C (PLC) family members are an N-terminal PH domain, which binds G$\beta\gamma$ subunits, and EF, X, Y and C2 motifs forming the catalytic core for phosphoinositide hydrolysis. PLC-β can be activated by Gα_q through a unique C-terminal (CT) domain [319], which also acts as a Gα_q GAP [111]. Unique to PLC-γ are two Src-homology-2 (SH2) domains and a Src-homology-3 (SH3) domain that bisect the PH domain. The SH2 domains confer sensitivity to stimulation by PDGF and EGF receptors, whereas the SH3 domain has been shown to act as a GEF for the phosphatidylinositol-3′ kinase (PI3K) enhancer, PIKE [320]. PLC-ε interacts with a variety of small GTPases through domains not found in other PLCs. An N-terminal CDC25 (cell division cycle protein 25-like) domain has been shown to promote guanine nucleotide exchange of Ras-family GTPases such as H-Ras and Rap1A, whereas the second Ras-associating (RA) domain (RA2) is reported to bind to H-Ras and Rap in a GTP-dependent fashion; the first RA domain (RA1) displays weak affinity for H-Ras and binds independent of nucleotide state. In addition, RhoA, RhoB and RhoC can activate PLC-ε through a unique 60–70-amino acid insert (shaded box) in the Y domain [161]; other Rho family members such as Rac1, Rac2, Rac3 and Cdc42 do not interact with PLC-ε.

the CDC25-homology domain at its amino terminus, functions as a GEF for Ras-family GTPases [159, 160, 165, 166]. In light of these findings, PLC-ε appears to be a candidate scaffold protein to integrate and mediate cross-talk between monomeric and heterotrimeric G-proteins [167].

PLC-ε contains tandem Ras-associating domains (RA1 and RA2) (fig. 4); thus, the observation that various monomeric G-proteins activate PLC-ε was not surprising. However, further examination of small GTPase activation of PLC-ε has revealed that both RA-dependent as well as RA-independent interactions can occur. Specifically, the Ras family G-proteins H-Ras, TC21, Rap1A, Rap2A and Rap2B stimulate PLC-ε in an RA2-dependent manner, whereas Ral, Rho and Rac activation of PLC-ε appears to be primarily RA independent [158, 161, 164]. The mechanism by which Ral and Rac activate PLC-ε is unknown; however, the interaction and mode of activation of PLC-ε by Rho has been elucidated [158, 161, 164, 168]. Wing and colleagues [161] identified a unique 65-amino acid insert within the catalytic core of PLC-ε, not present in other PLC isozymes, as the region within PLC-ε that imparts responsiveness to Rho. Interestingly, this region also appears to be essential for G$\alpha_{12/13}$ activation of PLC-ε. Thus, it is possible that G$\alpha_{12/13}$ activation of a Rho-GEF such as p115RhoGEF or LARG leads to activation of Rho and subsequently of PLC-ε. Heterotrimeric G-protein activation of PLC-ε by Gα_{12}, Gα_{13}, Gα_s and G$\beta\gamma$ has been demonstrated upon cellular co-transfection [106]; however, whether heterotrimeric G-protein-mediated activation requires direct interaction of these subunits with PLC-ε is unclear. Demonstration that PLC-ε activation occurs via monomeric GTPases known to be downstream of heterotrimeric G-proteins suggests that heterotrimeric G-protein-promoted PLC-ε stimulation is more likely indirect, and more closely resembles that of the novel PLC-β interactions described below.

Until recently, regulation of PLC-β isozymes by GPCRs was thought to occur primarily via direct interactions with either Gα subunits of the G_q family or G$\beta\gamma$ subunits [67]. However, the assumption that PLC-β signaling is solely regulated by heterotrimeric G-proteins was dramatically altered with the observation by Illenberger and colleagues that members of the Rho subfamily of small GTPases, specifically Rac1 and Rac2, activate PLC-β isozymes [169, 170]. This finding raises the question of how integrated regulation of these isozymes by small GTPases and heterotrimeric G-proteins occurs, and within what signaling cascades this phenomenon elicits specific cellular responses. In addition, these findings highlight the possibility that heterotrimeric G-protein activation of PLC-β isozymes might be synergistic via direct and indirect mechanisms involving G$\beta\gamma$. For instance, G$\beta\gamma$ subunits can activate Rac directly via the Rac-GEF P-Rex1 [109], as previously mentioned. Thus, it may be that in certain signaling cascades, G$\beta\gamma$ subunits from heterotrimeric G-proteins might stimulate PLC-β directly and activate a Rac-GEF such as P-Rex1 to increase Rac-GTP levels, thus activating PLC-β indirectly. Although PLC-β activation via this type of mechanism

has not been demonstrated, activation of PLC-ε by Gα_s-coupled receptors via a similar pathway has been described, as detailed below.

Schmidt and colleagues [162, 171, 172] made the observation that Gα_s-coupled receptors are capable of activating PLC-ε, and that this activation is dependent upon both heterotrimeric and monomeric G-proteins. Specifically, β_2-adrenergic-, M_3-muscarinic- and prostaglandin E_1 receptor-mediated activation of PLC-ε was reported [162, 171], with the mechanism of activation hypothesized as follows. Gα_s-coupled receptors stimulate adenylyl cyclase, which results in increased cyclic AMP levels and thus activation of the Rap-GEF EPAC (exchange protein activated by cAMP) [173, 174]. Once activated, EPAC is thought to catalyze GTP loading on Rap2B, leading to activation of PLC-ε. In addition to providing a potential mechanism by which GPCRs activate a PLC isozyme via integration of heterotrimeric and monomeric G-protein signaling, the findings of Schmidt and colleagues also provide evidence for a positive interaction between cAMP-promoted and PLC signaling pathways.

In addition to GPCR-mediated stimulation of PLC-ε, tyrosine kinase receptor-mediated regulation has been observed. Receptor tyrosine kinases such as those for the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) have been shown to activate PLC-γ enzymes by recruitment of the enzyme to the autophosphorylated receptor and subsequent tyrosine phosphorylation [67]. In contrast, the mechanism of PLC-ε activation by tyrosine kinase receptors appears to involve small GTPases. Specifically, Ras and Rap GTPases have been reported to participate in the activation of PLC-ε in a number of cell types [160, 164–166]. The mechanism of activation of PLC-ε by these GTPases appears to involve the RA2 domain, as mutations in RA2 either reduce or completely inhibit activation of the enzyme by the EGF receptor [164].

The direct contribution of PLC-γ to the activation of PLC-ε has also been examined. Song et al. found that a platelet-derived growth factor (PDGF) receptor mutant, deficient with respect to PLC-γ activation, still activates PLC-ε, via H-Ras and Rap1A as intermediaries [165]. Recently, however, Stope et al. reported that the mechanism of PLC-ε stimulation by the EGF receptor in HEK-293 cells involves not only small GTPase activation, but also PLC-γ mediated activation [175]. Specifically, the EGF receptor was identified as a 'platform' that assembles and activates two direct effectors, PLC-γ1 and the nonreceptor tyrosine kinase c-Src. Upon activation, PLC-γ1 and c-Src recruit and activates the Ca^{2+}/diacylglycerol-regulated guanine nucleotide exchange factor for Ras-like GTPases, RasGRP3, via second messenger formation and tyrosine phosphorylation, respectively. Once active, RasGRP3 catalyzes nucleotide exchange on Rap2B, inducing activation of this small GTPase. Active Rap2B then binds to PLC-ε and translocates the lipase to the plasma membrane where it can efficiently propagate signaling.

