HeterotrimERIC G-protein Go subunITS and GoLoco motif binding proteins are key members of a conserved set of regulatory proteins that influence invertebrate asymmetric cell division and vertebrate neuroepithelium and epithelial progenitor differentiation. GoLoco motif proteins bind selectively to the inhibitory subclass (Gαi) of Go subunits, and thus it is assumed that a Gαi-GoLoco motif complex plays a direct functional role in microtubule dynamics underlying spindle orientation and metaphase chromosomal segregation during cell division. To address this hypothesis directly, we rationally identified a point mutation to Gαi subunits that renders a selective loss-of-function for GoLoco motif binding, namely an asparagine-to-isoleucine substitution in the αD–αE loop of the Go helical domain. This GoLoco-insensitivity (“GLI”) mutation prevented Gαi association with all human GoLoco motif proteins and abrogated interaction between the Caenorhabditis elegans Go subunit GOA-1 and the GPR-1 GoLoco motif. In contrast, the GLI mutation did not perturb any other biochemical or signaling unit GOA-1 and the GPR-1 GoLoco motif. In contrast, the GLI mutation did not perturb any other biochemical or signaling unit GOA-1 and the GPR-1 GoLoco motif. This GoLoco-insensitivity (“GLI”) mutation prevented Gαi association with all human GoLoco motif proteins and abrogated interaction between the Caenorhabditis elegans Go subunit GOA-1 and the GPR-1 GoLoco motif. In contrast, the GLI mutation did not perturb any other biochemical or signaling unit GOA-1 and the GPR-1 GoLoco motif.

epithelial cells. This GLI mutation should prove valuable in establishing the physiological roles of Gαi-GoLoco motif protein complexes in microtubule dynamics and spindle function during cell division as well as to delineate potential roles for GoLoco motifs in receptor-mediated signal transduction.

Seven transmembrane-domain receptors (7TMRs) mediate the actions of various extracellular sensory, hormonal, and metabolic stimuli. Among the signaling components coupled to the intracytosolic side of 7TMRs are the heterotrimeric G-proteins: molecular switches composed of a guanine nucleotide-binding Go subunit and a Gβγ dimer that transduce 7TMR activation into intracellular modulation of multiple different effectors, including adenylyl cyclases, ion channels, cyclic nucleotide phosphodiesterases, and phospholipase C isoforms. 7TMR-promoted activation of Gβγ causes Go to exchange the more abundant GTP for bound GDP, which in turn causes GαGTP and Gβγ to dissociate. GαGTP and Gβγ are then free to regulate effector systems that alter cell physiology. This classical 7TMR-initiated G-protein nucleotide cycle is reset by intrinsic GTP hydrolysis activity possessed by the Go subunit.

An evolutionarily conserved role for Ga subunits of the adenylyl cyclase inhibitory (Ga) subfamily has recently been identified in the control of mitotic spindle orientation in cell divisions that generate cellular diversity during organismal development (6, 7). Studies of asymmetric cell division in Caen-
norhabditis elegans embryos and Drosophila melanogaster embryonic neuroblasts have identified initial steps of this process as generation of cell polarity and segregation of various cell fate determinants to different sides of the polarized cell (8); the mitotic spindle is then positioned to facilitate appropriate distribution of determinants to daughter cells during chromosomal segregation and cytokinesis. An integral part of the cellular machinery underlying accurate spindle positioning is the involvement of heterotrimeric G-protein Ga and Gβγ subunits in a manner considered independent of 7TMR activation and instead involving RIC-8 (a cytosolic guanine nucleotide exchange factor), GoLoco motif3 proteins (such as GPSM2/LGN, Pins, and GPR-1/2 that act as GDP dissociation inhibitors), and GTPase-accelerating proteins ("GAPs"; i.e. RGS proteins) (6–13). Vertebrate neuroepithelial progenitors use the same cellular machinery to modulate mitotic spindle orientation controlling the balance between asymmetric cell divisions that drive differentiation and planar divisions that favor maintenance and expansion of the neuroepithelial architecture (14–16). Similarly, an analogous mechanism appears to operate in the stratification and differentiation of mammalian skin (17).

