A Global Map of G Protein Signaling Regulation by RGS Proteins

Authors
Ikuo Masuho, Santhanam Balaji, Brian S. Muntean, ..., John J.G. Tesmer, M. Madan Babu, Kirill A. Martemyanov

Correspondence
kirill@scripps.edu

In Brief
Masuho et al. quantitatively define G protein selectivity of all of the canonical members of the regulator of G protein signaling (RGS) family. Based on this information, they determine the structural basis of selective RGS-G protein recognition and demonstrate how selectivity determinants evolved. They further show that the RGS-G protein selectivity is affected by the mutational genomic landscape and can be rationally altered.
**A Global Map of G Protein Signaling Regulation by RGS Proteins**

Ikuo Masuho,1 Santhanam Balaji,2,3 Brian S. Muntean,1 Nickolas K. Skamangas,1 Sreenivas Chavali,2,4 John J.G. Tesmer,5 M. Madan Babu,2,3 and Kirill A. Martemyanov1,6,*

1Department of Neuroscience, The Scripps Research Institute Florida, Jupiter, FL 33458, USA
2MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge CB2 0QH, UK
3Departments of Structural Biology and Center for Data Driven Discovery, St. Jude Children’s Research Hospital, Memphis, TN 38105, USA
4Department of Biology, Indian Institute of Science Education and Research (IISER) Tirupati, Karakambadi Road, Tirupati 517 507, India
5Departments of Biological Sciences and Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907-2064, USA
6Lead Contact

*Correspondence: kirill@scripps.edu

https://doi.org/10.1016/j.cell.2020.08.052

**SUMMARY**

The control over the extent and timing of G protein signaling is provided by the regulator of G protein signaling (RGS) proteins that deactivate G protein α subunits (Gα). Mammalian genomes encode 20 canonical RGS and 16 Gα genes with key roles in physiology and disease. To understand the principles governing the selectivity of Gα regulation by RGS, we examine the catalytic activity of all canonical human RGS proteins and their selectivity for a complete set of Gα substrates using real-time kinetic measurements in living cells. The data reveal rules governing RGS-Gα recognition, the structural basis of its selectivity, and provide principles for engineering RGS proteins with defined selectivity. The study also explores the evolution of RGS-Gα selectivity through ancestral reconstruction and demonstrates how naturally occurring non-synonymous variants in RGS alter signaling. These results provide a blueprint for decoding signaling selectivity and advance our understanding of molecular recognition principles.

**INTRODUCTION**

Heterotrimeric G proteins transduce a vast variety of extracellular stimuli, including hormones, ions, organic molecules, and light into the regulation of intracellular “effectors” to generate cellular responses (Neves et al., 2002). Collectively, G protein systems play a role in nearly every physiological process and in numerous pathologies (Heng et al., 2013; Kostenis et al., 2020; O’Hayre et al., 2014; Wang et al., 2018). G proteins are activated by the binding of GTP to the α subunits (Gα) that release them from inhibitory occlusion by the βγ dimer (Gβγ) (Glukhova et al., 2018; Lambert, 2008; Oldham et al., 2006; Syrovatkina et al., 2016). Mammalian genomes encode a conserved set of 16 Gα subunits, each possessing unique signaling properties and the ability to selectively engage a distinct set of effectors, including adenylate cyclases, phospholipase C isozymes, Rho guanine nucleotide exchange factors (GEFs), and ion channels (Hubbard and Hepler, 2006; Marinissen and Gutkind, 2001; Wettchereck and Offermanns, 2005).

The key determinant of G protein action in cells is their lifetime in an active state. Thus, the activation and deactivation of G proteins is tightly controlled and ought to occur with selectivity for individual G proteins to ensure the selectivity of downstream signaling (Siderovski and Willard, 2005; Syrovatkina et al., 2016; Wettchereck and Offermanns, 2005). Deciphering molecular mechanisms of this selectivity is of paramount importance for understanding how the signals are routed in the cells. A number of G protein activators have been described and demonstrated to act as GEFs on the Gα subunits with clear subtype selectivity (Cismovski et al., 1999; Garcia-Marcos et al., 2011; Tall et al., 2003). Among them, the largest class is the G protein-coupled receptor (GPCR) family (Fredriksson et al., 2003; Hilger et al., 2018; Mahoney and Sunahara, 2016). GPCRs exhibit clear preferences for activating particular Gα species, and there has been tremendous progress in understanding the molecular mechanisms in establishing this selectivity (Flock et al., 2017; Inoue et al., 2019; Masuho et al., 2015b; Okashah et al., 2019).

The opposing process of G protein deactivation occurs when G proteins hydrolyze guanosine triphosphate (GTP), a process assisted by the action of the GTPase-activating proteins (GAPs). The GAP action is essential for avoiding response saturation and for achieving temporal resolution dictated by individual physiological reactions (Ross, 2008). Most well-characterized GAPs for heterotrimeric G proteins belong to the regulator of G protein signaling (RGS) family, consisting of 20 canonical members in mammals (Dohliman and Thorner, 1997; Tesmer, 2009); RGS proteins bind to active Gα proteins and facilitate their GTPase action, thereby accelerating the termination of G protein signaling (Berman et al., 1996b; Hunt et al., 1996; Ross 1997).
Figure 1. Determining Gα Selectivity of All Canonical RGS Proteins in Living Cells

(A) Schematic of the BRET assay. Agonist-bound GPCR leads to the dissociation of inactive heterotrimeric G proteins into active GTP-bound Gα and Venus-Gbg subunits. The free Venus-Gbg interacts with the Gbg-effector mimetic masGRK3ct-Nluc-HA and increases the BRET signal. The application of the antagonist initiates the deactivation of G proteins and decreases the BRET signal.

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Studies in several members of the RGS family indicate that man diseases (Shamseldin et al., 2016; Squires et al., 2018) and is increasingly associated with human diseases (Shamseldin et al., 2016; Squires et al., 2018). Studies in several members of the RGS family indicate that they exert considerable selectivity in recognizing Gα (Heximer et al., 1997; Snow et al., 1998; Soundararajan et al., 2008; Tesmer, 2009; Wang et al., 1998). There has been significant progress documenting cases of selective RGS-Gα interactions (Hollinger and Hepler, 2002), analyzing the structural basis for this selectivity (Soundararajan et al., 2008; Taylor et al., 2016), and mapping amino acid residues involved in specific recognition (Kimple et al., 2009; Kosloff et al., 2011). Although these studies provide insights into the selectivity of RGS action for isolated cases, a comprehensive understanding of the complete landscape of Gα preferences of RGS proteins is still lacking.

This study presents a map of Gα selectivity for all canonical RGS proteins. We monitored the temporal regulation of GPCR-mediated G protein signaling and quantitatively characterized the GAP activity of the RGS proteins, testing nearly all of the theoretically possible Gα-RGS pairings (300 combinations). Using the functional activity as a readout in the context of a physiologically relevant cellular environment allowed us to document the preferences of RGS proteins for Gα substrates, revealing pairings and disallowed combinations. This information led to the identification of molecular determinants involved in the selectivity of Gα-RGS recognition. Applying computational algorithms, we also show how these determinants have evolved and can be used to create designer RGS proteins with novel selectivity profiles. Analysis of human genomic data further suggests that genetic variations in RGS selectivity determinants may contribute to non-disease traits, pathological dysregulation of GPCR signaling, and variable responsiveness to drug treatments.

RESULTS
Assaying Activity of All Canonical RGS Proteins on Gα Deactivation with a Real-Time Kinetic Approach in Living Cells
To test their possible RGS-Gα coupling systematically, we used a cell-based system that provides a cellular environment to study the action of RGS in the context of GPCR signaling. This assay monitors RGS-induced acceleration of G protein deactivation by real-time bioluminescence resonance energy transfer (BRET) strategy tracking the kinetics of heterotrimer re-association upon antagonizing GPCR, a reaction catalyzed by RGS proteins physiologically (Figure 1A). The key features of the assay include a “bystander” approach that allows the use of unmodified Gα subunits (Figure 1B) and full-length RGS proteins (Figure 1C).

Using a set of GPCRs with varying Gα selectivity, we recorded the deactivation kinetics of 15 Gα subunits (omitting sensory Gαt1, Gαt2, and Gαt3, but including the two common splice variants of Gαq and Gα11) in the absence of exogenous RGS proteins. A combination of intrinsic differences in Gα properties and the action of endogenous RGS proteins in HEK293T/17 cells yielded characteristic baseline deactivation rates (Figures 1D and 1E). Using a previously established approach (Masuho et al., 2013), we ensured that the deactivation kinetics were rate limited by the Gα GAPase activity. Disruption of RGS-Gα interactions by RGS-insensitive (DiBello et al., 1998; Lan et al., 1998) or GAP-deficient mutations (Druey and Kehrl, 1997; Srinivasan et al., 1998) substantially prolonged response recovery (Figure S1). These mutations interfere with the conserved interaction of RGS proteins with the switch I region of the Gα subunits. Further controls demonstrated that (1) the exogenous expression of RGS proteins does not alter the expression of signaling molecules and sensors (Figures S2A and S2B), (2) the different expression levels of GPCRs or the different amounts of active G proteins do not change the G protein deactivation rates (Figure S2C), and (3) deactivation rates are directly proportional to the amount of RGS (Figure S2D). These results confirm that RGS action dictates the kinetics of G protein deactivation. Analysis of the deactivation traces for a representative Gα (Gαsα) shows the varying impact of different exogenous RGS proteins on the kinetics of Gα termination (Figures 1F and 1G).

To quantify the activity of RGS proteins, the baseline deactivation rates (1/r) of each Gα were subtracted from the deactivation rates in the presence of exogenous RGS proteins, yielding the kGAP parameter (Figure 1H), a widely used metric of RGS catalytic activity (Ross, 2002). Plotting kGAP values for each of the Gα substrates provides a profile of relative activity for a given RGS protein. Analysis of the representative members of the RGS subfamilies using this strategy revealed differences in Gα preferences in a fingerprint-like fashion (Figure 1I). These Gα selectivity fingerprints were not affected by differences in the RGS expression levels (Figure S3A–S3D).

Principles of Gα Regulation by RGS Family
This strategy was applied to measure the activity of all 20canonical RGS proteins on the deactivation of each of 15 Gα subunits in a total of 300 possible combinations. We optimized RGS expression levels, ensuring at least 3-fold acceleration of the
deactivation rate for the preferred G \(z \)-type substrate to reliably assess even minor coupling. In particularly difficult cases (e.g., RGS13, RGS18), proteasomal blockade and codon optimization strategies were applied to augment RGS expression (Figures S3E–S3K). Given the differences in the expression levels of various RGS proteins, we did not attempt to compare their absolute activities and instead focused on elucidating the relative differences in G protein preferences. Collectively, our results provide a comprehensive G \(z \)-selectivity profile for the entire RGS family (Figures 1J and S4; Table S1).