The molecular mechanisms of PLC-ε regulation have been intensively studied; however, little is known about the function of PLC-ε in physiological processes. Studies indicate that the regional and temporal expression profile of each PLC isoform may account for its physiological function [67]. For example, PLC-β1 is highly expressed in the hippocampus and cerebral cortex [176], and PLC-β1 knockout mice exhibit minor developmental abnormalities in the hippocampus and develop epilepsy [177]. To begin to understand the physiological function of PLC-ε, Kataoka and colleagues examined the spatial and temporal expression patterns of PLC-ε in the central nervous system of mouse embryos and adults [178]. The induction of PLC-ε expression appears to be associated specifically with the commitment of neuronal precursor cells to the neuronal lineage, and seems to persist after terminal differentiation into neurons [178]. In contrast to PLC-β1, which exhibits region-specific expression [176], PLC-ε expression is observed in almost all regions containing mature neurons [178]. These results suggest that PLC-ε may be involved in a more general aspect of neuronal differentiation and neuronal function than a region-specific isoform such as PLC-β1, which is critical for very selective neuronal functions such as those associated with the hippocampus. It is possible that PLC-ε, via Ras and/or Rap regulation, may have a general role in fibroblast growth factor and neurotrophic factor signaling, both of which have been implicated in neuronal development.

Recently, the physiological function of PLC-ε in the nematode *C. elegans* was addressed. *C. elegans* ovulation and fertility are regulated by an Ins(1,4,5)P_3 signaling pathway activated by the receptor tyrosine kinase LET-23 [179, 180]. PI-PLCs generate Ins(1,4,5)P_3 by catalyzing the hydrolysis of PtdIns(4,5)P_2 into Ins(1,4,5)P_3; thus, it is possible that an enzyme involved in generation of Ins(1,4,5)P_3 would also play an important regulatory role in fertility and ovulation. Kataoka and colleagues used deletion mutants of the PLC-ε gene in *C. elegans*, *plc-1*, to investigate the role of the gene in ovulation. Two deletion alleles were generated that removed regions important for the catalytic activity of PLC-ε, and both exhibited reduced fertility as a result of ovulation defects [181]. This is the first genetic analysis of PLC-ε in an intact organism, and adds further complexity to our understanding of the potential role(s) PLC-ε is playing in physiological processes. Future studies examining the cellular function and regulation of PLC-ε both in vitro and in vivo will help to merge the gap between molecular and functional analyses of PLC-ε regulation, and thus provide evidence in support of PLC-ε as a critical player in mammalian physiology.

In addition to regulation of PLC-ε, other novel forms of PLC regulation have recently been revealed. PLC-L2 is a novel PLC-like protein that is most similar to PLC-δ but lacks lipase activity due to replacement of a conserved histidine residue in the X domain [182]. PLC-L2 is expressed in hematopoietic cells, where PLC-γ1 and -γ2 play important roles downstream of the T cell receptor (TCR) and B cell receptor (BCR), respectively [183]; however, the physiological role of this catalytically inactive PLC in these cells was unknown. Recently, Takenaka et al. generated mice with a targeted deletion of the *PLC-L2* gene to examine the role of PLC-L2 in hematopoietic cell signaling [184]. When PLC-L2 is absent, B cells exhibit a hyper-reactive phenotype which strongly suggests that the physiological role of PLC-L2 is to negatively regulate BCR signaling and immune responses.

The finding that PLC-L2 negatively regulates signaling indicates that PLCs may play more complex roles in signaling cascades than originally thought. With the recent discovery of two new PLCs, PLC-ε and PLC-ζ, the physiological functions of PLCs are constantly being redefined.

Receptor-independent guanine nucleotide exchange factors

In the past few years there have been several reports of receptor-independent activators of G-protein signaling. Cismowski and colleagues used a yeast-based screen to identify potential receptor-independent activators of heterotrimeric G-protein signaling [185]. One gene isolated in this screen was Dexras1 (renamed by Cismowski et al. as AGS1), a previously described dexamethasone-inducible Ras-family GTPase [186]. Dexras1 has been characterized as a putative GEF for Gα_i subunits [187] and can regulate heterotrimeric G-protein signaling pathways under certain circumstances [188, 189]. Intriguingly, Dexras1 was also shown to be activated by N-methyl-D-aspartate (NMDA) receptor-dependent nitric oxide (NO) production in vitro and in vivo [190]. A recent study has further characterized Dexras1 as a regulator of the circadian clock via NMDA and Gα_i dependent pathways [191]. Dexras1 is highly expressed in the suprachaismatic nucleus, the 'pacemaking' centre of circadian rhythm control [192]. Mice deficient in Dexras1 are abnormal in both photic (NMDA-dependent) and non-photic (neuropeptide Y-dependent) responses, probably via modulation of Gα_i signaling pathways [191].

Recently Tall and colleagues identified mammalian Ric-8A (Synembryn) as a receptor-independent GEF for G$\alpha_{q/i/o}$, but not Gα_s, using in vitro assays [193]. The inability of Ric-8A to activate Gα subunits bound within heterotrimeric G$\alpha\beta\gamma$ complexes has led to the hypothesis that Ric-8 proteins may act as signal amplifiers following initial heterotrimer activation by GPCRs [193]. More recently, we have shown that Ric-8 plays a fundamental role in regulating G-protein signaling during *C. elegans* asymmetric cell division in embryogenesis (discussed in detail below).

GoLoco motif-containing proteins

In a genetic screen in *Drosophila* to discover glial cell-specific targets of the transcription factor *pointed*, Granderath and colleagues cloned *Drosophila loco* (for 'locomotion defects'), the fly orthologue to *RGS12* [194]. This group also identified a Gα-interacting region in Loco that was distinct from the RGS domain. Using this information and the sequences of other described Gα-interacting proteins, we discovered a conserved 19-amino acid sequence motif, dubbed the G$\alpha_{i/o}$-Loco or GoLoco motif, that is present in Loco, RGS12, RGS14 and many other metazoan proteins [195]. A similar in silico discovery was independently made by Ponting [130], and GoLoco motifs have also been referred to as G-protein regulatory (GPR) motifs [196]. The GoLoco motif/Gα interaction is generally selective for Gα_i subunits in their GDP-bound form; the interaction results in a slowing of the spontaneous GDP release by Gα. The molecular determinants of GoLoco motif-mediated GDI activity [133], as well as the putative roles of these proteins as regulators of GPCR signaling and cell division processes are discussed at length in our recent review [134].

GoLoco motifs, either individually or in tandem repeats, have been discovered within several diverse proteins (fig. 5) including *C. elegans* GPR-1/2 [197–199], *Drosophila* Pins [200, 201] and the mammalian proteins Purkinje cell protein-2 (Pcp-2) [202, 203], Rap1GAPII [204, 205], G18 [206, 207], LGN [208–210] and AGS3 [196, 211, 212]. As many of the GoLoco motif-containing proteins have two or more names, the Human Genome Organization (HUGO http://www.gene.ucl.ac.uk/nomenclature/) has reclassified some of the human GoLoco motif proteins using a standardized nomenclature: AGS3 is now called G-protein signaling modulator-1 (GPSM1), LGN (also known as mammalian Pins; [213]) is called GPSM2, G18 (also called NG1 and AGS4) is now named GPSM3, and Pcp-2 (a.k.a. L7) is now GPSM4.