An essential feature of the various emerging models of G-protein nucleotide cycling in mitotic spindle positioning is the requirement for a Ga2GoLoco motif complex. For example, in our working model of C. elegans asymmetric cell division controlled by the Ga subunits GOA-1 and GPA-16 (18, 19), it is the GoGDP/GPR-1/2 complex that activates the generation of astral microtubule (MT) force on mitotic spindle poles, whereas in a competing model (3, 12, 20), the Ga-GDP/GoLoco motif complex is required for the nucleotide exchange ("GEF") activity for RIC-8, thereby generating Ga-GTP as the presumed active form of the G-protein (12, 21, 22). However, it has not been formally established that the Ga/GoLoco motif interaction is required per se for the function of Ga subunits and GoLoco motif proteins in mitotic spindle positioning. For example, both models of C. elegans asymmetric cell division have been generated primarily by correlating various genetic phenotype data, including loss of pulling forces upon RNA interference-mediated knockdown of goa-1/gpa-16 or gpr-1/2 expression (9–11, 18, 19). These phenotypic results, although suggestive of a critical function for a Ga/GoLoco protein complex, might alternatively reflect separate and distinct functions of Ga subunits and the multidomain GPR-1/2 proteins in parallel pathways culminating in MT force generation, given that both classes of proteins have other binding partners and established functions. Furthermore, it remains unresolved as to whether Gβγ is an independent signaling entity in this system or merely a buffer of free Ga-GDP levels (14, 25, 34, 35). The specific activities of wild type and N149I Ga1 were determined using [35S]GTPγS binding (mean ± S.E. of...
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mol of GTP·γS bound per mol of Gαi1) as follows: wild type, 0.93 ± 0.02; N149I 0.93 ± 0.02. C. elegans RGS-7, also with its His6 tag removed, was purified to homogeneity using methods standard for other RGS domains (41).

Surface Plasmon Resonance—Surface plasmon resonance analysis of GoLoco motif/Gα interactions was conducted as described in Refs. 25, 38.

Fluorescence Anisotropy—Fluorescence anisotropy assays of Go binding to FITC-labeled GoLoco motif peptides were conducted as described in Ref. 42 for Fig. 2 and Fig. 7 and as described in Ref. 40 for Fig. 3. A minor modification was the use of a 5 mM final concentration of the FITC-RGS14, FITC-RGS12, FITC-GPSM2(GL2), and FITC-KB-1753 peptides. FITC-RGS12 is described in Ref. 42. FITC-GPSM2(GL2) is described in Ref. 40. FITC-KB-1753 is described in Ref. 43. The FITC-RGS14 peptide included amino acids 496–531 of rat RGS14 (FITC-β-alanine-S-DIEGLVELLRQSSGAHDQRGLLR-KEDLVLEPEQ-NH2). Anisotropy data are presented as millipolarization units (mP) following data analysis as described in Ref. 42.

Nucleotide Binding and Hydrolysis Assays—[35S]GTP·γS binding and [γ-32P]GTP hydrolysis assays were conducted as described in Ref. 9, 44. [35S]GTP·γS binding was used to measure GPR-1/2-mediated GDI activity on GOA-1 as described in Refs. 40 for Fig. 3. A minor modification was the use of a 5 mM final concentration of the FITC-RGS14, FITC-RGS12, FITC-GPSM2(GL2), and FITC-KB-1753 peptides. FITC-RGS12 is described in Ref. 42. FITC-GPSM2(GL2) is described in Ref. 40. FITC-KB-1753 is described in Ref. 43. The FITC-RGS14 peptide included amino acids 496–531 of rat RGS14 (FITC-β-alanine-S-DIEGLVELLRQSSGAHDQRGLLR-KEDLVLEPEQ-NH2). Anisotropy data are presented as millipolarization units (mP) following data analysis as described in Ref. 42.

Dissociation of Superior Cervical Ganglion and cDNA Microinjection—Detailed methods of preparing rat superior cervical ganglion (SCG) neurons and cDNA microinjection were described previously (27). In brief, adult male Wistar rats were anesthetized by CO2 inhalation and decapitated as described previously (27, 45). Patch electrodes were fire-polished to final resistances of ~2 megohms when filled with internal solution. Uncompensated series resistance was <5 megohms and electronically compensated ~80%. Voltage protocol generation and data acquisition were performed using the custom-designed software S5. Current traces were filtered at 2 kHz and digitized at 10 kHz. All recordings were performed at room temperature (21–24 °C).

Electrophysiology Solutions and Chemicals—The external solution consisted of the following (in mM): 140 methanesulfonic acid, 145 tetraethylammonium hydroxide (TEA-OH), 10 HEPES, 10 glucose, 10 CaCl2, and 0.0003 tetrodotoxin, pH 7.4, with TEA-OH. The internal solution contained the following (in mM): 120 N-methyl-D-glucamine, 20 TEA-OH, 11 EGTA, 10 HEPES, 10 sucrose, 1 CaCl2, 4 MgATP, 0.3 Na2GTP, and 14 Tris creatine phosphate, pH 7.2, with methanesulfonic acid. The osmolalities of the external and internal solutions were adjusted with sucrose to 325 and 300 mosmol/kg, respectively. All drug and control solutions were applied to neurons via a custom-designed gravity-driven perfusion system as described previously (45).