Analysis of the RGS-G \(z \) interaction network provided several key insights. We found that RGS proteins vary markedly in the breadth of their selectivity, with some members (e.g., RGS1) key insights. We found that RGS proteins vary markedly in the breadth of their selectivity, with some members (e.g., RGS1) being very slow intrinsic deactivation rate (0.0003 s\(^{-1}\)) of basal GTPase activity of G \(z \), possibly underestimated the selectivity of its regulation by RGS proteins when assessed by the \(k_{\text{GA}}\) parameter (Figures S5G–S5I). Accordingly, we calculated a discrimination index (\(k_{\text{dis}}\)) defined by fold increase in the deactivation constant (1/\(k_{\text{d}}\)) upon the addition of RGS (Figure S5J). Although considering that \(k_{\text{d}}\) did not change the overall picture of G protein selectivity for most RGS members, it was useful in showing the unique ability of RZ subfamily members to uniquely regulate G \(z \) (Figures S5K and S5L) amidst their significant activity on virtually all of the other G \(z \) and G \(z \)-type proteins based on the \(k_{\text{GA}}\).

These data also revealed high selectivity in the regulation of the poorly studied G \(z_{15}\). This G protein is activated by a wide range of GPCRs and thus likely contribute to a variety of cellular responses (Offermanns and Simon, 1995). We found that it has a very slow intrinsic deactivation rate (0.0081 ± 0.0006 s\(^{-1}\)), making RGS regulation paramount for the temporal control of its signaling. Interestingly, G \(z_{15}\) can be deactivated by only a few RGS proteins (Figure S5D), mostly G \(z_{12}\)-type-prefering R4 members and an RZ subfamily member, RGS17 (Figures 2C and S5F).

These studies further revealed that no canonical RGS proteins could regulate the deactivation of G \(z_{2}\), G \(z_{9}\), G \(z_{12}\), or G \(z_{15}\) (Figure 1J). This outcome is perhaps not unexpected. Structural modeling shows that the switch I region of G \(z_{12/13}\) contains Lys-204 instead of a Thr present in all of the other G \(z \) subfamilies in the corresponding position, rendering it incompatible with RGS binding (Figures S5M and S5N). Furthermore, the structure of the \(z\)-B–C loop in the \(z\)-helical domain is also fundamentally different in G \(z_{12/13}\), contributing to the steric occlusion of canonical RGS protein binding (Sprang et al., 2007). Similarly, the presence of Asp229 in G \(x \), a position conserved as serine in all other G \(x \) subfamilies, renders it incapable of RGS binding in G \(x \) family members (Natochin and Artemyev, 1998) due to collisions with the \(z\)-A–B loop of RGS proteins (Figures S5O and S5P). The G \(x_{2}\) D229S mutation restores the ability of G \(x_{4}\) and G \(x_{16}\) to bind and the ability of G \(x_{16}\) to accelerate GTP hydrolysis on G \(x_{2}\) (Natochin and Artemyev, 1998).

**RGS-G \(z \) Recognition Patterns Selectively Shape Endogenous Secondary Messenger Signaling**

To study how global patterns of RGS-G \(z \) selectivity affect the processing of GPCR signals endogenously, we used striatal medium spiny neurons (MSNs) as a model (Figure 3A). The MSNs were chosen because of their undisputed physiological importance and the critical role of several well-defined GPCRs in processing neuromodulatory inputs to these neurons (Girault, 2012; Xie and Martemyanov, 2011) (Figure 3B). More important, several RGS proteins in the MSNs have been implicated in controlling behavioral responses to GPCR stimulation. The best-documented examples of these are RGS4 (Han et al., 2010; Michaelides et al., 2020), a member of the R4 subfamily, and RGS9 (Traynor et al., 2009), a member of the R7 subfamily.

We surveyed the expression landscape of RGS and G \(z \) proteins by curating the available quantitative RNA sequencing (RNA-seq) data (Gokce et al., 2016). This analysis revealed a significant expression of 12 RGS genes, with RGS4 and RGS9 being the most abundant. Three members of the R4 subfamily (RGS4, RGS2, and RGS8) and 3 members of the R7 subfamilies (RGS9, RGS11, and RGS7) were estimated to be more highly expressed by at least an order of magnitude than other striatal RGS proteins (Figure 3C). Interestingly, our dataset indicates that these RGS subfamilies have distinct patterns of G \(z \) selectivity; the R7 RGS proteins are narrowly tuned for G \(z_{9}\), whereas the R4 RGS members are capable of regulating a broad spectrum of G \(z \), including both G \(z_{9}\) and G \(z\) members (Figures 2B and 2C). Accordingly, transcripts encoding the members (G \(z_{z}\), G \(z_{9,14}\), G \(z_{9,13}\), G \(z_{9,12}\), and G \(z_{9,11}\)) of the G \(z_{9}\) and G \(z\) subfamilies were abundantly expressed by the MSNs (Figure 3C). Thus, we predicted that R4 RGS proteins would have a major influence on the processing of GPCR signals via both G \(z_{9}\) and G \(z\) pathways, whereas R7 RGS proteins would selectively affect only G \(z_{9}\)-mediated signals.

To test this prediction, we used biosensors to monitor the dynamics of second messenger pathway engagement downstream of both G \(z_{9}\) and G \(z\) while inactivating RGS proteins by CRISPR-Cas9 editing in the primary cultures of MSNs (Figure 3D). The G \(z_{9}\) activity was assessed by studying its inhibitory influence on cyclic AMP (cAMP) production in response to stimulation of the G \(z_{9}\)-coupled dopamine receptor D2 (D2R) by dopamine, whereas G \(z\) type activity was monitored by Ca \(^2+\) transients induced in response to the activation of the muscarinic M1/M3 receptors (M1/3R) by acetylcholine (Figure 3B). Considering the intra-class similarity of RGS-G \(z \) pairing and abundant expression of several members from each RGS class, we chose to simultaneously eliminate all MSN-expressed RGS proteins.
belonging to the same subfamily by CRISPR-Cas9 editing. The elimination of either the R4 or the R7 subfamily resulted in a significantly enhanced cAMP response, consistent with the role of these RGS members in the deactivation of the G{i/o} pathway (Figures 3E and 3F). In contrast, the elimination of R4 members but not R7 proteins augmented the Ca^{2+} response, which is in line with their observed G_{q} selectivity profiles (Figures 3G and 3H).

We next tested the effect of overexpressing individual RGS proteins. We chose to focus on RGS2, an abundantly expressed RGS protein, widely believed to be G_{q} selective based on biochemical measurements but able to regulate G{i/o} proteins.

**Figure 2. The Complete Network of RGS-G_{x} Interactions**

(A) Recognition patterns of G_{x} by RGS proteins. The width of lines connecting RGS and G_{x} indicates the strength of GAP activity. Nodes represent total GAP activity of RGS proteins (left side) or on G_{x} subunits (right side).

(B) G_{x} selectivity of RGS subfamilies obtained by dividing the total GAP activity on each G_{x} subunit by the number of RGS proteins with statistically significant GAP activity (see Figure S5F).

(C) RGS selectivity of G_{x} subunits obtained by dividing the total GAP activity of an RGS protein on all regulated G_{x} by the number of G_{x} subunits (see Figure S5C).
Figure 3. Implications of RGS Selectivity for GPCR Signaling in Striatal Neurons

(A) Schematic of the neurotransmitter inputs processing by medium spiny neurons (MSNs) in the striatum.

(B) Organization of striatal GPCR signaling cascades and the potential impact of RGS proteins.

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according to our data (Figures 1J and S4). The overexpression of RGS2 had an opposite effect on eliminating RGS proteins and dramatically suppressed the amplitudes of both cAMP and calcium responses (Figures 3I–3L). These observations indicate that the comprehensive RGS-Gz selectivity maps have predictive power in dissecting the logic of GPCR signal processing in an endogenous setting.

**Flexibility of Gz Selectivity Encoded in the RGS Homology Domains**

The analysis presented in this study revealed a wide range of Gz preferences across RGS proteins, which also feature considerable structural diversity (Riddle et al., 2005). This opens questions about the flexibility of recognition patterns across the family and the degree with which Gz selectivity is determined by the RGS domain shared by all RGS proteins. To address these questions in an unbiased way and gain insight into how the selectivity of mammalian RGS subfamilies may have evolved, we performed the reconstitution of ancestral RGS proteins (Figure 4A).

We traced the RGS family tree to reconstitute common ancestral RGS domains at three branch points before the diversification into the current four subfamilies and generated a series of chimeric RGS proteins (Figure 4B).

Examination of the Gz selectivity of the primordial ancestral RGS protein (AncR4/Z/12/7) revealed that it regulated all Gz subunits that RGS proteins can regulate, except Gz15 (Figures 4C and 4D). We next reconstructed two ancestral RGS proteins at the roots of the subfamily divisions (AncR4/Z and AncR12/7). Interestingly, AncR4/Z showed equally strong GAP activity toward Gz15 and Gz25 subfamilies, but not toward Gz9 (Figure 4D). Diversification of this precursor RGS subsequently generated various patterns of Gz15- and Gz25-selectivity observed in current R4 and R2 subfamilies. The other ancestral RGS protein, AncR12/7, showed Gz9 selectivity and was devoid of the ability to regulate the Gz9 subfamily. This ancestral RGS gave rise to Gz9-selective R12 and R7 RGS proteins. These results suggest that Gz selectivity patterns of extant human RGS proteins resulted from a combination of specialization along the Gz15 and Gz25 axes and de novo acquisition of Gz2 and Gz15 selectivity. This supports a predominantly evolutionary divergence model in which the primordial RGS precursor with balanced activity on different Gz substrates acquired various biases that followed different routes—for example, by suppressing the GAP activity toward the Gz9 subfamily in R7 and R12 RGS or re-gaining the activity on Gz15 subfamily by the R12 RGS. We thus conclude that the sequence composition of the RGS domain has considerable bearing on dictating the evolving Gz preferences of the RGS proteins, strongly suggesting that the major determinants of Gz selectivity are contained within the RGS domain.

**Structural Determinants Governing the Selectivity of Gz Recognition by RGS Proteins**

Elucidation of a Gz-RGS coupling map and demonstration of the crucial role of the RGS domain in determining the pairings prompted the identification of molecular determinants that govern their differential preferences. We compared the sequences of all human RGS domains, aligning them with reference to 20 available high-resolution structures that show the same conserved fold and preservation of key elements, with 9-z-helices and 10 loops (Figure S6A; Data S1). RGS11, RGS13, RGS20, and RGS21 were not included in this analysis because their structures have not been reported. This analysis allowed us to develop a Common RGS Numbering (CRN) system for labeling amino acids relative to their structural position similar to what was previously done for Gz (Flock et al., 2015) and GPCRs (Ballesteros and Weinstein, 1995; Isberg et al., 2015) (Figures S6B and S6C). This system helps to identify the position of every residue with reference to the secondary structure. For instance, RGS4 Asn128, which directly binds to Gz15, is denoted as L6.10, indicating that this residue is the 10th amino acid located in loop 6 of the RGS domain (Figure S6B). It should be noted that this nomenclature cannot be applied to the H6 region in the R12 subfamily because it is structurally distinct from other RGS subfamilies.