G-protein signaling in model organisms

Many eukaryotic organisms employ heterotrimeric G-proteins for signal processing and homeostasis. For instance, the budding yeast *Saccharomyces cerevisiae* responds to peptide 'pheromones', a-factor and α-factor, to accomplish mating (haploid cell fusion). Pheromone signaling in yeast is propagated and regulated via GPCRs, heterotrimeric G-proteins and a G$\beta\gamma$-mediated

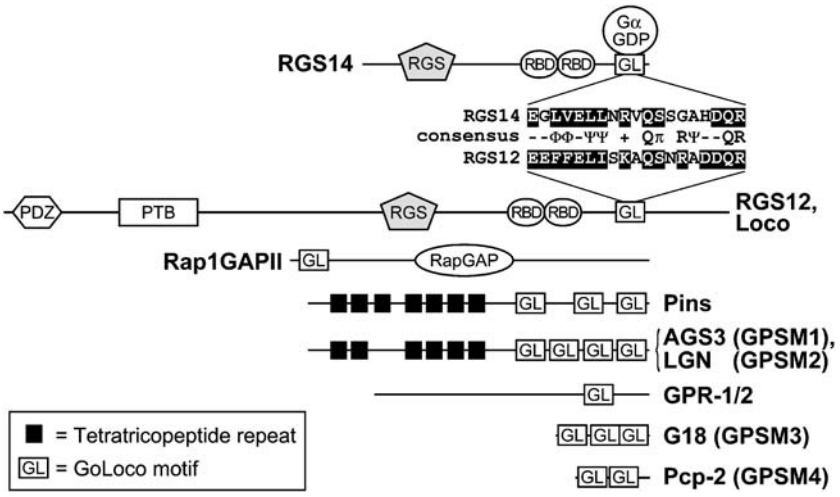

Figure 5. The 19-amino acid GoLoco motif is found in a diverse set of signaling regulatory proteins. Domain organization of single- and multi-GoLoco motif-containing proteins is illustrated. Abbreviations used are RGS (Regulator of G-protein Signaling domain), RBD (Ras binding domain), GoLoco or GL ($G\alpha_{i/o}$-Loco interacting motif), PDZ (PSD-95/Dlg/ZO-1 homology domain), PTB (phosphotyrosine binding domain), RapGAP (Rap-specific GTPase-activating protein domain), GPSM (G-protein-signaling modulator).

MAPK pathway [214]. The study of this system in yeast has provided enormous insight into G-protein-linked systems in mammals, mainly due to the tractability of the yeast system to genetic manipulation. For instance, the archetypal yeast RGS protein Sst2 was isolated in a genetic screen as a negative regulator of pheromone signaling [215], 14 years before the first functional identification of a mammalian RGS protein [112, 114]. Likewise, the slime mould *Dictyostelium discoideum* responds to bacterially secreted extracellular cyclic AMP (cAMP) [216] by chemotaxis and phagocytosis of the bacteria. This process is transacted by a canonical heterotrimeric G-protein signaling system and is akin to chemotactic and phagocytic processes in mammalian leukocytes [217]. This experimental system has provided superlative information about the cell biological mechanisms of directional sensing, polarization, cell motility and lipid metabolism controlled by G-protein-coupled pathways. The particulars of non-conventional G-protein signaling in *Drosophila* and *C. elegans* are discussed elsewhere in this review, while the genetic dissection of mammalian G-protein signaling via gene inactivation studies has recently been thoroughly reviewed in the literature [218].

A GPCR-RGS protein in plants?

An enigmatic, but potentially very enlightening, example of G-protein signaling exists in the *Plantae* model organism *Arabidopsis thaliana*. Heterotrimeric G-protein signaling in *Arabidopsis* controls both cell proliferation [219] and inhibition of stomatal opening by abscisic acid (via inhibition of guard cell inwardly rectifying K^+ channels) [220]. The *Arabidopsis* G-protein signaling repertoire contains an unusually restricted set of elements. At present only one prototypical $G\alpha$ subunit (AtGPA1), one $G\beta$ subunit (AGB1) and two $G\gamma$ subunits (AGG1 and AGG2) have been described [221]. Metazoan systems typically have hundreds to thousands of GPCRs, 10–20 $G\alpha$ subunits, 2–5 $G\beta$ subunits and 2–12 $G\gamma$ subunits. Intriguingly, no definitive report of either an *Arabidopsis* GPCR or a direct effector of AtGPA1 has been made, although candidates have been identified [222, 223]. Similarly, until recently, no RGS protein nor GAP of any kind for $G\alpha$ had been identified in *Arabidopsis*. We discovered the first plant RGS protein, subsequently named AtRGS1, as an anonymous open-reading frame with homology to mammalian RGS domains [224]. Provocatively, AtRGS1 contains an N-terminal region with the predicted topology and structure of a GPCR (fig. 6) [225], bringing forth the possibility that AtRGS1 is a conjoint guanine nucleotide exchange factor and GTPase-accelerating protein for AtGPA1. The RGS domain of AtRGS1 acts as a potent GAP for AtGPA1 in vitro [224, 225]. In vivo, the phenotype of AtRGS1 ablation or overexpression is consistent with the role of AtRGS1 as a negative regulator of AtGPA1 signaling, based on analyses of cell proliferation in the apical root meristem [224]. Currently, no evidence exists as to whether the 7TM component of AtRGS1 is a guanine nucleotide exchange factor for AtGPA1, and the identity of potential ligands remains elusive, although a sugar is a most likely candidate [226].

The ultimate receptor-selective RGS protein?

Evidence from mammalian systems has brought forth the hypothesis that RGS protein regulation of $G\alpha$ signaling can be 'receptor selective' [227–229]. For instance, RGS1 is a 1000-fold more potent inhibitor of muscarinic-

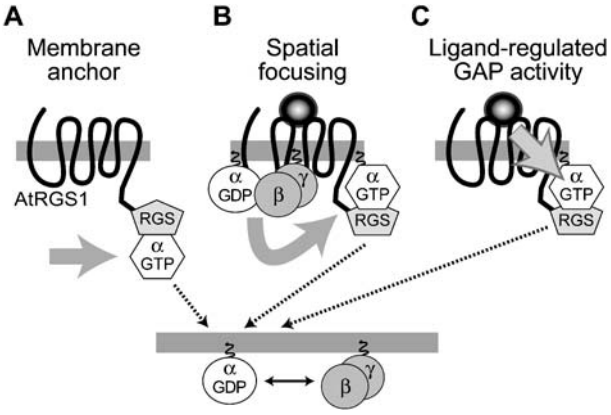

Figure 6. Proposed models of AtRGS1 signaling interactions. (*A*) In the 'Membrane anchor' scenario, AtRGS1 acts to recruit (grey arrow) activated AtGPA1 to specific membrane microdomains, allowing localized signaling and deactivation by AtRGS1. (*B*) In the 'Spatial focusing' model, AtRGS1 is a ligand-activated GPCR that coordinately catalyzes both guanine nucleotide exchange on the *Arabidopsis* heterotrimer (grey arrow) and GTP hydrolysis by the activated Gα subunit (dotted arrow). (*C*) In the third model, AtRGS1 is a ligand-regulated GAP: i.e. ligand-mediated agonism or inverse agonism regulates deactivation of AtGPA1·GTP via AtRGS1 RGS domain GAP activity.

versus cholecystokinin-receptor stimulated Ca^{2+} mobilization in pancreatic acinar cells; this is despite the receptor signaling pathways to $G\alpha_q$ being apparently indistinguishable [228]. The simplest mechanism for receptor selectivity would be direct interaction between GPCRs and RGS proteins. Alluring evidence supports the notion that some RGS proteins may be present in signaling complexes with GPCRs; for instance, the PDZ (PSD-95/Dlg/ZO-1 homology) domain of RGS12 directly interacts in vitro with peptides corresponding to the C-terminus of the interleukin-8 receptor B (CXCR2) [129]. In a similar fashion, a GST-fusion protein of the third intracellular loop of the M_1-muscarinic acetylcholine receptor can co-precipitate with ectopically expressed or purified recombinant RGS2 but not other RGS proteins [230], and this correlates with the high potency of RGS2 inhibition of M_1 versus other muscarinic acetylcholine receptor signaling. However, it is important to note that no cellular, nor even in vitro, interaction between a full-length GPCR and a full-length RGS protein has yet been described.