Cyclic AMP Accumulation Assay—HEK 293 cells stably expressing the rat D2l, dopamine receptor (46) were propagated in Dulbecco’s modified Eagle’s medium supplemented with 5% (v/v) bovine calf serum, 5% (v/v) FetalClone 1 serum (Thermo Fisher, Waltham, MA), 1 unit/ml penicillin, 2.5 ng/ml amphotericin B, and 2 μg/ml puromycin and maintained in a humidified incubator at 37 °C and 6% CO2. Cells were seeded into 24-well cluster plates and, upon reaching ~80% confluence, were transiently transfected with 200 ng of pcDNA3.1(+) pCI rat Gα11(Q204L, C352G), or Gα11(N149I, Q204L, C352G) together with 50 ng of pFLAG-YFP-A2A using Lipofectamine 2000 reagent (Invitrogen, 1 μl/well). At 24 h post-transfection, cAMP accumulation assays were carried out on ice in Earle’s balanced salt solution containing 15 mM Na+ -HEPES, 2% bovine calf serum, and 0.02% ascorbic acid following a 5-min preincubation in assay buffer. Cyclic AMP was stimulated by activation of the adenosine A2A receptor with the agonist 5’-N-methylcarboxamidoadenosine (MECA, 1 μM) at 37 °C for 15 min in the presence of the phosphodiesterase inhibitor 4-(3-butoxy-4-methoxybenzyl)imidazolizidin-2-one (Ro-20-1724, 100 μM). The stimulation medium was decanted, and the reaction was terminated by addition of ice-cold 3% trichloroacetic acid. The plate was stored at 4 °C for at least 1 h before cAMP quantification. Cyclic AMP was quantified using a competitive binding assay (47).

GPSM3 Membrane Recruitment Experiments—Similar to the transmembrane domain-anchored Gα subunits described in Ref. 48, a pcDNA3.1-based mammalian expression vector was generated to encode CFP-TM-Gα11 subunits consisting of (starting at the N terminus) a signal peptide, enhanced CFP (49), the N-terminal 103 amino acids of the rat µ-opioid receptor, the N-terminal 33 amino acids of human GαoA, and amino acids 34–354 of human Gα11. A pcDNA3.1-based mammalian expression vector was generated to encode YFP-GPSM3 consisting of the venus variant of enhanced YFP (30), a c-Myc amplifier (Molecular Devices, Sunnyvale, CA) was described in detail previously (27, 45). Patch electrodes were fire-polished to final resistances of ~2 megohms when filled with internal solution. Uncompensated series resistance was <5 megohms and electronically compensated ~80%. Voltage protocol generation and data acquisition were performed using the custom-designed software S5. Current traces were filtered at 2 kHz and digitized at 10 kHz. All recordings were performed at room temperature (21–24 °C).
epitope tag, a hexahistidine tag, and human GPSM3 fused in-frame (derived from pcDNA3.ImycHis human GPSM3 (26)).

Human embryonic kidney 293 cells (ATCC; Manassas, VA) were propagated in plastic flasks and seeded onto polylysine-coated glass coverslips according to the supplier’s protocol. Cells were transfected using polyethyleneimine and were used for experiments 12–48 h later. Coverslips bearing transfected cells were imaged using a Leica (Bannockburn, IL) SP2 scanning confocal microscope and a 63×, 1.4 NA objective; cells were excited using 458 nm (for CFP) or 514 nm (for YFP) laser lines. Images were acquired and analyzed by an experimenter who was blinded to the transfection condition. A 5-μm profile drawn normal to, and centered on, the plasma membrane was obtained for each cell.

**MDCK Cell and Spindle Rocking Experiments**—MDCK II cells were cultured, transfected, and processed for imaging as described (32). MDCK cells were transfected with either wild type or N149I Ga_i1-YFP. Time-lapse images of Hoechst 33342-stained chromosomal DNA condensation and segregation were recorded as described (32). The angles of the long axis of the metaphase chromosomal array in each frame were measured using Metamorph software (Molecular Devices, Sunnyvale, CA). The absolute angle changes of at least 100 sets of adjacent frames were binned into <5°, 5–10°, and >10° groups.

Co-immunoprecipitation—COS-7 cell culture, transfection, and immunoprecipitation was performed as described (32).

**Statistics and Curve Fitting**—Unless otherwise indicated, data analysis and curve fitting were performed using PRISM version 4.0 (GraphPad; San Diego). All data are representative of three or more independent experiments.

**Structural Analysis of the GoLoco Motif/Ga Interaction**—The crystallographic structure of Ga_i1-GDP bound to the GoLoco motif of RGS14 has been determined at 2.7 Å resolution (PDB code 1KJY (39)), and more recently at 2.2 Å resolution (PDB code 2OM2 (50)). The two structures share the same overall global architecture; however, there are appreciable differences between the two structural models (50). In this study, we confined our analysis to the 2OM2 structure, as it has higher overall resolution and better refinement statistics. Similarly, subtle but discrete differences exist between the two asymmetric units in both 2OM2 and 1KJY structures; for this reason, we have generally confined our analysis to the A and B chains of 2OM2 as the refinement of this asymmetric unit appeared superior. PyMol (DeLano Scientific; Palo Alto, CA) was used for analysis of structures and the generation of images. Amino acid interaction data were derived using SPACE (CMA) (51) and plotted using MATLAB (The MathWorks, Natick, MA).