We further analyzed eight currently available structures of RGS/Gz complexes and found that all RGS and Gz subunits interact in a very similar manner, with low root mean square deviation (RMSD) in the range of 0.46–1.42 Å. In the RGS domain, there are 11 residues directly contacting Gz that are almost 100% conserved in all structures (Figure S6B). In addition to these contacting positions, we found 20 residues on the RGS protein and 38 amino acids on Gz that contribute to the organization of binding interfaces based on their localization within the 5Å radius of any atom in the interface. On the RGS side, these...

(C) Analysis of single-cell RNA-seq of MSNs (Gokce et al., 2016) for RGS and Gz expression in alignment with experimentally derived GAP selectivity patterns from Figure 1.

(D) Experimental design involving primary striatal neurons from cAMP Encoder Reporter (CAMPER) mice transduced with lentiviral particles containing RGS-targeted single guide RNA (sgRNA) (3 per gene) for CRISPR-Cas9 editing. For cAMP imaging, the CAMPER cAMP sensor was activated by the delivery of Cre recombinase. For Ca2+ imaging, neurons were transduced with adeno-associated virus (AAV) particles encoding DIO-GCaMP7s along with the lentiviral particles for CRISPR-Cas9 editing.

(E) Average cAMP response to dopamine (1 μM) in CAMPER striatal neurons following CRISPR-Cas9 editing (n = 6–8 neurons).

(F) Quantification of maximum cAMP amplitude in (E).

(G) Average Ca2+ response to acetylcholine (10 μM) in neurons expressing jGCaMP7s following CRISPR gene editing (n = 14–27 neurons).

(H) Quantification of maximum Ca2+ amplitude from (G).

(I) Average cAMP response to dopamine (1 μM) in CAMPER striatal neurons following the overexpression of RGS2 (n = 8 neurons).

(J) Quantification of maximum cAMP amplitude from (I).

(K) Average Ca2+ response to acetylcholine (10 μM) in striatal neurons expressing jGCaMP7s following the overexpression of RGS2 (n = 16 neurons).

(L) Quantification of maximum Ca2+ amplitude from (K).

One-way ANOVA followed by Fisher’s least significant difference (LSD) (F and H). Unpaired t test (J) and (L). *p < 0.05 and **p < 0.01. Data are shown as means ± SEMs from 3–5 independent experiments.
residues are distributed across 3 structural elements, 2 loops (H3–H4 and L6–H6) and 1 helix (H7–L9) (Figures 5A and 5B). The surface on Gα is more distributed and involves both GTPase and α-helical domains.

To determine which elements most strongly contribute to the selectivity of Gα recognition, we analyzed these 31 RGS residues at the Gα-binding interface across all 20 human RGS paralogs in comparison with their orthologs from 21–65 animal species.
Figure 5. Selectivity Determinants of Gα Recognition by RGS Domain

(A and B) Gα-binding surface of RGS domain. GTPase and α-helical domains of Gα subunit are colored red and green, respectively. All of the RGS residues in structural elements within 5 Å from the Gα subunit are colored. The number of residues in each structural element is in parentheses.

(C) The selectivity and conserved residues on the Gα-binding surface according to common numbering nomenclature.

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This analysis revealed 14 highly conserved positions across orthologs and paralogs, suggesting that they likely serve as invariable architectural pillars that organize Gz binding and/or GAP activity. These residues included all of the direct Gz-contacting positions found in the RGS4/Gz1 complex (Figure S6B). A minor fraction of the scattered residues was ortholog variable and neutrally evolving (Figures 5C and 5E). The remaining fraction of ortholog-specific residues comprised 17 amino acids. Mapping them on the RGS domain structure showed that they are distributed at the periphery of the Gz-binding surface, surrounding the central positions of the conserved amino acids (Figure 5D), suggesting that they may contribute to Gz selectivity by modulating the interaction. We subsequently refer to these peripheral amino acid residues that are variable among paralogs but conserved within their respective orthologs as Gz selectivity bar codes for RGS proteins.

To identify motifs in the RGS domain that contribute to establishing Gz selectivity, we reconstructed and analyzed the RGS-Gz interaction network at a single amino acid resolution (Figures 5F and 5G). This analysis confirmed that the vast majority of selectivity bar code residues are engaged in non-conserved contacts that vary between different structures of the RGS-Gz complexes (Figure 5H). In contrast, the contacts involving the conserved residues were also predominantly conserved across RGS-Gz structures (Figure 5H). The highest degree of conserved residue-residue contacts is observed for the H3–H4 region with G.H2 and switch I in Gz and for the L7–L9 region with switch I (Figure 5F), indicating its crucial role as a structural backbone for RGS/Gz binding. In contrast, the interaction of the H7–L9 region with the z-helical domain showed the highest number of non-conserved contacts (Figure 5G), suggesting that these domains could significantly contribute to the RGS-Gz selectivity.

To better characterize the organization of the Gz-binding surface, we analyzed properties of the amino acids that form the Gz selectivity bar codes across different RGS subfamilies. This investigation revealed distinct patterns in accordance with the experimentally determined Gz selectivity patterns (Figure 5I). For example, R4 and RZ subfamilies that are dually selective for the Gz-q and Gz-q proteins showed a similar distribution of hydrophobic and positively charged residues in the H7–L9 region; hydrophobic and positively and negatively charged residues in L6–H6; and a nucleophilic residue in H3–H4. In contrast, the Gz-q-selective R12 family exhibited a different pattern featuring nucleophilic, aromatic, and amide residues in the H7–L9 region, and a unique positively charged patch in the L6–H6 lobe surrounded by the nucleophilic cluster. However, another pattern was observed in the narrowly tuned R7 proteins whose L6–H6 region is populated by small amino acids adjacent to the hydrophobic patch and a prominent positive charge in H7–L9. These findings reinforce the idea that the nature of amino acid properties at the selectivity bar code region on the Gz-binding interface of the RGS protein comprises major determinants of Gz recognition selectivity.

**Design Principles for Engineering RGS Protein Selectivity**

The identification of selectivity bar code residues in RGS proteins raises a question about their necessity and sufficiency in setting the selectivity of Gz recognition. This question was addressed experimentally, by transplanting the entire distributed pattern of selectivity residues (Figure 6A). For these experiments, we chose RGS13 and RGS18, which belong to the same R4 subfamily but differ in G protein selectivity (Figure 6C). RGS13 prefers Gz members over the Gz-o subfamily, whereas RGS18 equally regulates both Gz-o and Gz proteins. A comparison of their Gz selectivity bar codes indicates that they differ by 12 amino acid residues (Figure S7A). All of the amino acid residues of RGS13 were replaced with the ones from RGS18, resulting in RGS13/18-F chimera (Figure 6B). In agreement with the prediction based on our selectivity bar code model, RGS13/18-F protein exhibited RGS18-like Gz selectivity (Figure 6C).

These experiments were then extended to RGS8 and RGS14, a pair that belongs to different subfamilies and also have markedly different Gz selectivity and composition of Gz selectivity residues (Figures 6D and 6E). We identified 15 different amino acids within the Gz selectivity bar code different between these RGS proteins (Figures 6D and S7B) and transplanted all of these from RGS14 into corresponding positions of RGS8, generating a “full” chimera (RGS8/14-F) (Figure 6D). The RGS8/14-F chimera completely recapitulated the Gz fingerprint of RGS14 without gaining activity on G proteins not regulated by RGS8 or RGS14 (Figure 6E). We further probed whether the change in selectivity could be achieved by mutating fewer bar code residues (i.e., by replacing only nine amino acid residues) (Figures S7B). The resulting “partial” RGS8/14 chimera (RGS8/14-P) had the same Gz-q over Gz-o preference as parental RGS8 (Figure S7C). It thus failed to switch the Gz-selectivity fingerprint from the RGS8 to the RGS14 pattern, indicating that all of the bar code amino acids are required for establishing exact selectivity patterns of Gz-RGS recognition. Curiously, the RGS8/14-P mutant unexpectedly gained activity on Gz-q (Figure S7D), indicating that individual residues within the bar code can have an impact on the Gz selectivity of RGS proteins. Overall, these results indicate that identified selectivity bar codes are sufficient in dictating Gz substrate preferences.

(D) Mapping the conserved (blue) and selectivity (orange) residues on the Gz-interacting surface of the RGS domain.
(E) Quantitative analysis of the ortholog-specific, paralog-specific, neutrally evolving, and conserved residues.
(F and G) Interaction network between structural elements in RGS and Gz. The width of the lines indicates the number of non-covalent contacts. The nodes represent the total number of residue-residue contacts for each structural element. Common residue numbering (Flock et al., 2015) is used to indicate the structural elements in the Gz subunit.
(H) Quantitative analysis of the number of conserved and non-conserved contacts at the RGS-Gz binding interface.
(I) Amino acid properties of selectivity residues with >60% conservation. The asterisks indicate the conserved amino acid residues between R4 and RZ subfamilies. The PDB accession number 1AGR is used in (A), (B), (D), and (I).
Figure 6. Rewiring Gα-Selectivity by Overwriting Gα-Selectivity Bar Codes

(A) Scheme for rewiring Gα-selectivity.
(B) Gα-selectivity bar codes of RGS13 wild type (WT), RGS18 WT, and RGS13/18-F chimera.
Genomic Landscape of Variability in RGS Selectivity in the Human Population

To gain insight into how ongoing evolutionary diversification shapes Gz selectivity, we analyzed natural variation in RGS sequences. Prevalence analysis of missense variations (MVs) reported for 2,504 healthy individuals from the 1000 Genomes Project (Auton et al., 2015) revealed that, on average, an individual harbors 5 MVs within the canonical RGS proteins. Examination of the database (Tumer et al., 2017) indicated that a de novo MV occurs at approximately every 260 newborns, suggesting that RGS proteins are undergoing active evolution. We further analyzed the data on MVs within all of the canonical RGS proteins in 141,456 individuals (Data S3) from the gnomAD database (Karczewski et al., 2020). We found 106,521 rare MVs (minor allele frequency < 2%), with 79,167 MVs on the outside of the RGS domain, 27,354 MVs in the RGS domain, 1,220 MVs in conserved residues, and 1,757 MVs in selectivity residues (Figure 7A). In this analysis, the same variant type is counted multiple times if it occurs in multiple people, illustrating the scale of ongoing evolution (Figures 7A–7D). On average, 13 MVs exist in each amino acid residue of RGS proteins (Figure 7A). This density of MVs (14.8) was the highest outside of the RGS domain. In contrast, functionally important regions exhibited lower densities. The conserved and selectivity residues in RGS11 were the most variable among all of the RGS proteins (Figures 7B and 7C). The ratio of the MV density between selectivity and conserved residues revealed the highest MV frequency in the selectivity residues over the conserved residues in RGS17 (Figure 7D), suggesting likely extensive natural variation of Gz selectivity in RGS17.