Thus in the *Arabidopsis* G-protein signaling paradigm, it may be that AtRGS1 is the archetypal example of receptor selectivity by RGS proteins. By having conjoint GEF and GAP activities, the AtRGS1 protein potentially forms a precisely controlled and localized signaling complex: the so-called 'spatial focusing' hypothesis (fig. 6). Thus the concept has evolved that receptor selectivity of RGS proteins determines functional signaling outcomes, and evidence now exists that RGS proteins, first identified as negative regulators of GPCR signaling, may actually facilitate signal tranduction by 'spatial focusing', as outlined by Neubig and colleagues [231]. This concept stems from demonstrations that RGS proteins can positively (as well as negatively) modulate GIRK channels. RGS proteins can accelerate both the activation and deactivation kinetics of GIRK channels without altering the current amplitude or dose-response relationship to agonist application [116, 117]. Similarly, in the presence of GTP, RGS proteins can potentiate receptor-mediated GTPγS binding by Gα subunits [231]. Thus, RGS proteins may add a level of selectivity to GPCR action by permitting effector activation exclusively within the proximity of the GPCR while providing (via GAP activity) a constant supply of Gα·GDP for continued GPCR coupling [231].

Fat-free RGS protein membrane association

An alternative explanation for the domain structure of AtRGS1 is that the N-terminal 7TM segment acts solely as a membrane anchor for the C-terminal RGS domain. AtGPA1 is predicted to be N-terminally myristoylated and, therefore, plasma membrane localized. Thus, forced membrane localization will enhance interaction between the cognate Gα and RGS domain pair. It is important to note that no mammalian RGS proteins have demonstrable transmembrane domains, although the RGS domain-containing sorting nexins, SNX13, -14 and -25, are reported to have one or two potential transmembrane-spanning sequences [232]. However, it has been established that phospholipid binding by RGS domains [233–235] and palmitoylation of RGS domains [236, 237] each can negatively affect the ability of RGS domains to serve as GAPs for Gα subunits. Thus, it appears that interactions between lipids and RGS domains may be intimately linked to physiological function [238], and independent methods to evoke the membrane localization of RGS proteins may have evolved in plants versus mammals. In mammalian cells, the membrane translocation of RGS proteins can be induced by GPCRs [239] and constitutively activated Gα subunits [240]; however, recent evidence suggests that significant differences exist between endogenous and ectopically overexpressed RGS proteins [241]. This can include the mislocalization, mistranslation and altered half-life of RGS proteins. Thus, the physiological relevance of the transcription and localization of ectopically expressed RGS proteins needs to be carefully evaluated.

Turning on the off signal?

An alternative, and provocative, hypothesis to explain the convergence of seven transmembrane and RGS domains in the same polypeptide is that AtRGS1 is a ligand-regulated GAP for AtGPA1 (fig. 6) whereby a soluble ligand serves to activate (agonist), or to repress (inverse agonist), AtRGS1 GAP activity. The kinetic parameters

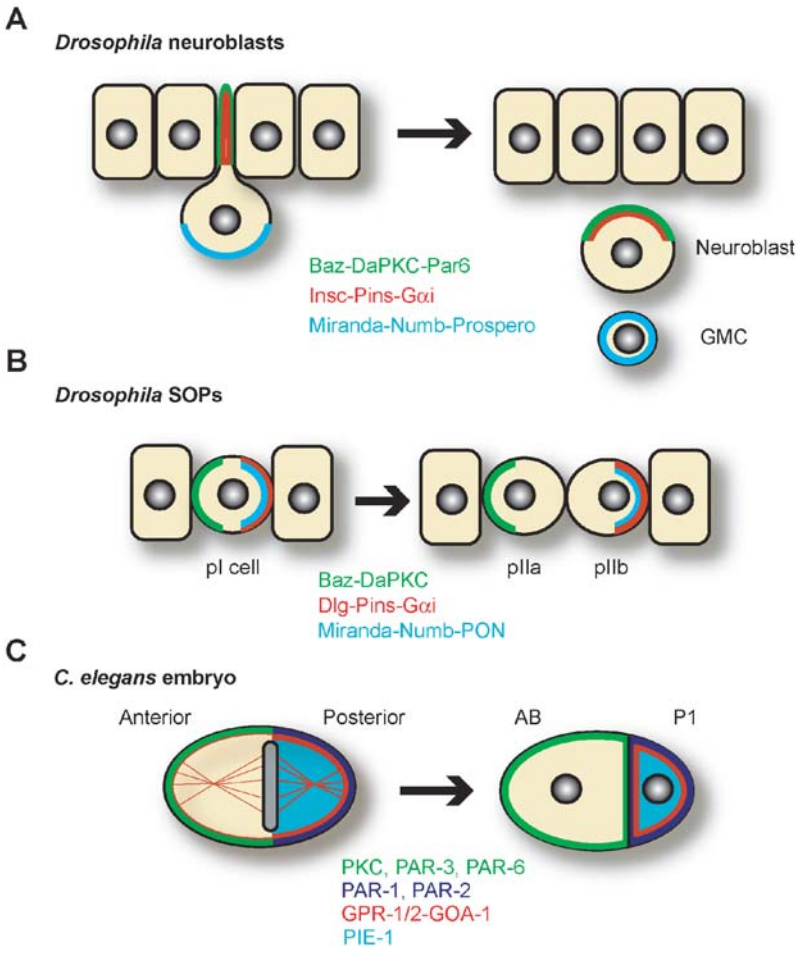

Figure 7. Models of asymmetric cell division in *Drosophila* and *C. elegans*. (A) In delaminating neuroblasts, two apical complexes (Bazooka [Baz], atypical protein kinase C [DaPKC] and Par6; Inscuteable [Insc], Partner of Inscuteable [Pins] and Gαi) facilitate the localization of cell-fate determinants to the basal lateral membrane and the orientation of the mitotic spindle. (*B*) In sensory precursor (SOP) cells, planar polarity is established by counteracting complexes of Baz-DaPKC towards the posterior and Discs Large (Dlg)-Pins-Gαi towards the anterior. (*C*) In *C. elegans* one-cell zygotes, PAR-1/-2 proteins enrich GPR-1/2-GOA-1 complex localization towards the posterior, resulting in greater astral microtubule pulling forces on the posterior spindle pole and a resultant smaller P1 daughter cell.

of the *Arabidopsis* G-protein cycle support this scenario, given that AtGPA1 has a rapid nucleotide exchange rate but slow intrinsic GTP hydrolysis activity [225]. Unfortunately, for a definitive answer to these questions, a ligand for AtRGS1 needs to be discovered. Deorphaning putative GPCRs is inherently problematic [242]. Despite massive effort, a wealth of knowledge about mammalian signal transduction, and a broad range of techniques to measure well-characterized G-protein effector systems, only a small quotient of orphan GPCRs have had ligands identified for them [242]. Accordingly, more information needs to be ascertained about AtGPA1 signaling through the use of genetic and biochemical approaches. The biochemical characterization of direct effectors, such as the putative AtGPA1 effector phospholipase-D α1 (PLDα1) [223], and the creation of robust cell biological assays, is a necessity for any deorphaning effort. Conversely, genetic studies may serve to elucidate a ligand for AtRGS1. To this end, a recent report suggests that sphingosine 1-phosphate (S1P) is a potential plant-GPCR ligand. Abscisic acid inhibits stomatal opening caused by the activation of sphingosine kinase and, consequently, the production of S1P [243]. In mammalian systems, S1P is a well-described intracellular and extracellular messenger that activates a large family of GPCRs [244]. Thus it is possible that S1P is a ligand for AtRGS1 or other candidate plant GPCRs. Future studies should be directed towards fully elucidating the signaling components of this pathway. The potential for a novel receptor-activated GAP activity to occur in vivo seems highly likely as plants are the only sessile organisms known to utilize heterotrimeric G-protein signaling. Thus their mechanisms of signal perception and response to environmental conditions are likely to be dramatically different than that of the paradigmatic model organisms used to study G-protein signal transduction.