**RESULTS AND DISCUSSION**

**Rational Design of a Loss-of-Function Point Mutation in Ga_i1 to Prevent GoLoco Motif Interaction**—A major feature of the Ga_i1-GDP/RGS14 GoLoco motif complex (39, 50) consists of the GoLoco motif N terminus forming an α-helix that binds in the pocket formed by the α2 helix (“switch II”) and the α3 helix of the Ras-like domain of Ga_i1 (Fig. 1A). The invariant glutamine residue (Gln_515) of the GoLoco motif (D/E)QR triad terminates this helical portion of the GoLoco motif. The GoLoco motif peptide continues to transit across the surface of Ga_i1 to contact the all α-helical domain of Ga_i1. The conserved Asp_514→Gln_515→Arg_516 triad is responsible for turning the GoLoco motif peptide and positioning Arg_516 into the nucleotide binding pocket of Ga_i1, so that the Arg_516 side chain is able to make direct contact with the α- and β-phosphates of GDP, thus stabilizing the bound nucleotide and conferring GDPi activity (39). The C-terminal segment of the GoLoco motif makes a sharp hydrogen-bonded turn (Lys_521→Glu_522→Asp_523→Leu_524) as it enters the central groove between the αA- and αB-helices of the G-protein helical domain where it makes an extensive net-
GoLoco-insensitivity Mutation in \( \alpha \) Subunits

Asn149 to Ile Mutation in \( \alpha_{11} \) Prevents Interaction with GoLoco Motifs in Vitro—Mutation of the \( \alpha_{11} \) amino acid Asn149 to isoleucine dramatically attenuated GDP-dependent binding of \( \alpha_{11} \) to the GoLoco motif of RGS14, as assessed by SPR spectroscopy (Fig. 2B). We verified this attenuated affinity with an independent measurement of binding that employed fluorescence anisotropy; the N149I mutation reduced the calculated binding affinity of \( \alpha_{11} \)-GDP for the RGS14 GoLoco motif by 300-fold (Fig. 2C). The signature biochemical activity of GoLoco motifs is GD activity (7). We therefore also tested GD activity of the RGS14 GoLoco motif on wild type and N149I \( \alpha_{11} \) (Fig. 2, D and E). We were unable to observe significant GoLoco motif-mediated GD activity using \( \alpha_{11} \) (N149I), whereas wild type \( \alpha_{11} \) was a substrate for RGS14 GoLoco motif GD activity in the nanomolar range (Fig. 2, D and E), as observed previously (25).

To test the universality of this GoLoco-insensitivity point mutation, we analyzed the binding of N149I \( \alpha_{11} \) to all human GoLoco motifs using SPR. A graphical representation of all known GoLoco motif proteins, as well as the purified protein constructs used in these SPR analyses, is presented in supplemental Fig. S2.4 Wild type \( \alpha_{11} \) (at 1 mM) exhibited robust, GDP-selective binding to all known GoLoco motifs (Fig. 3, A–G); at a 10-fold higher concentration, N149I \( \alpha_{11} \) did not demonstrate any binding to GSPM2 (Fig. 3B) nor to PCP-2 (Fig. 2A).

4 The GoLoco motif of Rap1GAP2 was not tested in these experiments as it is devoid of GD activity and incapable of functional interactions with \( \alpha_{11} \), \( \alpha_{2} \), \( \alpha_{15} \), and \( \alpha_{3} \) (40).
GoLoco-insensitivity Mutation in Ga Subunits

C. elegans Ga$_{i0}$-like G-protein GOA-1, known to functionally interact with GoLoco motifs to regulate asymmetric cell division in the one-cell embryo (9). We measured RGS-7$_{5}^{5}$-mediated acceleration of GTP hydrolysis by GOA-1. We observed that the N150I mutation had no appreciable effect on the ability of RGS-7 to stimulate the GTPase activity of GOA-1 in a dose-dependent fashion (Fig. 4C). Additionally, we verified that GOA-1(N150I) is indeed resistant to GoLoco motif-mediated GDi activity (Fig. 4D), suggesting that the Asn to Ile mutation can be transferred across species, consistent with the conserved evolutionary relationships among metazoan G-proteins and GoLoco motif proteins (6, 7).

Functional Properties of the GoLoco-insensitive Ga—Ca$^{2+}$ channel modulation in sympathetic neurons was used to examine the ability of GoLoco-insensitive Ga mutants to complex with endogenous G$_{i0}$ y subunits. N-type Ca$^{2+}$ channels respond to both tonic and 7TMR-mediated G-protein activation with a characteristic voltage-dependent modulation mediated by G$_{i0}$ y subunits (55). Ca$^{2+}$ channel currents in whole-cell voltage-clamped neurons were evoked with a double-pulse voltage protocol consisting of two 25-ms test pulses to + 10 mV separated by a depolarizing conditioning pulse to +80 mV (56). In control neurons under basal conditions (Fig. 5A, open circle), the amplitude during the first test pulse (prepulse) is slightly smaller than that evoked by the second test pulse (postpulse) resulting in a mean facilitation ratio (postpulse/prepulse amplitude) greater than 1 (Fig. 5F, open bar). Basal (in the absence of agonist) facilitation has been shown to arise from tonic modulation by G$_{i0}$ y subunits (28, 57). Application of norepinephrine (NE, 10 $\mu$M) activates endogenous $\alpha_{2}$-adrenergic receptors, resulting in a large increase in basal facilitation (Fig. 5F, filled bar). Activation of tonic modulation (Fig. 5B–D) abolished both tonic and agonist-mediated modulation as indicated by decreases in mean basal facilitation (Fig. 5F) and agonist-mediated inhibition of the prepulse amplitude (Fig. 5E). The decreases were comparable with those produced by heterolo-