To understand the functional implications of the observed variations, we investigated the impact of randomly chosen seven mutations across various positions in the selectivity bar code region of six RGS proteins by testing their activity on the panel of six Gz subunits (Figure 7E). We found that all of the evaluated amino acid changes affected Gz selectivity. Notably, changes at L7.13 in RGS19 (R190W) increased the GAP activity toward Gz1 but decreased the activity on Gz2, Gz11, and Gz20 without any influence on Gz2. Alterations in L6.8, H6.2, H7.6, and H7.9 selectively augmented the regulation of Gz10 without diminishing the activity on other Gz. The balance between Gz2 and Gz20 regulation can also be affected by these mutations—for example, E98G (L6.8) in RGS13 preferentially increased activity toward Gz20 over Gz2, while R351Q (H6.2) in RGS11 and N164S (H7.9) in RGS12 augmented Gz2 regulation more than Gz20. Altering the H6.4 position in RGS9 M370K resulted in a net loss of activity across Gz regulated by this RGS.

Interestingly, variants in RGS proteins are also increasingly viewed as possibly contributing to pathological conditions due to generally disruptive effects (DiGiacomo et al., 2020; Squires et al., 2018). However, the exact mechanisms of functional alterations and implications for Gz selectivity for a vast number of cases remain unexplored. For instance, RGS16 has been recently implicated in insomnia (Hu et al., 2016; Lane et al., 2016), and knockout of this gene in mice disrupts circadian regulation (Doi et al., 2011). The genetic variation (rs1144566) in human RGS16 reported in the genome-wide association study (GWAS) catalog (Buniello et al., 2019) affects selectivity bar code residue H6.4 (Figures 7F and 7G). We experimentally evaluated the functional implication of minor allele variations in H6.4 of RGS16 prevalently occupied by arginine. Our data showed that the R137P mutation nearly completely abrogated the GAP activity of RGS16 for both of its representative preferred substrates, Gz20 and Gz29, indicating a strong loss of function (Figure 7H). Curiously, the R137L substitution selectively compromised the activity of RGS16 only on Gz20 without significant effects on the regulation of Gz29. These results indicate that mutations in the selectivity bar code may lead to RGS dysfunction associated not only with the complete loss of function but also with a more subtle alteration in the Gz selectivity.

DISCUSSION

In this study, we present a nearly complete map of Gz recognition selectivity for all 20 canonical human RGS proteins. The wealth of accumulated evidence in the past 2 decades since their discovery revealed that members of the RGS family exert two distinct effects on the G protein signaling. First, they accelerate G protein deactivation and thus control the duration of signaling. The slow intrinsic GTPase activity of Gz subunits rate limits the termination of the response and does not permit the rapid signaling cycles often demanded by the physiological processes (e.g., in neuronal communication and cardiac activity). By accelerating the Gz GTPase, RGS proteins speed up termination of the response and thereby increase the temporal fidelity of GPCR-initiated signaling. This function is best exemplified by studies on photoreceptors in which the loss of RGS protein in the visual cascade initiated by rhodopsin diminishes the temporal resolution of visual signals, preventing the detection of moving objects (Chen et al., 2000). Second, by deactivating G proteins and/or competing with the effector molecules, RGS proteins interfere with signal propagation, thus taming the extent of signaling (Hepler et al., 1997; Lambert et al., 2010) and allowing adjustment of the signaling volume, depending on the physiological needs. The loss of this RGS function is well noted to sensitize responses causing cellular overreactivity (Lamberts et al., 2013; Neubig, 2015; Xie et al., 2012). From this perspective, RGS proteins could be considered endogenous genetically encoded antagonists of GPCR signaling.

The results of our systematic profiling of RGS substrate preferences prompt reconsideration of the mechanisms involved in cellular signaling diversification. Despite their large numbers, GPCRs can only signal through the same limited number of G proteins that they can activate. Previous studies indicated that signaling diversity is in part dictated by a combination of G proteins activated by individual GPCRs (Inoue et al., 2019; Masuho...
Figure 7. Impacts of Genetic Variation on Gα Selectivity of RGS Proteins

(A) The density of MVs as calculated by the number of MVs divided by the number of amino acid residues in each structure.

(B and C) The density of MVs in selectivity and conserved residues. If the density is >1, then >1 MV exists in each amino acid residue in the structural element on average.

(legend continued on next page)
The negative regulation of individual Gz by RGS proteins, if sufficiently selective, would greatly contribute to signaling diversification to allow much more refined signaling characteristics with cellular specificity depending on the available RGS and G proteins. Whereas recent large-scale efforts have provided tremendous system-level insights into the selectivity of G protein activation by GPCRs (Flock et al., 2015; Inoue et al., 2019; Masuho et al., 2015b), the information about the selectivity of RGS has been missing. We fill this gap by establishing Gz selectivity profiles for the entire family of RGS proteins. Based on this information, we propose that RGSs and GPCRs work in synergy to generate diverse cell-type-specific signaling.

Although the experiments presented in this study demonstrate the importance of the bar code residues on the Gz-interacting interface of RGS proteins in dictating Gz preferences, the sufficiency of this residue-residue contact network in dictating precise selectivity patterns across the entire RGS family remains to be tested. It appears quite likely that the secondary network of residues that make contact with the Gz-binding residues on the surface can further adjust and/or reinforce the stringency of Gz recognition. In support of this possibility, members of the R4 subfamily show more diverse functional properties than sequence similarity, suggesting contributions of additional residues within the RGS domain outside of the Gz-interacting surface in shaping Gz selectivity. This is consistent with the results of our ancestral reconstruction experiments, that shuffling wider group of the amino acid residues in the entire RGS domain can also modulate Gz selectivity. Furthermore, elements outside of the RGS domains may further contribute to the Gz recognition preferences of RGS proteins. Such a possibility is suggested by studies on complex multi-modal members of the R7 family, in which interaction partners (Gαs and R7BP) (Levay et al., 1999; Masuho et al., 2013) and domains (DEP, PGL) (Martemyanov et al., 2003; Skiba et al., 2001) have been shown to regulate Gz recognition. Many RGS genes also produce multiple splice isoforms that alter the structure of RGS proteins by adding or eliminating functionally important motifs without changing the RGS domain (Barker et al., 2001; Chatterjee et al., 2003; Granneuman et al., 1998; Saitoh et al., 2002) and may further fine-tune Gz selectivity. Finally, several RGS proteins also interact with GPCRs, G protein effectors, and scaffold proteins (Abramow-Newerly et al., 2006), and this event may further alter Gz specificity. Although these possibilities were not addressed in this study, our experiments with shuffling determinants, mutagenesis, and ancestral reconstructions all within the RGS domain indicate that these additional mechanisms may contribute to establishing the Gz selectivity but are unlikely to completely overwrite it.

Previous biochemical studies used purified recombinant proteins to examine the preferences of RGS proteins on Gz substrates selected ad hoc yielding important information that has served as a reference for RGS-Gz pairing. For example, RGS4 was shown to regulate both Gα16 and Gα2 subfamilies, but not Gα12 or Gα13 (Berman et al., 1996a; Berman et al., 1996b; Hepler et al., 1997). In contrast, RGS2 was found to have no appreciable GAP activity toward Gαib and to be selective for Gαib in both solution GTPase assays and pull-down experiments (Heximer et al., 1997; Kimple et al., 2009). R7 RGS family members were reported to be Gα selective, with weaker GAP activity on Gαi (Hooks et al., 2003; Posner et al., 1999a; Snow et al., 1998).

The selectivity of RGS7 for Gαo over Gαi was observed with the purified RGS domain (Lan et al., 2000), which is consistent with our conclusion that its RGS domain encodes a Gz selectivity bar code. GzR selectivity of RZ subfamily members RGS17 (RGSZ2), RGS19 (GAIP), and RGS20 (RGSZ1) was also observed (Glick et al., 1998; Wang et al., 1998). An R12 RGS member, RGS10, has been shown to regulate Gαo, Gαs, and Gαq, but not Gαz (Hunt et al., 1996; Popov et al., 1997). Our investigation confirms many of the previously noted Gz preferences of RGS proteins, while additionally refining them to include G proteins not previously studied. However, in some cases, our results contradict previously documented coupling. One of the notable examples of this is GzR selectivity of RGS2. Although our investigation shows that RGS2 can indeed regulate several members of the Gαz subfamily, we also find that it exhibits strong activity on the Gαs proteins comparable to that on Gαo. We think that the discrepancy is largely related to the choice of the assay system. Most of the previous studies used purified RGS and Gz proteins and measured GTP hydrolysis rates using biochemical assays conducted in solution. This approach has limited sensitivity and is devoid of the membrane environment where GPCRs, RGS, and G proteins normally operate under physiological context. In fact, the activity of RGS proteins has been shown to be significantly modulated by the membranes and lipid modification on Gz subunits (Tu et al., 1997). Furthermore, the proteoliposome-based assay was found to yield ~100-fold higher sensitivity as compared to the solution-based assay (Posner et al., 1999b). RGS2, in particular, was noted to act on Gαzib in the presence of lipid bilayer (Ing et al., 1998). Thus, the cellular BRET assay strategy that we chose provides physiologically relevant information on RGS-Gz coupling as it exploits the endogenous environment and appropriate context of RGS action.

One of the key insights provided by this work is the delineation of the determinants involved in RGS-Gz recognition. Establishing principles involved in the selectivity of protein-protein interaction has been a major goal of many investigations (Flock et al., 2017; Nooren and Thornton, 2003). Interaction between RGS and Gz provides an excellent model for interrogation of the underlying principles with possible general implications.
Both protein families are well represented by numerous members with clearly defined orthologs and paralogs, and conservation of the structural organization (Baltoumas et al., 2013; Tesmer, 2009). Thus, the experimental definition of the Gα preferences of all of the RGS proteins naturally prompted dissection of the underlying selectivity determinants. This study was focused on examining the contribution of the Gα-binding surface of the RGS domain. A combination of gene orthology/paralogy analysis with structural mapping identified a set of 17 variable amino acids that surround the core critical for forming direct contacts with the Gα subunits. We found that mutations in these amino acids significantly change the Gα preferences of RGS proteins. Interestingly, transplanting sets of variable amino acids from one RGS protein to another completely overwrites the Gα selectivity of the recipient. These observations support the idea that the selectivity of Gα recognition is, at least in part, encoded by the property of the amino acids that form this bar code region on the surface.