A novel role for heterotrimeric G-protein subunits in mitotic spindle force generation and asymmetric cell division

A non-canonical G-protein cycle is emerging from studies of asymmetric cell division. Asymmetric cell division (ACD) is a mechanism, used by metazoan organisms to create cellular diversity, in which two unique daughter cells are generated from a single precursor. In this process, cell-fate determinants are localized to one pole or another and the mitotic spindle is orientated such that, upon division, these cell-fate determinants are asymmetrically partitioned. Heterotrimeric G-proteins are associated with protein complexes that control cell polarity, and play an integral role in mitotic spindle pulling force generation (reviewed in [134, 245–247]). The fruit fly *D. melanogaster* and the nematode worm *C. elegans* are two model organisms commonly used for the study of asymmetric cell division. Both delaminating neuroblasts and sensory organ precursors in *Drosophila,* and the *C. elegans* early embryo, utilize a similar set of proteins to control polarity and spindle pulling forces (fig. 7A–C). The following sections detail the roles of heterotrimeric G-proteins in these two model systems and reviews what is known of related proteins in mammals.

Asymmetric cell division in *Drosophila*

Delaminating neuroblasts

In the *Drosophila* embryo, the central nervous system is derived from epithelial neuroprogenitor cells or 'neuroblasts' that divide asymmetrically into a smaller ganglion mother cells (GMCs) and larger neuroblasts (fig. 7A) [248]. After division, daughter GMCs terminally differentiate into neurons, whereas daughter neuroblasts retain their neural pluripotency. Neuroblast ACD is an intricate process that begins with delamination of cells from the neuroectoderm, followed by establishment of apical-basolateral cell polarity and localization of cell-fate proteins, and finally orientation of the mitotic spindle for division.

Cell-fate determinants Miranda, Prospero and Numb are localized at the basolateral membrane of the dividing neuroblast where they segregate into the smaller GMCs. Prospero is a transcription factor that activates GMC-specific genes and inhibits neuroblast-specific genes [249–253]. *prospero* RNA is asymmetrically localized by Staufen, an RNA-binding protein [254–256]. The cortical localization of both Staufen and Prospero during mitosis are in turn controlled by the coiled-coil protein Miranda [257, 258]. Finally, the cell-fate determinant Numb, which is localized by partner of numb (PON), inhibits Notch signaling after the first division by polarizing the distribution of α-adaptin, resulting in enhanced endocytosis of Notch at one pole [259–262].

Orientation of cell polarity and mitotic spindle positioning for proper segregation of these cell-fate determinants requires a network of proteins that localize to the apical membrane at the beginning of mitosis as neuroblasts delaminate. At the apical membrane, a complex of Gαi, Partner of Inscuteable (Pins), *Drosophila* atypical protein kinase C (DaPKC), *Drosophila* partitioning defect protein 6 (DmPAR6), Bazooka (Baz) and Inscuteable establish polarity cues and the axis of division. Inscuteable, a key player in this apical complex, is required for proper spindle orientation and localization of cell-fate determinants [263, 264]. Inscuteable binds to both Pins [201] and Bazooka [265], serving as the linchpin between Pins/Gαi and Baz/DaPKC/DmPAR6 complexes (discussed below). Binding to both Baz and Pins occurs through a central asymmetry domain [265, 266] comprising a series of putative Armadillo repeats.

Pins is a multi-domain protein consisting of seven tetratricopeptide repeats (TPRs) at its N-terminus and three GoLoco motifs at its C-terminus (fig. 5). Consistent with other GoLoco motif proteins, Pins binds the GDP-bound form of *Drosophila* Gαi, and the addition of GTPγS to neuroblast lysates strongly inhibits coimmunoprecipitation of Gαi with Pins [267]. While a biochemical analysis of Pins has not been performed, closely related mammalian GoLoco motif proteins such as GPSM2 (LGN) and GPSM1 (AGS3) display GDI activity [209, 212, 268]. It appears that Gαi is the physiologically relevant G-protein, as Gαo is not detected in complex with Pins [267], and in general, the majority of GoLoco proteins display a strong preference for $G\alpha_i$ over $G\alpha_o$ subunits. Loss of Pins, Inscuteable or other apical components results in an increased rate of spindle misorientation and loss or mislocalization of cell-fate determinants. However, none of these result in the loss of asymmetric division. Partial recovery, known as telophase rescue, can occur in some cells lacking individual components of the apical cortex complex [269, 270]. This may be explained by recent studies suggesting that the two complexes (Baz/DaPKC/Par6 and Pins/Gαi) are at least partially redundant. While mutation of a single component results in spindle mislocalization, simultaneous mutation of components in both complexes results in symmetric division as well as the characteristic spindle misorientation and cell-fate determinant mislocalization [270]. Genetic studies by Izumi and colleagues suggest that the two complexes perform both overlapping and unique roles, where Bazooka localizes Miranda and partially contributes to asymmetry, and Pins/Gαi orients the spindle and contributes to asymmetry [271]. The redundancy of the system suggests that asymmetry and spindle position are extremely important for viability.

The Gβ subunit Gβ13F is also involved in regulating asymmetric cell division in *Drosophila* neuroblasts. Unlike Gαi, Gβ13F has a uniform cortical distribution

[267]. Either elimination of Gβ13F or overexpression of Gαi results in symmetric division [267, 272]. Until recently, it was unknown how Gα- and Gβ-subunits individually contributed to asymmetric division, as RNA-interference (RNAi) of *Gβ13F* expression also resulted in a concomitant loss of Gα. To ameliorate this, Yu et al. used Gαi mutants to establish the relative roles of Gαi and Gβ13F [273]. Gαi mutants demonstrate similar phenotypes to those of Baz, Pins, DaPKC or Inscuteable mutants, where a fraction of cells still undergo asymmetric division. However, loss of Gβ expression results in near complete (96%) loss of asymmetric division, similar to mutations to both the Gαi/Pins and Baz/DaPKC pathways, leading to the proposal that Gβ13F acts upstream of the other components [273]. Cell-fate determinants are still localized accurately in *Gβ* and *Gγ* double mutants, suggesting that the G$\beta\gamma$ dimer is primarily involved in spindle positioning rather than determinant localization. Furthermore, an increase in either *Gβ* or *Gγ* expression results in small spindles, while a decrease in *Gβ* expression results in large symmetric spindles [272]. Given the uniform cortical expression of G$\beta\gamma$, additional regulation by apical components must control G$\beta\gamma$ subunits in order for an asymmetric spindle to form [274]. The precise nature of the hierarchy between individual apical membrane complex components and G$\beta\gamma$ subunits remains to be elucidated.