5 There is considerable confusion with regard to cross-organism RGS protein nomenclature. Our experiments used C. elegans RGS-7 (GenBank accession number AY569308), which is likely the nematode ortholog of mammalian RGS3, a PDZ- and C2-domain-containing RGS protein (3, 80).
GoLoco-insensitivity Mutation in Ga Subunits

FIGURE 4. Biochemical properties of GoLoco-insensitive Ga subunits. A, spontaneous nucleotide exchange rates ($k_{ex}$) of wild type (black) and N149I (gray) Gaαi1-GDP were measured. A time course of specific binding of 100 nM Gaαi1 subunit to 1 μM GTPγS was determined using an [35S]GTPγS filter-binding assay. Data were fit to single exponential functions with rate constants as follows: wild type Gaαi1, 0.013 ± 0.002 min⁻¹ and N149I Gaαi1, 0.009 ± 0.0004 min⁻¹. B, spontaneous GTP hydrolysis rates ($k_{hyd}$) of wild type (black) and N149I (gray) Gaαi1 were measured using [γ-32P]GTP hydrolysis assays. A time course of [γ-32P]GTP hydrolysis was determined using charcoal filtration. Data were fit to single exponential functions with rate constants as follows: wild type Gaαi1, 0.40 ± 0.003 min⁻¹ and N149I Gaαi1, 0.30 ± 0.003 min⁻¹. C, GTPase-accelerating protein (GAP) activity of C. elegans RGS7 on 200 nM wild type and N150I-mutated C. elegans GOA-1 was measured using 100 nM BODIPYFL-GTP and fluorescence spectroscopy. Data were fit to the four parameter logistic equation to determine EC₅₀ values of RGS-7 GAP activity (95% confidence intervals in parentheses) as follows: wild type GOA-1, 830 (570–1300) nM; N149I GOA-1, 670 (580–790) nM. D, GDI effect of the C. elegans GPR-1/2 GoLoco motif on C. elegans GOA-1 was quantified using [35S]GTPγS filter binding. Time courses were obtained by preincubating 100 nM GOA-1 (wild type or N150I) with either buffer or 10 μM GPR-1/2 GoLoco motif peptide for 5 min. Samples were then added to 1 μM GTPγS, and specific [35S]GTPγS binding was quantified by filtration and scintillation counting. Data were fit to exponential association functions (95% confidence intervals in parentheses) as follows: wild type GOA-1 alone, 0.202 (0.160–0.240) min⁻¹; wild type GOA-1 + GoLoco peptide, 0.068 (0.055–0.081) min⁻¹; N150I GOA-1 alone, 0.178 (0.150–0.210) min⁻¹; N150I GOA-1 + GoLoco peptide, 0.194 (0.140–0.250) min⁻¹.

The ability of the GoLoco-insensitive Gaαi1 subunit to inhibit the Goa effector adenyl cyclase was assessed by measuring the inhibition of agonist-stimulated cAMP accumulation in cells co-expressing constitutively active Gaαi1(Q204L) subunits and the A2A adenosine receptor. Co-transfection of Gaαi1(Q204L) inhibited MECA-stimulated cAMP accumulation by more than 50% when compared with cells co-transfected with the vector control (Fig. 7B). Cells co-expressing Gaαi1(N149I,Q204L) also reduced MECA-stimulated cAMP accumulation, indicating that GLi Gaαi1 retains the canonical Gaαi inhibitory function on adenylyl cyclase.

Effect of the GoLoco-insensitivity Mutation on GoLoco Motif-dependent Properties of Gaα Subunits—To examine the effect of the GLi mutation on Ga regulation of GoLoco motif protein biology, we undertook multiple approaches. Gaα subunits facilitate the membrane localization of GoLoco motif proteins in various model systems, including Drosophila neuroblasts and...
mammalian cell lines (23, 32). Using MDCK cells, we measured the ability of exogenously expressed KT3 epitope-tagged wild type and GLi Gαi subunits to regulate the cellular distribution of endogenous GPSM2 (Fig. 8). We consistently observed that wild type Gαi1 expression promoted the plasma membrane recruitment of GPSM2, whereas GLi Gαi1 had no effect on GPSM2 cellular distribution. Analogous results were observed using mRFP-tagged Gαi1 and endogenous GPSM2 (supplemental Fig. S3). We also observed that exogenous expression of GPSM2 frequently resulted in the accumulation of GPSM2 in “vesicle-like” intracellular organelles. These structures were eliminated by co-transfection with wild type Gαi1, presumably by Gαi-mediated recruitment of GPSM2 to the plasma membrane (supplemental Fig. S4). However, co-transfection of YFP-GPSM2 with Gαi1(N149I) did not alter the morphology of YFP-GPSM2-containing vesicular structures (supplemental Fig. S4).