Previous studies explored the role of electrostatic interactions in specifying the selectivity of Gα recognition by several RGS proteins across all of the subfamilies (Asli et al., 2018; Israeli et al., 2019; Kosloff et al., 2011; Salem-Mansour et al., 2018). Collectively, these studies reported 12 amino acid residues in RGS proteins that influence their ability to recognize Gα. Mutation of these residues either alone or in combination (up to 7 simultaneously) was shown to either increase or decrease the GAP activity of RGS proteins on the Gα substrates of choice. These studies examined one Gα substrate at a time, thus making it unclear whether the manipulations resulted in switching relative Gα preferences for a given RGS as opposed to overall gain or loss of substrate recognition. Nevertheless, these studies convincingly demonstrate that changes in electrostatic properties of amino acids at the RGS-Gα interface can alter the efficiency of the Gα recognition. Interestingly, all but two (H4.4 and H5.14) of these residues mapped on the Gα selectivity bar code region identified in this study, supporting the idea that electrostatic interactions play an important role in shaping the selectivity of RGS-Gα recognition. Similarly, mutations in RGS2 at the interface with the α-helical domain of Gα subunit diminished GAP activity on GαD (Nance et al., 2013). In agreement with a large number of contacts made by the α-helical domain with the RGS domain, our analysis shows that variants mapping to this domain in several RGS proteins (H7.6, H7.9, H8.3) affect their Gα selectivity. Taken together with our observations that even single amino acid substitutions within the selectivity bar code can change the Gα preferences of RGS proteins, these results point to critical determinants of RGS-Gα recognition. Curiously, we found that altering these determinants can generate RGS proteins with novel selectivity profiles not displayed by canonical members of the family (e.g., RGS8/14-P, AncR4/2/12/7; see Figures 4 and 6). Thus, we believe that the Gα-selectivity determinants identified here may pave the way for the de novo creation of RGS proteins with rationally designed G protein selectivity.

Our findings also have implications for pharmacogenomics and understanding disease mechanisms associated with the disruption in RGS-mediated G protein control. We uncovered a significant variation affecting nearly all of the RGS proteins. More importantly, many of these variants occurred in selectivity bar code domains and were found experimentally to affect the Gα selectivity of RGS proteins. These genetic alterations are expected to change the profiles of signaling pathways engaged by the GPCRs, creating a situation that the same drug targeting the same receptor would produce varying effects due to RGS heterogeneity. Such a situation may be cryptic in the population if one only profiles variation within GPCRs (Hauser et al., 2018), but it may still lead to interindividual variability in drug response. Therefore, understanding the impact of RGS proteins and their genetic variability on GPCR signaling is expected to be important for individualizing drug prescriptions in the implementation of precision medicine.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cell.2020.08.052.

ACKNOWLEDGMENTS

We thank Dr. Dipak Patil for helpful discussions, Ms. Hideko Masuho for technical support and data analyses, and Ms. Natalia Martemyanova for assistance with mouse husbandry. This work was supported by NIH grant DA036596 (to K.A.M.), the UKRI Medical Research Council (MC_U105185859, to M.M.B., S.B., and S.C.), and by HL122416, HL071818, and CA221289 (to J.J.G.T.). M.M.B. is a Lister Institute Research Prize Fellow. J.J.G.T. was also supported by the Walther Cancer Foundation, and M.M.B. and S.B. are supported by funding from ALSAC. S.C. was supported by the Ramalingaswami Re-entry Fellowship (BT/RLF/Re-entry/05/2018; SC) from...
AUTHOR CONTRIBUTIONS

I.M. participated in project design, performed experiments and data analysis, interpreted the data, and drafted the manuscript; S.B. participated in project design, performed experiments and revised the manuscript; B.S.M. performed experiments and data analysis and drafted the manuscript; N.K.S. performed experiments and data analysis; S.C. performed data analysis; J.J.G.T. performed experiments and data analysis and drafted the manuscript; K.A.M. was responsible for the project design, data interpretation, and manuscript writing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-GAPDH antibody | MilliporeSigma | Cat# MAB374; RRID:AB_2107445 |
| Anti-HA tag antibody (clone 3F10) | MilliporeSigma | Cat# 11867423001; RRID:AB_390918 |
| Anti-GFP antibody (clones 7.1 and 13.1) | MilliporeSigma | Cat# 11814460001; RRID:AB_390913 |
| Anti-GFP, N-terminal antibody | MilliporeSigma | Cat# G1544; RRID:AB_439690 |
| Anti-c-myc antibody (clone 9E10) | MilliporeSigma | Cat# 11867423001; RRID:AB_390912 |
| Anti-muscarinic acetylcholine receptor m3 antibody | MilliporeSigma | Cat# AB9018; RRID:AB_2080197 |
| Anti-Gi0 antibody | MBL life science | Cat# 551; RRID:AB_591430 |
| Anti-Gi1 antibody | Santa Cruz Biotechnology | Cat# sc-392; RRID:AB_631537 |
| Anti-dopamine D2 receptor antibody | Santa Cruz Biotechnology | Cat# sc-9113; RRID:AB_2094973 |
| Anti-RGS13 antibody | Novus Biologicals | Cat# H00006003-B01; RRID:AB_1049627 |
| Anti-RGS18 antibody | Novus Biologicals | Cat# NB81-92329; RRID:AB_11002698 |
| HRP-conjugated anti-rabbit antibody | Jackson ImmunoResearch | Cat# 211-032-171; RRID:AB_2339149 |
| HRP-conjugated anti-mouse antibody | Jackson ImmunoResearch | Cat# 115-035-174; RRID:AB_2338512 |
| HRP-conjugated anti-rat antibody | Jackson ImmunoResearch | Cat# 112-035-175; RRID:AB_2338140 |
| **Bacterial and Virus Strains** |        |            |
| pGP-AAV9-syn-FLEX-jGCaMP7s-WPRE | Dana et al., bioRxiv 434589 | Addgene Plasmid #104491 |
| One Shot Stbl3 E. coli | Thermo Fisher Scientific | Cat# C737303 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Dulbecco’s modified Eagle’s medium | Thermo Fisher Scientific | Cat# 11965-092 |
| Fetal bovine serum | Genesee Scientific | Cat# 25-550 |
| Sodium pyruvate | Thermo Fisher Scientific | Cat# 11360-070 |
| MEM non-essential amino acids | Thermo Fisher Scientific | Cat# 11140-050 |
| Penicillin-streptomycin | Thermo Fisher Scientific | Cat# 15140-122 |
| Matrigel | Corning | Cat# 356230 |
| Lipofectamine LTX and Plus reagent | Thermo Fisher Scientific | Cat# 15338-100 |
| Dulbecco’s phosphate-buffered saline | MilliporeSigma | Cat# D5652 |
| Dopamine hydrochloride | MilliporeSigma | Cat# H8502 |
| Haloperidol | MilliporeSigma | Cat# H1512 |
| SCH 39166 hydrobromide | Tocris | Cat# 2299 |
| Acetylcholine chloride | MilliporeSigma | Cat# A2661 |
| Atropine monohydrate sulfate | MilliporeSigma | Cat# A0257 |
| Bradykinin | Tocris | Cat# 3004 |
| B-9430 | BACHEM | Cat# H-7556 |
| Neurobasal-A Medium | Thermo Fisher Scientific | Cat# 10888-022 |
| GlutaMAX | Thermo Fisher Scientific | Cat# 35050-061 |
| B-27 Supplement | Thermo Fisher Scientific | Cat# 17504-044 |
| Penicillin-Streptomycin | Thermo Fisher Scientific | Cat# 15140-122 |
| DNase I | Thermo Fisher Scientific | Cat# 18047019 |
| Poly-D-lysine hydrobromide | MilliporeSigma | Cat# P6407 |
| Papain | Worthington Biochemical | Cat# LS003126 |
| BsmBI | New England Biolabs | Cat# R0580 |
| T4 PNK | New England Biolabs | Cat# M0201 |

(Continued on next page)
## Continued

| REAGENT or RESOURCE Source | SOURCE | IDENTIFIER |
|----------------------------|--------|------------|
| T4 Ligase                 | New England Biolabs | Cat# M0202 |
| Lipofectamine 2000        | Thermo Fisher Scientific | Cat# 11668019 |
| HBSS 10X                  | Thermo Fisher Scientific | Cat# 14175095 |
| Dopamine hydrochloride    | MilliporeSigma | Cat# H8502 |
| Acetylcholine chloride    | Tocris | Cat# 2809 |
| Picrotoxin                | Tocris | Cat# 1128 |
| CGP 55845 hydrochloride   | Tocris | Cat# 1248 |
| DNOX disodium salt        | Tocris | Cat# 2312 |

### Critical Commercial Assays

| Nano-Glo Luciferase Assay Substrate (furimazine) | Promega | Cat# N1120 |

### Deposited Data

| gnomAD | Karczewski et al., 2020 | https://gnomad.broadinstitute.org/ |
| denovo-db | Turner et al., 2017 | https://denovo-db.gs.washington.edu/denovo-db/index.jsp |
| GWAS catalog | Buniello et al., 2019 | https://www.ebi.ac.uk/gwas/home |
| Human proteome map | Kim et al., 2014 | https://www.humanproteomemap.org/ |
| OMA database | Altenhoff et al., 2018 | https://omabrowser.org/oma/home/ |
| Quantitative RNaseq data related to the expression landscape of RGS and Gα | Gokce et al., 2016 | https://www.sciencedirect.com/science/article/pii/S2211124716308130 |

### Experimental Models: Cell Lines

| HEK293T/17 | ATCC | ATCC: CRL-11268 |

### Experimental Models: Organisms/Strains

| Mouse: C57BL/6J | The Jackson Laboratory | JAX: 000664 |
| Mouse: C57BL/6-Gt(ROSA)26Sortm1(CAG-ECFP*/Rapgef3/Venus*)Kama/J | The Jackson Laboratory | JAX: 032205 |

### Oligonucleotides

| Table S2 | This paper | N/A |

### Recombinant DNA

| Plasmid: M3R | cDNA Resource Center | Cat# MAR0300000 |
| Plasmid: D1R | cDNA Resource Center | Cat# DRD0100000 |
| Plasmid: BDKRB2 | cDNA Resource Center | Cat# BDKB200000 |
| Plasmid: Flag-D2R | Dr. Abraham Kovoor | N/A |
| Plasmid: GαIA | Dr. Hiroshi Itoh | N/A |
| Plasmid: GαIA, G184S | Dr. Osamu Saitoh | N/A |
| Plasmid: GαAB | cDNA Resource Center | Cat# GNA0OB0000 |
| Plasmid: Gαi4 | Dr. Hiroshi Itoh | N/A |
| Plasmid: Gαi1, G183S | This paper | N/A |
| Plasmid: Gαi2 | Dr. Hiroshi Itoh | N/A |
| Plasmid: Gαi2, G184S | This paper | N/A |
| Plasmid: Gαi3 | Dr. Hiroshi Itoh | N/A |
| Plasmid: Gαi3, G183S | This paper | N/A |
| Plasmid: Gαi4 | Dr. Hiroshi Itoh | N/A |
| Plasmid: Gαi4, G184S | This paper | N/A |
| Plasmid: Gαi4, G183S | This paper | N/A |
| Plasmid: Gαq | cDNA Resource Center | Cat# GNA0Q0000 |
| Plasmid: Gαq, G183S | This paper | N/A |
| Plasmid: Gαq, G184S | This paper | N/A |
| Plasmid: Gαq11 | cDNA Resource Center | Cat# GNA1100000 |
| Plasmid: Gαq11, G188S | This paper | N/A |
| Plasmid: Gαq12, G188S | This paper | N/A |
| Plasmid: Gαq14 | cDNA Resource Center | Cat# GNA1400000 |