Sensory organ precursor cells

A contrasting example of heterotrimeric G-protein signaling in the context of spindle positioning is found in *Drosophila* sensory organ precursor (SOP) cells (fig. 7B). Parts of the peripheral nervous system in *Drosophila* are derived from SOP cells [275], and involve Gαi- and G$\beta\gamma$-subunit control of spindle positioning and cell-fate determinant localization [267]. Spindle orientation and cell-fate determinants in neuroblasts are oriented around an apical-basolateral axis, whereas SOP cells exhibit planar polarity along the anterior-posterior axis. In this system, the pI (primary precursor) cell divides asymmetrically into an anterior pIIa, which inherits cell-fate determinants such as Numb and PON, and a posterior pIIb [276, 277]. In contrast to neuroblasts, SOP cells have two counteracting complexes, (i) Gαi/Pins towards the anterior and (ii) DmPar6/DaPKC/Baz towards the posterior [278]. Furthermore, Inscuteable is not expressed in pI cells [279], and its ectopic expression reverses polarity, bringing Bazooka into the anterior complex with Pins, such that cell-fate determinants such as Numb mislocalize to the posterior [280]. Division of SOP cells is asymmetric only in the context of cell-fate determinant distribution to daughter cells, as the cells and spindle sizes are equal. Asymmetric division only occurs in these cells during simultaneous loss of components from each of the Gαi/Pins and DaPKC/Baz/DmPar6 complexes, a situation mimicked by loss of Gβ subunit function [278]. Finally, in another contrast to the neuroblast, expression of the constitutively-active Gαi^{Q205L} mutant perturbs spindle orientation in SOP cells [267], suggesting that there may be some GPCR-mediated signal in this context (i.e. generation of active Gαi·GTP). To this end, the Frizzled receptor, proposed to be a de facto GPCR [281], modulates spindle rotation and polarity in the pI to pIIa/b division [276, 282].

Asymmetric cell division in *C. elegans*

In *Caenorhabditis elegans* embryos, the first division is asymmetric (fig. 7C) [283–285]. The zygote divides into a larger AB anterior cell and a smaller P1 posterior cell. Polarity is established by the sperm at fertilization [285], and as with *Drosophila* neuroblasts, spindle positioning and the expression and localization of cell-fate determinants are coordinated by a complex array of proteins. At the top of the hierarchy are the PAR (Partitioning defective) proteins, a group of structurally unrelated proteins isolated in a screen for regulators of asymmetric cell division [286]. There are six PAR proteins, which, in combination with atypical protein kinase C-3 (aPKC-3) and the small G-protein Cdc42, establish the anterior-posterior axis of cell polarity. PAR-3/-6 and aPKC-3 localize to the anterior cortex [287–289], while PAR-1/-2 define the posterior end [290, 291]. Mutation of any of the PAR proteins or aPKC results in symmetric division [283, 286, 289, 292].

As previously discussed, heterotrimeric G-protein subunits and modulators such as Pins are directly involved in establishing cell polarity in *Drosophila* ACD systems. In contrast, in the *C. elegans* zygote, G-protein subunits, GoLoco proteins and other modulators appear to act downstream of polarity determinants (such as aPKC-3 and the PAR proteins) in positioning the mitotic spindle and regulating pulling forces on this spindle during the first zygotic division. There are four G-protein subunits relevant to asymmetric cell division in *C. elegans*: GPA-16 and GOA-1 are Gα subunits (most similar to mammalian Gα_i and Gα_o, respectively) and GPB-1 and GPC-2 are Gβ and Gγ subunits, respectively. Concurrent inactivation of GOA-1 and GPA-16 leads to a loss of asymmetric pulling force (fig. 8), causing daughter cells to be the same size [291]; loss-of-function mutations or RNAi of either *gpb-1* or *gpc-2* results in improper centrosome rotation, leading to spindle misorientation [291, 293]. The hierarchy of PAR proteins being upstream of G-protein subunit involvement is confirmed by the lack of any defect in the localization of PAR proteins or cell-fate determinants in response to reduction of Gα expression [197, 291].

A functional genomic screen by Gönczy and colleagues identified the single GoLoco motif-containing proteins

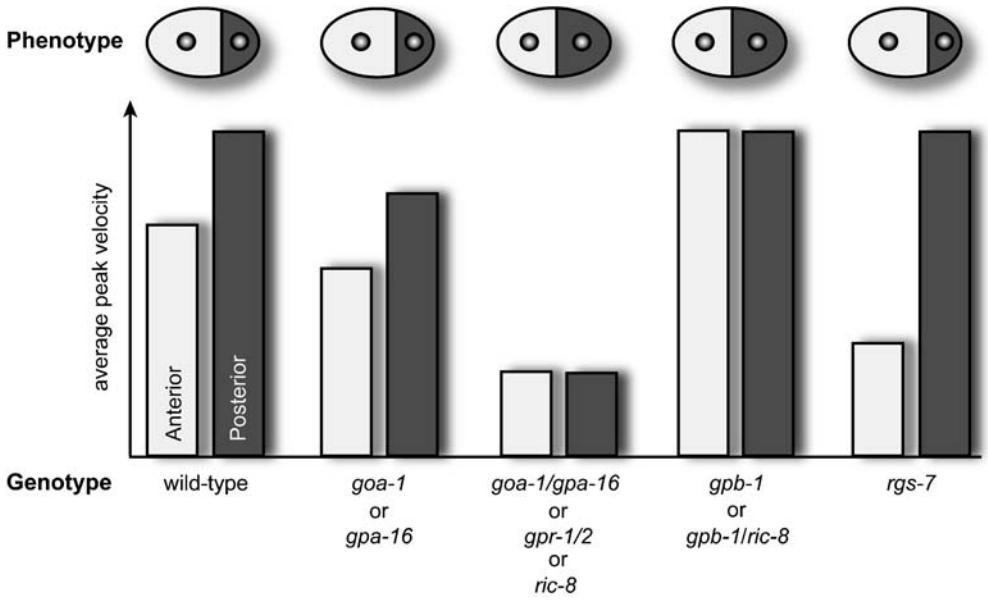

Figure 8. Phenotypes and relative spindle pulling forces of *C. elegans* embryos in various genetic backgrounds. In wild-type embryos, posterior enrichment of Gα and GPR-1/2 are associated with stronger posterior pulling forces resulting in asymmetric division (light grey, AB daughter cell; dark grey, P1 daughter cell). Loss-of-function mutation or RNAi of either *goa-1* or *gpa-16* Gα subunit leads to reduction in force magnitude and force asymmetry, but no change in the overall asymmetry of the cell division [294]. Mutation or RNAi of both G-protein subunits, both GoLoco motif proteins *gpr-1/2* or the receptor-independent Gα GEF *ric-8* causes symmetric division due to loss or mislocalization of pulling force generators. Simultaneous loss of *ric-8* and *gpb-1* leads to an enhancement of anterior pulling forces indistinguishable from *gpb-1* RNAi alone [294]. In contrast, *rgs-7* mutants display reduced anterior pulling forces, resulting in exaggerated asymmetry and a smaller P1 cell [305]. In all cases, pulling forces were determined by laser ablation of central mitotic spindles and direct measurement of resultant peak velocities of spindle poles.

GPR-1 and GPR-2 (fig. 5) as crucial for asymmetric cell division [197]. We and others have shown that the single GoLoco motif of GPR-1 acts as a GDI towards the *C. elegans* Gα subunit GOA-1 [198, 294]. As GPR-1 and GPR-2 are nearly identical at their protein and nucleotide sequence levels, a single interfering RNA is able to knock down expression of both proteins; RNAi-mediated knock down of *gpr-1/2* results in a loss of asymmetric division, and mislocalization of spindles in two-cell embryos – a phenotype identical to that of concomitant *goa-1* and *gpa-16* RNAi [197]. GPR-1/2 proteins colocalize and interact with the Gα subunit GOA-1 to regulate *C. elegans* asymmetric division [197, 199, 291]. RNAi of either *gpr-1/2* or *gpa-16/goa-1* was found to significantly reduce both anterior and posterior spindle-pole peak migration velocities in laser-mediated spindle-severing experiments (fig. 8), whereas wild-type embryos display a 40% higher peak velocity at the posterior spindle pole [197]. This greater net posterior pulling force in the wild-type embryo correlates well with (i) the prediction by Grill et al. of a ~50% enrichment of force generators in the posterior, as obtained in ultraviolet (UV) laser-induced centrosome disintegration studies [295] and (ii) the higher levels of GPR-1/2 seen at the posterior cortex [197]. Collectively, these findings suggest that the Gα GDI proteins GPR-1/2 and their target Gα subunits either directly modulate the actions of astral microtubule force generators or are the force generators themselves; one current model of how these proteins might act during asymmetric cell division, in conjunction with other newly discovered G-protein regulators, is discussed below.