We also examined the effect of the GLi mutation on interactions between Gαi1 and the triple GoLoco motif protein GPSM3 (26). We used a CFP-tagged and transmembrane domain-immobilized chimeric GαOA/Gαi1/subunit6 (“CFP-TM-GoLoco”) to demonstrate the ability of Gα subunits to specify the membrane localization of GPSM3 (48). Expression of YFP-

6 The chimeric GαOA/Gαi1 subunit, comprising the N-terminal 33 amino acids of GαOA and the remainder of the polypeptide sequence from Gαi1, was originally created to facilitate kinetic imaging and functional assays not described in this manuscript (G. J. Digby and N. A. Lambert, unpublished data). Of the 33 GαOA-derived amino acids present within this Gα chimera, 22 are identical to those found in GαOA, and 8 more are conservative substitutions (i.e., only three positions represent nonconservative differences in side chain character). This 33-amino acid N-terminal region composes the flexible first α-helix of Gα that does not participate in the GoLoco motif interaction (7).
GoLoco-insensitivity Mutation in Gα Subunits

![Image](310x401 to 563x737)

FIGURE 7. GoLoco-insensitive Gαi1, has normal interactions with the effector adenyl cyclase and the effector-mimetic peptide KB-1753. A, affinity of wild type and N149I Gαi1, proteins for the Gα-effector mimic peptide KB-1753 (43) was measured using fluorescence anisotropy. S1 mM FITC-KB-1753 peptide was mixed with increasing amounts of Gαi1, proteins, and equilibrium fluorescence anisotropy was measured. Data are presented as the mean ± S.E.M. of triplicate determinations. Dissociation constants were determined by nonlinear regression: wild type Gαi1, GDP (24.1 ± 4 μM), wild type Gαi1,-GDP-AIF4 (294 ± 40 μM), N149I Gαi1,-GDP (13.0 ± 2 μM), N149I Gαi1,-GDP-AIF4 (311 ± 40 μM). B, cells were transiently transfected with cDNA encoding Gαi1(Q204L), Gαi1(N149I,Q204L,C352G), or pcDNA3.1(+) as a vector control, with the adenosine A2A receptor. Cyclic AMP accumulation was stimulated with 1 μM MECA for 15 min at 37 °C. Data represent the mean ± S.E.M. of four independent experiments in duplicate. *p < 0.05; **p < 0.01 compared with A2A-R + empty vector transfection under matched stimulation (basal or MECA), one-way analysis of variance followed by Dunnett’s post hoc test.

tagged GPSM3 in the absence of co-expressed Go subunits is characterized by a uniform distribution of the GoLoco motif through the cell (Fig. 9, left panel). Expression of GPSM3-YFP in the presence of membrane-tethered, wild type CFP-TM-GoLocoα5 results in a predominantly cytoplasmic distribution of GPSM3 (Fig. 9, right panel). As yet another alternative technique to monitor Go/GoLoco motif interaction in cells, we used co-immunoprecipitation from lysates of co-transfected COS-7 cells; both GPSM1 and GPSM2 interacted robustly with wild type but not GLi, Gαi1 (Fig. 10). In summary, these data are all consistent with the GLi mutation being a loss-of-function with respect to GoLoco motif binding in cells.

Structural Basis of the GoLoco-insensitivity Mutation—Our data illustrate that the GLi mutation abrogates the ability of Gαi subunits and GoLoco motif proteins to interact in vitro and in cells. Despite such an extreme loss-of-function in this one aspect of Gα biology, the GLi Gαi subunits behave normally in all other biochemical and cellular assays we have conducted. There is ample precedent for finding such mutations within Gαi subunits; the RGS-insensitivity mutation (G183S within Gαi1) was first isolated in Saccharomyces cerevisiae Gpa1 (60), shown to be transferable to mammalian Gαi5, Gαi4, and Gαq subunits (60, 61), and validated as affecting only the Gα/RGS domain interaction without affecting nucleotide, receptor, Gβγ, or effector interactions (62). To better understand the potent and highly selective nature of the GLi mutation, we re-analyzed the previously described Gαi5,-GDP/RGS14 GoLoco motif structure (50). Structural analysis of this GαiGoLoco peptide complex indicates that the predominant role of Asn149 within Gαi1 is to directly contact Gln515 of the RGS14 GoLoco motif. The side chain oxygen of Gln515 forms a hydrogen bonding network with both the side chain terminal amine (distance of 3.1 Å) and the backbone amine (distance of 2.9 Å) of Asn149 (Fig. 1B). Asn149 appears to be an important node in a network of Gαi1.
amino acid residues, including Glu43, Asn76, Gln79, Ser80, Gln147, Leu148, and Arg178 that act to stabilize the position of Gln515 in the GoLoco motif (Fig. 1C and supplemental Fig. S5). Gln515 of the GoLoco motif is crucial in positioning Arg516 into direct contact with GDP, and to accomplish this positioning, Gln515 makes a number of stabilizing interactions with G/H residues in the P-loop, αA helix, switch I, and the αD/αE loop (supplemental Figs. S2 and S5). This network of residues, in which Asn149 is involved, is also important in stabilizing the “seatbelt” between Glu43 and Arg178 hypothesized to restrain the bound nucleotide within its binding pocket (supplemental Fig. S5) (63, 64).