(Continued on next page)
| REAGENT or RESOURCE IDENTIFIER | SOURCE | IDENTIFIER |
|-------------------------------|--------|------------|
| Plasmid: Gα14 G184S          | This paper | N/A |
| Plasmid: Gα15               | cDNA Resource Center | Cat# GNA1500000 |
| Plasmid: Gα15 G188S         | This paper | N/A |
| Plasmid: Gα5S               | Dr. Hiroshi Itoh | N/A |
| Plasmid: Gα4L               | cDNA Resource Center | Cat# GNA0SL0000 |
| Plasmid: Gα15                | cDNA Resource Center | Cat# GNA0L0000 |
| Plasmid: Gα12               | cDNA Resource Center | Cat# GNA1200000 |
| Plasmid: Gα13               | cDNA Resource Center | Cat# GNA1300001 |
| Venus-156-239-Gβ1           | Hollins et al., 2009 | N/A |
| Venus-1-155-γγ2            | Hollins et al., 2009 | N/A |
| masGKrK3ct-Nluc-HA         | Gulati et al., 2018 | N/A |
| masGKrK3ct-Nluc-myc       | This paper | N/A |
| Plasmid: GIβ5S              | cDNA Resource Center | Cat# GNB0500000 |
| Plasmid: GIβ5L              | cDNA Resource Center | Cat# GNB05L0000 |
| Plasmid: RGS1               | This paper | N/A |
| Plasmid: RGS2               | cDNA Resource Center | Cat# RGS0200000 |
| Plasmid: RGS3-2             | cDNA Resource Center | Cat# RGS0300002 |
| Plasmid: RGS4               | cDNA Resource Center | Cat# RGS0400000 |
| Plasmid: RGS5               | cDNA Resource Center | Cat# RGS0500000 |
| Plasmid: RGS6               | cDNA Resource Center | Cat# RGS0600000 |
| Plasmid: RGS6 N401V         | This paper | N/A |
| Plasmid: RGS7               | cDNA Resource Center | Cat# RGS0700000 |
| Plasmid: RGS8               | cDNA Resource Center | Cat# RGS0800000 |
| Plasmid: RGS8 N122A         | This paper | N/A |
| Plasmid: RGS9-1             | This paper | N/A |
| Plasmid: RGS10              | cDNA Resource Center | Cat# RGS1000000 |
| Plasmid: RGS10 E52K         | This paper | N/A |
| Plasmid: RGS11              | cDNA Resource Center | Cat# RGS1100002 |
| Plasmid: RGS12              | cDNA Resource Center | Cat# RGS1200003 |
| Plasmid: RGS13              | cDNA Resource Center | Cat# RGS1300000 |
| Plasmid: RGS13 with codon optimization | This paper | N/A |
| Plasmid: RGS14              | cDNA Resource Center | Cat# RGS1400000 |
| Plasmid: RGS16              | cDNA Resource Center | Cat# RGS1600000 |
| Plasmid: RGS17              | This paper | N/A |
| Plasmid: RGS18              | cDNA Resource Center | Cat# RGS1800000 |
| Plasmid: RGS18 with codon optimization | This paper | N/A |
| Plasmid: RGS19              | cDNA Resource Center | Cat# RGS1900001 |
| Plasmid: RGS19 S156A        | This paper | N/A |
| Plasmid: RGS20              | cDNA Resource Center | Cat# RGS2000002 |
| Plasmid: RGS21              | This paper | N/A |
| Plasmid: AncR4/Z/12/7       | This paper | N/A |
| Plasmid: AncR4/Z            | This paper | N/A |
| Plasmid: AnxR12/7           | This paper | N/A |
| Plasmid: RGS13/18-F         | This paper | N/A |
| Plasmid: RGS13/18-P         | This paper | N/A |
| Plasmid: RGS8/14-F          | This paper | N/A |
| Plasmid: RGS9-1 M370K       | This paper | N/A |
| Plasmid: RGS11 R351Q        | This paper | N/A |

(Continued on next page)
Mice

All experimental work involving mice was approved by The Scripps Research Institute’s IACUC committee in accordance with NIH guidelines. Mice were housed under standard conditions in a pathogen-free facility on a 12:12 light:dark hour cycle with continuous access to food and water. Male and female CAMPER (Gt(ROSA)26Sor<sup>tm1(CAG-ECFP/Rapgef3/Venus)<sub>Kama</sub></sup>) and wild-type C57/Bl6 mice of both sexes aged from postnatal day 0 to postnatal day 3 were utilized in these studies and were not subjected to any prior experiments.
Cultures of clonal cell lines

HEK293T/17 cells were obtained from ATTC (Manassas, VA) and grown in DMEM supplemented with 10% FBS, minimum Eagle’s medium non-essential amino acids, 1mM sodium pyruvate, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified incubator containing 5% CO2.

Primary cultures of striatal medium spiny neurons

Primary striatal neurons were cultured similar to previous work (Muntean et al., 2018). The striatum from either wild-type or homozygous CAMPER pups were rapidly isolated at age P0 in ice-cold HBSS supplemented with 20% FBS, 4.2 mM NaHCO3, and 1 mM HEPES. Striatal tissue was then washed in HBSS without FBS prior to digestion at 37 °C for 15 minutes in a buffer (pH 7.2) containing 137 mM NaCl, 5 mM KCl, 7 mM Na2HPO4, 25 mM HEPES, and 0.3 mg/ml papain. Striatal tissue was washed three times with HBSS (20% FBS), three times with HBSS, and three times with growth media (Neurobasal-A containing 2 mM GlutaMAX, 2% B27 Supplement serum-free, and 1% Penicillin-Streptomycin). Striatal tissue was then dissociated through pipetting ~15 times with a standard P1000 pipette in the presence of DNase I (0.05 U/μL) and plated on poly-D-lysine coated glass coverslips. The cells were maintained in a humidified incubator at 37° C and 5% CO2. Half of the growth media was replenished every three days. For Ca2+ imaging, neuronal cultures from wild-type mice were incubated for 14-18 days with lentiviral-containing supernatant and AA9-syn-FLEX-JGCaMP7s-WPRE. For CAMPER imaging, neuronal cultures from CAMPER mice were incubated for 14-18 days with lentiviral-containing supernatant. Lipofectamine 2000 was used to transfect RGS2 along with control pSECC (1 μg each/cover slip) in wild-type or CAMPER neurons as indicated in the text for overexpression experiments.

METHOD DETAILS

cDNA constructs

M3 muscarinic acetylcholine receptor (AF498917), dopamine D1 receptor (GenBank: NM_000794 with one silent SNP (A1263G)), bradykinin B2 receptor (GenBank: AY275465), Gzα1b (GenBank: AH002708), Gzα2 (GenBank: J03260), Gzα11 (GenBank: AF493900), Gzα14 (GenBank: NM_004297), Gzα15 (GenBank: AF493904), Gζα long isofrom (GζαL) (GenBank: NM_000516), GζαL (GenBank: AF493893), GζαL (GenBank: NM_007353), Gζα13 (GenBank: NM_006572), RG2 (GenBank: AF493926), RGS3-2 (GenBank: NM_00128922), RGS4 (GenBank: AF493928), RGS5 (GenBank: AF493929), RGS6 (GenBank: NM_000496), RGS7 (GenBank: AY587875), RGS8 (GenBank: AF300649), RGS10 (GenBank: AF493934), RGS11 (GenBank: NM_003834), RGS12 (GenBank: NM_198227), RGS13 (GenBank: NM_000297), RG14 (GenBank: NM_006480), RGS16 (GenBank: AF493937), RGS18 (GenBank: NM_130782), RGS19 (GenBank: NM_005873), RGS20 (GenBank: NM_001165933), codon-optimized RGS13, RGS17 (GenBank: NM_016194) in pcDNA3.1(+) were purchased from cDNA Resource Center (https://www.cdna.org), masGRK3ct-Nluc-myc, RGS1 (GenBank: NM_000292), RGS9 (GenBank: NM_001165933), codon-optimized RGS13, RGS17 (GenBank: NM_012419), codon-optimized RGS18, RGS21 (GenBank: NM_001039152), AncR4/Z/12/F, AncR4/Z, AncR12/7, RGS13/18-F, RGS13/18-P, RGS14/15-F, RGS14/15-I, RGS18/190, RGS18/190L, and RGS19 R190W proteins in pcDNA3.1(+) were synthesized by GenScript. Flag-tagged dopamine D2 receptors (GenBank: NM_000795) containing the hemagglutinin signal sequence (KTIIALSYIFCLVFA) at the N terminus was a gift from Dr. Abraham Ko.

Antibodies

Anti-GAPDH antibody (MAB374), anti-HA tag antibody (clone 3F10) (11867423001), anti-GFP antibody (clone 9E10) (11667149001), and anti-muscarinic acetylcholine receptor m3 antibody (AB9018) were purchased from MilliporeSigma. Anti-Gzα2 antibody (551) was purchased from MBL life science. Anti-Gzα2 antibody (sc-392) and anti-D2R antibody (sc-9113) were purchased from Santa Cruz Biotechnology. Anti-RGS3 antibody (H0000603-B01) and anti-RGS18 antibody (NB1-92329) were purchased from Novus Biologicals. HRP-conjugated anti-rabbit antibody (211-032-171), HRP-conjugated anti-mouse antibody (115-035-174), and HRP-conjugated anti-rat antibody (112-035-175) were purchased from Jackson ImmunoResearch.
**Transfection**

For transfection, cells were seeded into 3.5-cm dishes at a density of 2 × 10^6 cells/dish. After 2 h, expression constructs (total 5 μg/dish) were transducted into the cells using PLUS (5 μl/dish) and Lipofectamine LTX (6 μl/dish) reagents. The GPCR (dopamine D2 receptor (D2R) (1) for Gi/o, M3 muscarinic acetylcholine receptor (M3R) (1) for Gq, dopamine D1 receptor (D1R) (1) for Gs, and bradykinin B2 receptor (BDKRB2) (1) for G12/13), Gα (GαsA (2), GαsB (1), Gαi1 (1), Gαi2 (2), Gαs (1.5), Gαz (1.5), Gαq (2), Gα11 (2), Gα14 (4), Gα15 (2), Gαs short (6), Gαs long (4), Gαzoff (6), Gα12 (3), or Gα13 (4)), Venus 156-239-Gβγ (1), Venus 1-155-Gγ2 (1), masGRK3ct-Nluc-HA (1) were transfected with different amounts of RGS construct (the number in parentheses indicates the ratio of transfected DNA (ratio 1 = 0.21 μg)). RGS1 (12), RGS2 (12), RGS3-2 (6), RGS4 (12), RGS5 (12), RGS6/Gβ3 (1), RGS7/Gβ3 (2), RGS8 (6), RGS9-1/Gβ3 (2), RGS10 (6), RGS11/Gβ3 (6), RGS12 (6), RGS13 (6), RGS14 (6), RGS16 (6), RGS17 (6), RGS18 (12), RGS19 (6), RGS20 (6), and RGS21 (12) were transfected to examine comprehensive G protein selectivity. Gα14/15 and Gαzoff were transfected with Ric-8A (1) and Ric-8B (1), respectively. A construct carrying catalytic subunit of pertussis toxin PTX-S1 were transfected with Gα2, M3R, D1R, or BDKRB2 to inhibit the possible coupling of endogenous Gi/o to GPCRs. An empty vector (pcDNA3.1(+)) was used to normalize the amount of transfected DNA.