Involvement of heterotrimeric G-proteins in mammalian cell division

In contrast to the considerable wealth of studies in *C. elegans* and *D. melanogaster*, information regarding the role of heterotrimeric G-proteins in mammalian cell division is relative scarce. There are, however, mammalian orthologues to the G-protein subunits, GoLoco motif proteins and even cell-fate determinants with cognate roles in asymmetric cell division in vertebrates (e.g. Numb; [296, 297]). For example, GPSM2 (LGN) [208] has 47% overall sequence identity with *Drosophila* Pins [274], displaying 67 and 32% identity to fly Pins in the TPR and GoLoco repeat regions, respectively. Several studies have demonstrated subcellular translocation of GPSM2 during cell division, including movement from the cytoplasm to the midbody [298], the spindle pole [213] or the cortex [268]. Either ectopic expression or RNAi-mediated knockdown of GPSM2 results in spindle disorganization and abnormal chromosome segregation [213], leading to cell cycle disruption [268]. Detailed studies by Du and colleagues have revealed that GPSM2

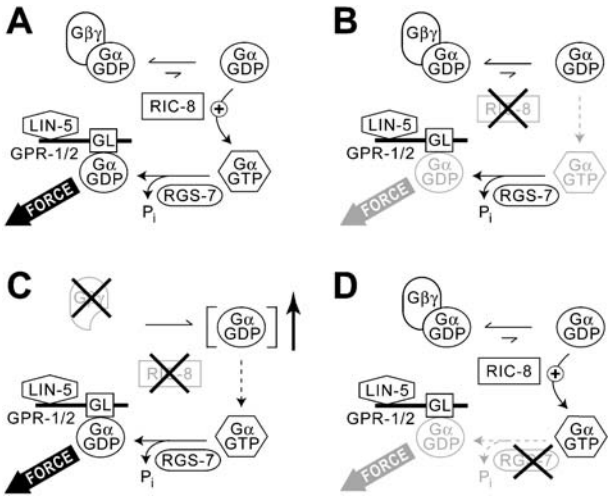

Figure 9. Working model of Gα activation during asymmetric cell division of *C. elegans* embryos. (*A*) In the wild-type embryo, RIC-8 GEF activity generates Gα·GTP. (It is still unclear whether *C. elegans* RIC-8 can act directly on G$\beta\gamma$-complexed Gα·GDP, since rat Ric-8A has been shown, at least in vitro, to act only on free Gα subunits [193]. An alternate possibility is that a distinct pool of free Gα exists or is generated from Gα·GDP/G$\beta\gamma$ heterotrimers by some as-yet unidentified player in this pathway.) The intrinsic GTPase activity of Gα·GTP, possibly accelerated by RGS-7 GAP activity, then generates Gα·GDP that binds the GoLoco motif of GPR-1/2 (the latter protein in complex with its critical co-factor LIN-5; [199]). The GPR-1/2/Gα·GDP complex is presumed to either modulate the (as-yet undefined) astral microtubule force generator or directly generate force (black arrow). (*B*) In the absence of RIC-8 activity, Gα·GTP levels are reduced, resulting in significantly lower levels of Gα·GDP required to form the GPR-1/2/Gα·GDP complex. This is consistent with the observations of reduced GPR-1/2/Gα·GDP co-immunoprecipitation from *ric-8 (md1909+RNAi) C. elegans* embryos [294]. (*C*) Combining the elimination of RIC-8 activity with RNAi-mediated knockdown of *gpb-1* (Gβ) is observed to restore the levels of GPR-1/2/Gα·GDP complex [294] and the magnitude of force applied to the spindle poles (fig. 8). Gα·GDP freed from its normal heterotrimeric state has a higher spontaneous nucleotide exchange rate [5] and therefore, in this model, can cycle through the GTP-bound state, GTP hydrolysis and GPR-1/2 interaction (or can directly bind to GPR-1/2; not shown). (*D*) In this model, loss of RGS-7 GAP activity leads to slower conversion of Gα·GTP to the Gα·GDP form required for the GPR-1/2/Gα·GDP complex. This is consistent with the reduced anterior forces observed in loss-of-function *rgs-7* mutants, although it is not known if RGS-7 is restricted in expression to the anterior cortex [305].

localizes to the spindle poles during cell division where it binds to the nuclear mitotic apparatus protein (NuMA) [213]. NuMA is involved in microtubule stabilization and organization at spindle poles; it is believed to nucleate microtubule bundles as a multimeric complex [299]. NuMA association with microtubules occurs through a C-terminal domain, and GPSM2 binds directly to NuMA through an overlapping region of the same C-terminal domain. Thus GPSM2 affects spindle organization by limiting the amount of NuMA available for microtubule nucleation [210].

The GoLoco domains of GPSM2 display GDI activity on $G\alpha_{i1}$ and $G\alpha_o$, although binding affinity and GDI activity towards $G\alpha_o$ are an order of magnitude lower than towards $G\alpha_{i1}$ [209]. During cell division, GPSM2 localizes to the cell cortex, and the multiple GoLoco motif C-terminus is sufficient for this distribution [268] as is the case with the *Drosophila* homologue Pins [300]. It is likely that binding to Gα subunits directs this membrane association, as ectopic expression of $G\alpha_o$ is reported to induce cortical localization of GPSM2 in non-dividing cells [268]. Another closely-related TPR- and GoLoco motif-containing protein present in metazoans is GPSM1 (AGS3); however, this protein has not been functionally analyzed with respect to its involvement in cell division to the same degree as GPSM2.

The usual suspects: GEF, GDI and GAP activities in asymmetric cell division

The discovery of Gα subunits as key constituents in the protein machinery of asymmetric cell division has led to the proposal that heterotrimeric G-protein signaling in ACD could occur in the absence of any canonical GPCR-mediated signal [200]. This is supported by circumstantial evidence that the in vitro culturing of fly neuroblasts, which effectively eliminates external signaling cues, does not perturb spindle positioning or segregation of cell-fate determinants [301, 302]. In a corresponding fashion, the shell surrounding *C. elegans* embryos makes it improbable that the first zygotic division receives or requires any extrinsic cue.

RIC-8 might act in lieu of receptor-mediated GEF activity in *C. elegans* embryo division. As mentioned previously, mammalian Ric-8A is a receptor-independent GEF for $G\alpha_{i/o}$ and $G\alpha_q$ subunits in vitro [193]. In *C. elegans*, *ric-8* mutations cause defects in spindle orientation and lead to a frequency of embryonic lethality of 15–30% [303]. *ric-8* mutant lethality can be augmented to 100% with concomitant mutation to *goa-1*, suggesting that these gene products might act in the same pathway [303]. Indeed, in collaboration with Pierre Gönczy, we have shown that *C. elegans* RIC-8 interacts with GOA-1 (selectively with its GDP-bound form) and acts as a GEF for GOA-1 as observed by RIC-8-dependent increases in GTPγS binding and steady-state GDP hydrolysis [294]. RNAi-mediated elimination of RIC-8 function (in a background of loss-of-function *ric-8* alleles) leads to reduced anterior and posterior pulling forces on the mitotic spindle of the one-cell zygote [294] – a phenotype identical to that of concomitant *goa-1* and *gpa-16* RNAi and of *gpr-1/2* RNAi (fig. 8). Elimination of RIC-8 function also reduces the level of GOA-1·GDP/GPR-1/2 complex observed in *C. elegans* embryonic extracts [294]; however, concomitant inactivation of G$\beta\gamma$ (via *gpb-1* RNAi) along with *ric-8* RNAi restores levels of the GOA-1·GDP/GPR-

1/2 complex as well as restoring robust anterior and posterior pulling forces on the mitotic spindle (fig. 8).