Although the only amino acid residue of the RGS14 GoLoco motif that directly contacts GDP is Arg516 (Fig. 1B), GoLoco motif binding to G/H induces a tighter fit of GDP into the nucleotide binding pocket (39). The salt bridge interaction between the P-loop residue Glu43 and the switch I residue Arg178 likely stabilizes bound GDP (39, 63, 65) and, in cooperation with Arg516, accounts for the structural determinants of GDI activity. We also observed that the backbone amine of Asn149 contacts the side chain of Ala512 (distance of 3.8 Å); however, this interaction was not observable in all crystallographic models7 and so may be of uncertain significance.

Through multiple experimental methods, we have demonstrated that the N149I mutation in G/H does not perturb in vitro biochemical nor in cellulo signal transduction properties of G/H subunits. Based on the structural analysis described above, substitution of the amide side chain of Asn with the aliphatic side chain of Ile would disrupt the hydrogen bonding network between the side chain nitrogen of Asn149 and the side chain carbonyl of Gln515 of the GoLoco motif. This is most likely responsible for the majority of the loss-of-function phenotype of the GLi mutation, as it appears that orientation of this highly conserved glutamine is critical to GoLoco motif function. The only two amino acid positions completely con-

7 The RGS14 GoLoco motif residue Ala512 was observed to interact with Asn149 of G/H, in both asymmetric units in the PDB 2OM2 structure and the chain A/chain B asymmetric unit of the PDB 1KJY structure.
GoLoco-insensitivity Mutation in Ga Subunits

![Image](image.png)

**FIGURE 11.** Wild type, but not GoLoco-insensitive, Ga11(YFP) destabilizes metaphase chromosomes and spindle orientation. Time-lapse images of Hoechst 33342-stained chromosomes during metaphase alignment and segregation were recorded as described (32); supplemental movies are available. Representative consecutive fluorescence images taken from time-lapse sequences showing the motion of Hoechst-stained chromosomes in MDCK II cells expressing Ga11, (wild type)-YFP (upper panel) and Ga11(N149I)-YFP (lower panel). The images were taken every 3 s as described (32). To show the movements of the metaphase chromosomal arrays, the position of the long axis of the chromosomal array along the metaphase plate in each image was marked by red (upper panel) or blue lines (lower panel), and positions of the axis in previous adjacent images are marked with white lines.

**TABLE 1**

Ectopic expression of wild type, but not GoLoco-insensitive, Ga11(YFP) destabilizes spindle orientation

| Angle change during spindle rocking | Relative frequency of observation (%) |
|-----------------------------------|---------------------------------------|
| Wild type Ga11-YFP | N149I Ga11-YFP |
| 0°–5° | 45 (3) | 94 (2) |
| 5°–10° | 38 (4) | 6 (2) |
| >10° | 17 (2) | 0 |

served in all functional GoLoco motifs (supplemental Fig. S1) (7) are the Gln and the Arg residues of the DQR triad. In light of this, we examined the role of Asn149 in Ga11 class subunits. As described above, it has been noted that Asn149 is involved in stabilizing the seabeat configuration between Ga residues Glu43 and Arg178 that is partially responsible for GoLoco motif and Gβγ-mediated GDI activity (39, 63). However, in our studies, interaction between N149I mutant Ga11 subunits and Gβγ subunits appeared to be normal, and this is consistent with Gβγ subunits having GDI activity toward Ga11 despite the Ile substitution at this position in Ga11 (66). Although Asn149 is conserved in all Ga11_o subunits, the closely related Ga11 subunit contains a histidine at this position (Fig. 2A). Interestingly, Ga11 is unique among Ga11 subunits in that it reportedly interacts with the truncated GoLoco motif of Rap1GAP1a in a GTP-selective manner (67) unlike the canonical Ga/GoLoco motif interaction, which is GDP-selective (7).

Ga11/GoLoco Motif Interaction Is Crucial for the Modulation of Microtubule Dynamics —In mammalian cells, overexpression of either GPSM2 or wild type Ga11 has previously been shown to destabilize the processes of mitotic spindle orientation and metaphase chromosome segregation (32). In MDCK cells, this is characterized by an increase in the amplitude of spindle oscillations during metaphase (32). The presumed mechanism of action of Ga11 or GPSM2 overexpression on spindle oscillations is an increased recruitment of force-generating Ga11/GoLoco motif complexes to the plasma membrane (32). Independently, it has also been observed that overexpression of Ga13 or GPSM2 alters spindle pole positioning in mammalian cells (68). However, these observations are only suggestive of a critical function for a Ga11/GoLoco protein complex, given that these results might alternatively reflect separate, distinct functions of Ga and GoLoco proteins in parallel pathways.