**Cell-based GAP assay**

Cellular measurements of BRET between Venus-Gα1γ2 ε and masGRK3ct-Nluc-HA were performed to examine GAP activity of RGS protein in living cells (described in detail in Masuho et al., 2015a, 2015b). Sixteen to twenty-four hr post-transfection, HEK293T/17 cells were washed once with BRET buffer (Dulbecco’s Phosphate-Buffered Saline (PBS) containing 0.5mM MgCl2 and 0.1% glucose) and detached by gentle pipetting over the monolayer. Cells were harvested by centrifugation at 500 g for 5 min and re-suspended in BRET buffer. Approximately 50,000 to 100,000 cells per well were distributed in 96-well flatbottomed white microplates (Greiner Bio-One). The NanoLuc (Nluc) substrate, furimazine (Hall et al., 2012), were purchased from Promega and used according to the manufacturer’s instruction. BRET measurements were made using a microplate reader (POLARstar Omega; BMG Labtech) equipped with two emission photomultiplier tubes, allowing us to detect two emissions simultaneously with the highest possible resolution of 20 ms per data point. All measurements were performed at room temperature. To activate and then deactivate, the final concentration of 100 μM ligands were used. Specifically, dopamine and haloperidol for D2R, dopamine and SCH39166 for D1R, acetylcholine and atropine for M3R, and bradykinin and B-9430 for BDKRB2 were applied on the transfected cells to control the activity of those GPCRs. The BRET signal is determined by calculating the ratio of the light emitted by the Venus- Gα1γ2 ε (535 nm with a 30 nm band path width) over the light emitted by the masGRK3ct-Nluc-HA (475 nm with a 30 nm band path width). The average baseline value (basal BRET ratio) recorded prior to agonist stimulation was subtracted from the experimental BRET signal values and the resulting difference (ΔBRET ratio) was normalized against the maximal ΔBRET value recorded upon agonist stimulation. The rate constants (1/τ) of the deactivation phases were obtained by fitting a single exponential curve to the traces with Clampfit 10.3. kGAP rate constants were determined by subtracting the basal deactivation rate (kapp) from the deactivation rate measured in the presence of exogenous RGS protein. Obtained kGAP rate constants were used to quantify GAP activity.

**Western blotting**

For each 3.5-cm dish, transfected cells were lysed in 1 mL of sample buffer (62.5 mM tris-HCl, pH 6.8, 2 M urea, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, bromophenol blue (0.08 mg/ml)). Western blotting analysis of proteins was performed after samples were resolved by SDS–polyacrylamide gel electrophoresis and transferred onto PVDF membranes. Blots were blocked with 5% skim milk in PBS containing 0.08% Tween 20 for 1 h at room temperature, which was followed by 90 min incubation with specific antibodies diluted in PBS. Primary antibodies used were RGS1 (1), RGS2 (1), RGS3 (6), RGS4 (12), RGS5 (12), RGS6 (6), RGS7 (1), RGS8 (6), RGS9-1 (1), RGS10 (6), RGS11 (1), RGS12 (6), RGS13 (6), RGS14 (6), RGS16 (6), RGS17 (6), RGS18 (12), RGS19 (6), RGS20 (6), and RGS21 (1) for Gi/o; RGS2 (1), RGS4 (12), RGS5 (12), RGS6 (6), RGS7 (1), RGS8 (6), RGS9-1 (1), RGS10 (6), RGS11 (1), RGS12 (6), RGS13 (6), RGS14 (6), RGS16 (6), RGS17 (6), RGS18 (12), RGS19 (6), RGS20 (6), and RGS21 (12) for Gq; RGS1 (1), RGS2 (1), RGS3 (6), RGS4 (12), RGS5 (12), RGS6 (6), RGS7 (1), RGS8 (6), RGS9-1 (1), RGS10 (6), RGS11 (1), RGS12 (6), RGS13 (6), RGS14 (6), RGS16 (6), RGS17 (6), RGS18 (12), RGS19 (6), RGS20 (6), and RGS21 (12) for Gi; and RGS1 (1), RGS2 (1), RGS3 (6), RGS4 (12), RGS5 (12), RGS6 (6), RGS7 (1), RGS8 (6), RGS9-1 (1), RGS10 (6), RGS11 (1), RGS12 (6), RGS13 (6), RGS14 (6), RGS16 (6), RGS17 (6), RGS18 (12), RGS19 (6), RGS20 (6), and RGS21 (12) for Gs. Blots were then incubated with HRP-conjugated secondary antibodies (anti-D2R antibody (1:1,000), anti-M3R antibody (1:1,000), anti-Ga1ε antibody (1:1,000), anti-Ga1γ2 ε antibody (1:1,000), anti-Gαi1 ε antibody (1:1,000), anti-Gαi2 ε antibody (1:1,000), anti-Gαq antibody (1:1,000), anti-c-myc antibody (1:1,000), anti-Gα11 ε antibody (1:1,000), anti-GRS13 antibody (1:1,000), or anti-RGS18 antibody (1:5,000), and anti-GAPDH antibody (1:10,000)). Blots were washed in PBST and incubated for 45 min with a 1:10,000 dilution of secondary antibodies conjugated with horseradish peroxidase (HRP) in PBST containing 1% skim milk. Western blotting was performed with BlotCycler automated western blot processor (Precision Biosystems). Proteins were visualized with Kwik Quant imager (Kindle Biosciences).

**Lentivirus preparation for CRISPR-Cas9 mediated knockout of RGS proteins**

As previously described (Doyle et al., 2019; Muntean et al., 2018), sgRNA sequences targeting RGS proteins were designed with CHOPCHOP (https://chopchop.cbu.uib.no/). According to the design, oligo DNAs were synthesized by Integrated DNA Technologies. The oligo DNAs were treated by T4 polynucleotide kinase and annealed in a thermal cycler. Finally, the oligo DNAs were ligated into the BsmBI site of the pSECC vector with T4 DNA Ligase. Three sgRNA constructs were made for each target gene. The plasmids were purified from Stbl3 E. coli. Lentiviruses were generated by Lipofectamine LTX-mediated transfection of HEK293T/17 cells with the packaging vectors, pSECC, pCMV-VSV-G, pMDLg/pRRE, and pRSV-Rev. The supernatant containing the lentiviral particles was collected at 48 hours post-transfection.
Live-imaging of cAMP and Ca²⁺ dynamics

Primary neuronal cultures were imaged under a Leica TCS SP8 confocal microscope through a 25x objective lens. Changes in cAMP were recorded from CAMPER neurons, as previously described (Doyle et al., 2019; Muntean et al., 2018). Briefly, excitation of mTurquoise FRET donor with a 442 nm diode laser was paired with simultaneous acquisition of XYZ image stacks at 10 s intervals collected through two HyD detectors tuned to 465–505 nm (mTurquoise FRET donor) and 525–600 nm (Venus FRET acceptor). Quantification of fluorescence intensity was performed on neuronal cell bodies using ImageJ (Schneider et al., 2012) to calculate FRET from the donor/acceptor ratio. The FRET ratio was converted to the concentration of cAMP using a dose-response curve to cAMP standards in permeabilized neurons. Segregated dopamine receptor subtype expression in striatal neurons enabled the identification of D2R-expressing neurons according to the directionality of cAMP response to dopamine. Dopamine was added in phasic puffs during continuous perfusion (2 mL/minute) of a pH 7.2 buffer consisting of 1.3 mM CaCl₂, 0.5 mM MgCl₂, 0.4 mM MgSO₄, 0.4 mM KH₂PO₄, 4.2 mM NaHCO₃, 138 mM NaCl, 0.3 mM Na₂HPO₄, 5.6 mM D-Glucose, and 20 mM HEPES. Changes in intracellular calcium concentration were recorded from wild-type neurons expressing GCaMP7s. Excitation was performed with a 488 nm laser, and the acquisition of XYZ image stacks at 1 s intervals was collected through a HyD detector tuned to 494–593 nm. Quantification of fluorescence intensity was performed on neuronal cell bodies using ImageJ. Acetylcholine was added in phasic puffs during continuous perfusion (2 mL/minute) of a pH 7.3 buffer consisting of 2.2 mM CaCl₂, 1 mM MgCl₂, 138 mM NaCl, 11 mM D-Glucose, 10 mM HEPES, 50 μM picrotoxin, 300 nM CGP55845, and 10 μM DNQX.

Alignment of human RGS paralogs and orthologs

Whole protein sequences of human RGS proteins were downloaded from the UniProt database (https://www.uniprot.org/). The core RGS domain in each of these human RGS proteins was assigned based on HMMER searches conducted on pfam database domain profiles using human RGS proteins. Then the core RGS domains assigned in all of the human RGS paralogs were aligned using MSAProbs (Liu et al., 2010) and this alignment was termed as human RGS domain alignment (HRDA). Animal orthologs of RGS proteins were obtained from the OMA database (https://omabrowser.org/oma/home/) (Altenhoff et al., 2018) and equivalent regions to the core RGS domain of human RGS were only considered for further investigations. We aligned the core RGS domain regions in the animal orthologs with human ones. For each human RGS, i.e., RGS1 to RGS21, we constructed multiple sequence alignments of the given RGS with its corresponding animal orthologs.

RGS common numbering scheme

We developed a common RGS numbering scheme (CRN), by integrating consensus secondary structure information of available crystal structures of the RGS domain on to HRDA sequence alignment. This allowed us to uniquely assign an alignment position to a combination three types of information: 1) Secondary structural element i.e. “H” for helix, “S” for strand and “L” for loop, along with the index of the secondary structural element i.e. “H1” stands for helix number 1 and “L2” stands for loop number 2, etc. 2) Residue number of the alignment position within the index of the given structural element i.e. “H1.12” denotes 12th position in helix number 1 or helix H1 or L3.2 denotes 2nd position in loop number 3 or loop L3.