As a whole, these genetic and biochemical observations have led to the idea that RIC-8 functions in cell division upstream of GPR-1/2 – a function that somehow counteracts the entrapment of Gα·GDP in the G$\alpha\beta\gamma$ heterotrimer and leads to production of a GOA-1·GDP/GPR-1/2 complex, as illustrated in the working model of figure 9. This model considers the GOA-1·GDP/GPR-1/2 complex as the active species in signaling to pulling force generation. It is important to note that some of our findings regarding *C. elegans* RIC-8 have been independently confirmed by Gotta and co-workers [304], although this group interprets the involvement of RIC-8 GEF activity in asymmetric cell division as evidence that Gα·GTP is the active species in force generation required for this process.

Remarkably, as in GPCR-stimulated heterotrimeric G-protein signaling, RGS proteins are emerging as critical regulators of Gα action in cell division. For example, Hess and colleagues recently reported that the *C. elegans* RGS-7 protein can act to accelerate GTP hydrolysis by GOA-1 [305]; loss of RGS-7 function leads to hyper-asymmetric spindle movements in the one-cell zygote, resulting from a decreased anterior spindle pulling force (summarized in fig. 8). In the working model of Gα involvement in pulling force generation (fig. 9), the findings of Hess et al. could be explained by RGS-7 acting selectively at the anterior cortex to accelerate conversion of Gα·GTP to Gα·GDP for interaction with GPR-1/2; loss of RGS-7 GAP activity would therefore lead to less anterior GOA-1·GDP/GPR-1/2 complex and less force generation from the anterior cortex (fig. 9D). Unfortunately, the initial studies by Hess and colleagues did not include an examination of the distribution of endogenous RGS-7 protein in the dividing one-cell zygote, and thus future studies are required to ascertain whether RGS-7 function is indeed restricted to the anterior cortex.

Intriguingly, recent evidence supports a similar role for RGS proteins in mammalian cell division. With our colleagues Josef Penninger and Tony D'Souza, we generated *Rgs14* knockout mice; lack of RGS14 expression in the mouse zygote leads to an early embryonic lethality, specifically at the first zygotic division [306]. RGS14 was found to be one of the earliest proteins expressed by the mouse embryonic genome immediately prior to the first division; the protein was observed to co-localize with microtubules forming the anastral mitotic apparatus of the dividing one-cell zygote. Immunofluorescence microscopy of mouse embryos lacking RGS14 revealed misaligned chromatin and a dearth of microtubule organization or diffuse tubulin and DNA staining, the latter phenotype suggestive of chromosomal fragmentation. In all mammalian cell types examined, RGS14 segregated to the mitotic spindle and centrosomes during mitosis [306]; alteration of RGS14 levels in exponentially proliferating cells, either by RNAi-mediated knockdown or constitutive expression, was found to be deleterious to continued cell proliferation – a phenomenon very similar to that observed by Du and colleagues with GPSM2/LGN overexpression or knockdown [210, 213]. We have also recently reported that RGS14 is a microtubule-associated protein and its depletion from mitotic cell extracts prevents aster formation normally catalyzed by the addition of ATP and taxol [307]. Our findings implicate RGS14 (and its Gα targets) as critical players in cell division processes from the very first zygotic division and suggest that heterotrimeric G-protein regulation of microtubules may be a conserved mechanism by which metazoans control spindle organization and force generation during chromosomal DNA segregation into daughter cells.

Unanswered questions and future directions

Many questions remain unanswered as far as the detailed mechanism of G-protein regulation of spindle pulling forces during cell division. It has been proposed that tubulin may be a direct downstream target of G-proteins in the context of cell division [134]. This is supported by evidence that both Gα and G$\beta\gamma$ subunits can regulate tubulin assembly and microtubule dynamics [308–313]. In particular, GTP-bound $G\alpha_{i1}$ can bind directly to tubulin, transactivate its intrinsic GTPase activity and modulate microtubule assembly [308, 310]. Using a novel form of microscopy, Labbé and colleagues have demonstrated that microtubule residence at the cell cortex is significantly longer on the anterior versus posterior side of the *C. elegans* early embryo [314]. In contrast, upon RNAi-mediated elimination of *goa-1* and *gpa-16* expression, microtubule residence time is equivalent at the anterior and posterior cortex (i.e. both equal to that of the posterior cortex in wild-type embryos), thus reinforcing the evidence that Gα subunits are responsible for asymmetric force generation. It is of note that microtubule residence time was not changed in general [314], indicating that force generation does not involve changes in microtubule cortical dynamics but, more likely, in the machinery regulating microtubule polymerization and depolymerization.

Whereas the GTP-bound form of Gα subunits is the active species in canonical GPCR signaling pathways, it remains to be proven if this is the case in asymmetric cell division. With the potential exception of Rap1GAP [204], GoLoco motif-containing proteins such as Pins, GPSM2 and GPR-1/2 only bind to the GDP-bound form of Gα subunits. Thus it remains to be established if the active species responsible for controlling spindle pulling forces is Gα·GDP, Gα·GTP, GoLoco-bound Gα·GDP (as suggested in our working model; fig. 9), or something else entirely. In addition, it is unclear how the G$\alpha\beta\gamma$ het-

erotrimer is dissociated to allow Ric-8 and GoLoco proteins unfettered access to the GDP-bound Gα subunit. In in vitro studies with rat Ric-8A, Tall and colleagues have suggested that Ric-8 GEF activity cannot operate on G$\beta\gamma$-complexed Gα·GDP [193]; perhaps *C. elegans* RIC-8 does not share this restriction or a cellular context with appropriately membrane-targeted G-protein subunits is required to observe GEF activity on the heterotrimer. Some have proposed that GoLoco motif proteins can disrupt G$\alpha\beta\gamma$ heterotrimeric complexes [199, 267, 315]; however, in electrophysiological studies of the influence of GoLoco motif peptides on GPCR coupling to G$\beta\gamma$-gated GIRK potassium channels, we have been unable to observe GoLoco motif-mediated activation of G_i-heterotrimers in a fully integrated cellular context [316]. Moreover, our recent evidence that RIC-8 acts upstream of GPR-1/2 in the cycle underlying GOA-1-mediated spindle pulling force generation ([294]; fig. 9) disavows this possibility. Other proteins are clearly involved in spindle dynamics and may be directly engaged in generating Gα free from G$\beta\gamma$. LIN-5 has been identified as a binding partner of GPR-1/2 in *C. elegans* (fig. 9), and disruption of *lin-5* results in a symmetric zygotic division phenotype akin to that of *gpr-1/2* or *gpa-16/goa-1* RNAi [198, 199]. LIN-5 is a coiled-coil protein that localizes GPR-1/2 to the posterior cortex and is thus paramount for correct pulling force distribution. Another protein, LET-99, appears to counteract the Gα/GPR-1/2 pathway, such that loss-of-function *let-99* mutations result in increased pulling forces and a hyperactive rocking motion during spindle rotation [317]. The apparent multiple levels of control and complexity of this system are not surprising in light of the essential nature of correct asymmetric division for embryo viability. Further studies will be required to identify the precise role of each of the heterotrimeric subunits in cell division, delineate the complex interactions between polarity cues and spindle positioning, and identify the mechanism by which heterotrimeric G-proteins regulate pulling forces.

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