To delineate the precise role of Ga11/GoLoco motif interactions in ectopically induced spindle oscillations, we used MDCK cells transfected with either wild type or GLi Ga11_YFP and measured simple spindle oscillations using time-lapse video microscopy. We observed that MDCK cells transfected with wild type Ga11 underwent vigorous mitotic spindle oscillations during mitosis, as described previously (32) (Fig. 11 and supplemental movies 1 and 2), whereas at comparable expression levels, Ga11(N149I)-transfected cells did not exhibit enhanced spindle rocking relative to untransfected cells (Fig. 11 and supplemental movies 3 and 4). To quantify these results, we measured the change of the long angle of metaphase chromosomal arrays during mitosis using image analysis. The amplitude of spindle oscillations induced by wild type Ga11 expression was substantially higher than that found upon GLi Ga11 expression (Table 1). To our knowledge, this result represents the first unambiguous demonstration that direct protein/protein interaction between Ga11 subunits and GoLoco motifs is responsible for the modulation of cortical MT dynamics controlling mitotic spindle orientation.

The precise mechanism of Ga11/GoLoco motif complex-mediated regulation of cortical MT dynamics and spindle positioning during cell division is not clear. The use of our newly described GLi Ga11 mutant in various model systems of symmetric and asymmetric cell division should help to clarify some of the molecular mechanisms of these processes. In particular, there are several important questions that remain unresolved. First, what is the nature of the Ga11 nucleotide binding/hydrolysis cycle that occurs during cell division? What is the active Ga11 species (Ga11-GDP, Ga11-GTP/GoLoco complex, or Ga11/GTP), and what is the hierarchy of participating Ga regulatory proteins such as Gβγ, RIC-8, RGS proteins, and GoLoco motif proteins? A consensus within the field appears to be that Ga11-GDP/GoLoco motif complexes represent the “active” species during MT dynamics in cell division (69). However, the order in which the nucleotide binding and hydrolysis cycle of Ga11 progresses has not been resolved. A recent paper has described RIC-8 as being able to act as a GEF on GoLoco motif liganded Ga11 subunits, thereby implying that GoLoco motif-

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8 The GDP-binding arginine residue is a lysine in Rap1GAP2b/c GoLoco motifs (40). However, these GoLoco motifs have no functional activity (40).
bound Go may be the physiological substrate for RIC-8 (22). However, this same paper also demonstrated that the GoLoco motif is a noncompetitive inhibitor of RIC-8 GEF activity (22), in concordance with earlier results observed within the nematode system (9).

Second, what is the direct mechanism by which Go subunits modulate MT dynamics? This latter question is beginning to be understood. It appears that Go proteins act to relieve intramolecular auto-inhibition of “Pins-like” GoLoco motif proteins (e.g. GPSM1/AGS3 and GPSM2/LGN). Go-GDP binding to the GoLoco motifs of these multidomain proteins is believed to act as a conformational switch, allowing the subsequent binding of members of the nuclear mitotic apparatus/mushroom body defect (NuMA/MUD) family of proteins (21, 32, 70). The nuclear mitotic apparatus/mushroom body defect (NuMA/MUD) proteins are MT-binding and -regulating proteins; thus, their association with GoGoLoco motif protein complexes at the centrosome most likely modulates the dynamics of plus-end astral MTs (71–73).

Conclusion—Our data presented here describe a single point mutation in Go subunits that selectively abrogates the ability of Gq and GoLoco motifs to interact in vitro and in a cellular context. This Asn to Ile mutation in the αD/αE loop of the helical domain of Gα prevents the conserved GoLoco motif glutamine residue from properly orienting the GDP-binding arginine of the GoLoco motif. We have demonstrated the utility of this mutant in interrogating the role of Gα proteins in the modulation of mitotic spindle orientation. We anticipate the widespread use of this GoLoco-insensitivity mutation in both cell culture and in vivo settings to address the physiological roles of GqGoLoco motif complex formation in diverse cell division processes, akin to how the RGS-insensitivity mutation of Gq subunits has been used to identify the physiological roles of endogenous RGS proteins in 7TMR signaling strength and duration (62, 74). A particularly important ancillary use of the GoLoco-insensitivity mutation will be to delineate a potential role for GoLoco motif proteins in 7TMR-mediated signal transduction. Gain-of-function studies suggest that GPSM1/AGS3 can modulate the cellular levels of Gα subunits and thus indirectly affect 7TMR signaling strength (75); other studies have suggested that 7TMR signaling in vivo may be modulated by GPSM1 and GPSM2 function (76–78). Application of the GLi mutant to such studies will surely provide biochemical and structural insights into the biological function of this important class of Gα regulatory proteins.

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