Normalized BLOSUM scores

For any given alignment position n in the alignment, amino acid residues at this position for across organisms Rᵢ, where i = 1 to m, where m is the total number of sequences in the alignment.

\[
\text{Normalized BLOSUM score (NBS)} = \sum_{i=1}^{m} \frac{\sum_{j=1}^{m} BS_{ij}}{mC_2}
\]

Where BSᵢ = BLOSUM score (Rᵢ → Rⱼ)/Maximum [BLOSUM score (Rᵢ → Rⱼ) OR BLOSUM score (Rⱼ → Rⱼ)] and “ → “ refers to amino-acid residue substitution

Evaluate mean of all over all the “l” positions in the alignment:

\[
\text{MeanNBS} = \frac{\sum_{n=1}^{l} NBS_n}{l}
\]

Orthology/paralogy analysis

To identify the ortholog specific conserved residues and commonly conserved residues between paralogs of human RGS in the core RGS domain. We developed a strategy, by comparing assigning the CRN to each of the RGS alignments and we then categorized the residue at a given CRN position is: (a) Ortholog-specifically conserved if the normalized BLOSUM score for this CRN is 1.5 times higher in a given RGS alignment than in the equivalent CRN of HRDA alignment position and the given CRN position also displays above average normalized BLOSUM score within the RGS alignment. (b) Paralog-specifically conserved if the normalized BLOSUM score for this CRN in the HRDA alignment is 1.5 times higher than in the equivalent CRN of RGS alignment and the given position displays above average normalized BLOSUM score within the HRDA alignment. (c) Conserved in both if CRN in RGS alignment and the HRDA display comparable normalized BLOSUM scores, i.e., within 1.5 times normalized BLOSUM score of either of...
them. The given position displays above average normalized BLOSUM score within the HRDA and RGS alignments. (d) Neutrally evolving if the above three conditions were not met. The alignment of RGS domain from orthologs is provided as Data S1 and S2. In the datasets, the residue numbers following the accession OMA database ID and UniProt ID or Ensembl database ID are presented.

**Reconstitution of recombinant ancestral RGS proteins**

The reconstitution of ancestral RGS proteins based on the computational algorithm using FastML was performed (Ashkenazy et al., 2012) on different groups of RGS alignments *i.e.*, for *e.g.*, R4, RZ, R12, and all RGS proteins. Ancestral reconstruction methods identify most likely sequences, including indels, in a specific ancestral node in a phylogenetic tree for given multiple sequence alignment.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Multiple t tests with correction for multiple comparison using the Holm–Sidak method was conducted to determine the effect of RGS on the deactivation rates of Gα subunits with GraphPad Prism Ver. 6. Only statistically significant values are plotted. Values represent means ± SEM from three independent experiments each performed with three replicates.
Figure S1. The Effect of Introducing RGS-Insensitive and GAP-Deficient Mutations on the Deactivation Rates, Related to Figure 1

(A) The time course of deactivation of wild-type Go subunits and RGS-insensitive mutants. Each trace represents the mean of the responses measured in three independent experiments. (B) Deactivation rate constants of Go WT and RGS-insensitive mutants. Data are represented as mean ± SEM (n = 3 independent experiments). (C) The time course of deactivation of GoA and Goq with RGS WT or N122A mutant. Each trace represents the mean of the responses measured in three independent experiments. (D) Deactivation rate constants of GoA and Goq with RGS WT or GAP-deficient mutants. Data are represented as mean ± SEM (n = 3 independent experiments). (E) Western blot analysis of 3xHA-RGS proteins were performed.
Figure S2. Effects of the Expression Levels of GPCR Signaling Molecules and RGS Proteins on G Protein Deactivation Rates, Related to Figure 1

(A and B) Expression levels of GPCR signaling molecules and RGS proteins were examined with western blotting. Overexpression of RGS proteins does not change the expression levels of GPCRs, G proteins, and sensors. (C) Effects of increasing GPCR on activation and deactivation rates of G proteins (left). Increasing amount of GPCR cDNA for transient transfection increased G protein activation rates but did not alter G protein deactivation rates. Effects of increasing active G proteins on deactivation rates of G proteins (right). Increasing concentration of agonist produced more active G protein but maintain consistent G protein deactivation rates. (D) Effects of increasing RGS on G protein deactivation rates. Increasing amount of RGS cDNA for transient transfection increased deactivation rates.
Figure S3. Effect of RGS Expression Level on Gα Selectivity, Related to Figure 1

(A-D) Gα-selectivity fingerprints ($k_{ GAP}$) of RGS18 (A), RGS19 (B), RGS10 (C), and RGS6 (D) with low or high expression levels. (A) GAP activity of RGS18 before and after codon optimization was compared. High expression condition had 14-fold higher $k_{ GAP}$ activity relative to low expression (see I). (B) HEK293T/17 cells
were transfected with 0.42 μg or 1.3 μg of RGS19 for low or high expression, respectively. (C) HEK293T/17 cells were transfected with 0.21 μg or 1.3 μg of RGS10 for low or high expression, respectively. (D) HEK293T/17 cells were transfected with 0.11 μg or 0.21 μg of RGS6 for low or high expression, respectively, with consistent amount of Gβ5S for both conditions (0.21 μg). The GAP activity on 15 different G proteins was normalized to the largest value to obtain relative kGAP. The thickness of the lines connecting each data point represents the SEM of three independent experiments. The relative values are plotted on a linear scale. (E-K) Optimizing the expression of RGS13 and RGS18. (E) Effects of protease inhibitor (MG-132) were examined by western blotting. Cells were treated with 1 μM MG-132 for 4 hours prior to lysing the cells. (F) and (G) Codon optimization of RGS13 and RGS18. Codon adaptation index (CAI) and codon usage frequency distribution before (blue) and after (red) codon optimization are shown. (H) and (I) The activity of RGS13 and RGS18 before and after codon optimization. Each trace represents the mean of the responses measured in three wells. (J) Data are represented as mean ± SEM (n = 3 wells). (J) and (K) Western blot analysis was performed to examine the expression levels of RGS13 and RGS18 with specific antibodies. Western blotting with anti-GAPDH antibody was performed as a loading control.
Figure S4. Gα Selectivity of All Canonical RGS Proteins, Related to Figures 1 and 2
Gα-selectivity fingerprints (kGAP) of all canonical RGS proteins are shown. The GAP activity on 15 different G proteins was normalized to the largest value to obtain relative kGAP, as shown in Figure 1J. The thickness of the lines connecting each data point represents the SEM of three independent experiments. The relative values are plotted on a linear scale.
Figure S5. Selectivity of RGS Regulation of Gα Subunits, Related to Figure 2

(A) Promiscuity of RGS proteins. The number of Gα subunits affected by each RGS protein was obtained from Figure 2A to determine the range of substrates (promiscuity) for each RGS protein. (B) Impact of RGS proteins. The sum of normalized $k_{GAP}$ values from Figure 2C was used to quantify the overall impact of each RGS protein.

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RGS protein. (C) Selectivity of RGS proteins. Impact (B) was divided by promiscuity (A) to obtain normalized Gα selectivity of each RGS protein (C). (D) Promiscuity of Gα subunits. The number of impacting RGS proteins was obtained from Figure 2B to determine the number of RGS regulating each Gα subunit (promiscuity). (E) The sum of normalized k_{GAP} values over all impacting RGS proteins was obtained from Figure 2B to determine the sensitivity of Gα subunits to RGS proteins. (F) RGS selectivity of Gα subunits. Sensitivity (E) was divided by promiscuity (D) to obtain normalized RGS subfamily selectivity of each Gα subunits (F). (G-L) The activity of R2 subfamily on Gs2. (G) and (H) Effects of RGS17 on the deactivation of GαoA (G) and Gαz (H). (I) and (J) The k_{GAP} (I) and k_{dis} (J) of RGS17 on GαoA and Gαz. Data are represented as mean ± SEM (n = 3 independent experiments). (K) Representative Gα selectivity fingerprints of R4, R2, R12, and R7 subfamilies. The maximum activity (k_{dis}) from the 15 different G proteins was normalized to the largest value to obtain comparative k_{dis} activity and was plotted at corresponding vertices in the wheel diagram. The thickness of the lines connecting each data point represents the SEM of three independent experiments. (L) Heatmap of k_{dis} of all RGS proteins. (M-P) RGS insensitive mechanisms of Gα12/13 and Gαs. Gα12/13 and Gαs have unique surface features that preclude their interaction with RGS proteins. Panels (M) and (O) depict regions of the RGS4-Gαi1 interface from PDB entry 1AGR (RGS4 with gray, Gαi1 with pink), whereas panels (N) and (P) depict Gα12 and Gαs (both with green) docked onto Gαi1 from the 1AGR structure to highlight their incompatibilities with binding RGS proteins, as represented by RGS4. (M) Val179 and Thr182 in switch I of Gαi1 and a short αβ-αC loop in the helical domain is replaced by Lys204, Lys207, and an extended αβ-αC loop, respectively, in Gα12 (N). These features are conserved in the Gα12/13 subfamily and would lead to profound steric collisions with the backbone of a bound RGS domain. (O) Ser206 in switch II of Gαi1 is replaced by Asp229 in Gαs (P) which would introduce van der Waals collisions (dashed lines with numbers corresponding to distances in Å) as well as charge repulsion with an adjacent carboxylate in the α5-α6 loop of RGS4. The Gαs-D229S mutation confers the ability of RGS4 and RGS16 to bind Gαs, and the ability of RGS16 to accelerate GTP hydrolysis on Gαs.
Figure S6. The Common RGS Numbering (CRN) System, Related to Figure 5
(A) The structure of the RGS4 RGS domain with color-code for each helix. (B) The alignment of all human RGS paralogs with CRN. The common residue numbers are shown on top of the alignment. Directly contacting residues based on the structure of the RGS4/Gαi complex are highlighted with red asterisks at the bottom.

(legend continued on next page)
of the alignment. The gray indicates the residues with conserved property and black indicate the conserved residues. Of note, there are two insertion/deletion regions in this alignment of the RGS domain. First, there are four amino acid residues in loop 5 in the most of RGS proteins. Instead, there are six amino acids in RGS12 and RGS14, but only three amino acid residues in all R7 RGS members in this structural element. Second, all three R12 RGS proteins are missing an amino acid residue in the H6 region. It is not possible based on existing structural alignments to say where this gap actually occurs, because the H6 region is conformationally heterogeneous in R12 structures and cannot be structurally aligned with other RGS proteins other than to say it has helical character as detected by NMR. The disorder of this region in R12 subfamily members has in fact been proposed to play a role in selecting against the Gαq family due to loss of beneficial interactions with SwII (Taylor et al., 2016). The conserved and selectivity residues identified by ortholog/paralog analysis (Figure 3C) are highlighted in blue and orange, respectively. The sequence alignments were generated with T-Coffee (http://tcoffee.crg.cat/apps/tcoffee/do:regular) and colored by BoxShade (https://embnet.vital-it.ch/software/BOX_form.html). (C) Reference table of the definitions of the secondary structure elements used in the CRN nomenclature. PDB accession number 1AGR is used in panel (A).
Figure S7. RGS-Gα Selectivity of RGS8, RGS14, and Mutants in kGAP and kdis, Related to Figure 6
(A) Sequence pattern of the RGS13, RGS18 and RGS13/18 chimera are shown. Identical amino acid residues between RGS13 and RGS18 were indicated by asterisks. (B) Sequence pattern of the R4 and R12 subfamilies, their representative RGS proteins (RGS8 and RGS14), and mutant RGS proteins are shown. Identical amino acid residues between RGS8 and RGS14 were indicated by asterisks. (C) and (D) The Gα-selectivity fingerprints (kGAP (C) and kdis (D)) of RGS8, RGS14, and two mutants are shown. The thickness of the lines connecting each data point represents the SEM of three independent experiments. The relative values are plotted on a linear scale.