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**An Interhelical Salt Bridge Controls Flexibility and Inhibitor Potency For  
Regulators of G-protein Signaling (RGS) Proteins 4, 8, and 19**

Vincent S. Shaw<sup>1</sup>, Mohammadjavad Mohammadi<sup>1</sup>, Josiah A. Quinn, Harish Vashisth, and  
Richard R. Neubig

Department of Pharmacology and Toxicology (V.S.S., J.A.Q., R.R.N.) and “Nicholas V.  
Perricone, M.D.” Division of Dermatology, Department of Medicine (R.R.N.), Michigan State  
University, East Lansing, MI; Department of Chemical Engineering, University of New  
Hampshire, Durham, NH (M.M., H.V.)

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## **A Salt Bridge Drives RGS Flexibility and Inhibitor Selectivity**

Corresponding authors:

Richard R. Neubig  
1355 Bogue St, Room B440  
East Lansing, MI 48824  
[rneubig@msu.edu](mailto:rneubig@msu.edu)  
(517) 353-7145

Harish Vashisth  
33 Academic Way, Room S344B  
Durham, NH 03824  
[harish.vashisth@unh.edu](mailto:harish.vashisth@unh.edu)  
(603) 862-2483

Text pages: 15  
Number of figures: 6  
Number of references: 33  
Words in abstract: 247  
Words in introduction: 535  
Words in discussion: 797

### Abbreviations:

CCG-50014, 4-[(4- fluorophenyl)methyl]-2-(4-methylphenyl)-1,2,4-thiadiazolidine-3,5-dione; DCC, dynamic cross-correlation; DI, deuterium incorporation; FCPIA, flow cytometry protein interaction assay; GPCR, G-protein coupled receptor; HDX; hydrogen-deuterium exchange; MD, molecular dynamics; RGS: regulator of G-protein signaling; TDZD, thiadiazolidinone; WT, wild type

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## ABSTRACT

Regulators of G-protein Signaling (RGS) proteins modulate receptor signaling by binding to activated G-protein  $\alpha$ -subunits, accelerating GTP hydrolysis. Selective inhibition of RGS proteins increases G-protein activity and may provide unique tissue specificity. Thiadiazolidinones (TDZDs) are covalent inhibitors that act on cysteine residues to inhibit RGS4, RGS8 and RGS19. There is a correlation between protein flexibility and potency of inhibition by the TDZD CCG-50014. In the context of a single conserved cysteine residue on the  $\alpha_4$  helix, RGS19 is the most flexible and most potently inhibited by CCG-50014, followed by RGS4 and RGS8. In this work we identify residues responsible for differences in both flexibility and potency of inhibition among RGS isoforms. RGS19 lacks a charged residue on the  $\alpha_4$  helix that is present in RGS4 and RGS8. Introducing a negative charge at this position (L118D) increased the thermal stability of RGS19 and decreased the potency of inhibition of CCG-50014 by 8-fold. Mutations eliminating salt bridge formation in RGS8 and RGS4 decreased thermal stability in RGS8 and increased potency of inhibition of both RGS4 and RGS8 by 4-fold and 2-fold respectively. Molecular dynamics (MD) simulations with an added salt bridge in RGS19 (L118D) showed reduced RGS19 flexibility. Hydrogen-deuterium exchange (HDX) studies showed striking differences in flexibility in the  $\alpha_4$  helix of RGS4, 8, and 19 with salt bridge modifying mutations. These results show that an  $\alpha_4$  salt bridge-forming residue controls flexibility in several RGS isoforms and supports a causal relationship between RGS flexibility and the potency of TDZD inhibitors.

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## **SIGNIFICANCE STATEMENT**

Inhibitor potency is often viewed in relation to the static structure of a target protein binding pocket. Using both experimental and computation studies we assess determinants of dynamics and inhibitor potency for three different RGS proteins. A single salt bridge-forming residue determines differences in flexibility between RGS isoforms; mutations either increase or decrease protein motion with correlated alterations in inhibitor potency. This strongly suggests a causal relationship between RGS protein flexibility and covalent inhibitor potency.

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## INTRODUCTION

Drug specificity is often considered to be like a key fitting into a complementary shaped lock. It has become clear recently that protein dynamics can play an important role in drug discovery (Feixas *et al.*, 2014). Regulators of G-protein Signaling (RGS) proteins bind to activated G $\alpha$  subunits of G-proteins, thereby accelerating GTP hydrolysis and attenuating G-protein signaling. In regulating G-Protein Coupled Receptor (GPCR) signaling, RGS proteins play a role in the physiology of numerous systems. By inhibiting RGS proteins, signaling via a GPCR may be enhanced. There are twenty RGS isoforms, each with a different tissue distribution. Combination of GPCR agonists with inhibitors specific for a single RGS isoform should limit effects on GPCR signaling to the subset of target tissues with intersecting distributions of the RGS isoform and the GPCR. This has the potential to reduce agonist off-target effects and makes RGS proteins an attractive target for modulation of GPCR signaling.

The potent RGS inhibitors discovered to date are all covalent modifiers of cysteine residues and are selective for RGS4 and RGS1 (Roman *et al.*, 2010; Turner *et al.*, 2011; Hayes *et al.*, 2018). These proteins have four and three cysteines, respectively, in the RGS homology domain, which is more than most other RGS proteins. RGS4 has been linked to nervous system related disease states in which RGS4 inhibition may be desirable, including seizures (Chen *et al.*, 2012) and Parkinson's disease (Lerner and Kreitzer, 2012; Blazer *et al.*, 2015; Shen *et al.*, 2015). Continued efforts to seek non-covalent inhibitors are worth pursuing because the lower risk associated with non-covalent inhibitors is considered safer and may facilitate further development (Potashman and Duggan, 2009). In addition, it would be valuable to discover RGS inhibitors with other specificities since other RGS proteins which are not potently inhibited by covalent modifiers have been

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implicated as potential targets, including RGS17 in cancer (James *et al.*, 2009; Bodle *et al.*, 2013) and RGS19 in depression (Wang *et al.*, 2014). To identify noncovalent inhibitors with novel specificities, it will be useful to understand what factors apart from the number of cysteines in the RGS domain drive selectivity of RGS inhibitors.

The RGS homology domain contains nine alpha helices. A cysteine residue on  $\alpha_4$ , which faces the interior of the  $\alpha_4$ - $\alpha_7$  helical bundle, is conserved among 18 of the 20 RGS isoforms, excepting only RGS6 and RGS7 (Tesmer, 2009). Interestingly, when RGS proteins are mutated to contain only this single, shared cysteine, there are still dramatic differences in the potency by which different isoforms are inhibited (Shaw *et al.*, 2018). RGS19, which contains only the shared  $\alpha_4$  cysteine, is more potently inhibited than single-cysteine versions of RGS4 and RGS8 (Mohammadiarani *et al.*, 2018; Shaw *et al.*, 2018).

Previously, we found using molecular dynamics (MD) simulations that RGS19 is more flexible than RGS4 and RGS8 (Shaw *et al.*, 2018). In these modeling studies, we also found that salt bridge interactions were perturbed in response to inhibitor binding (Mohammadi *et al.*, 2018). In this work, we sought to identify residue interactions responsible for flexibility differences among these isoforms and we predicted that mutations that alter salt bridge interactions will both enhance RGS protein flexibility and increase the potency of RGS inhibitors such as CCG-50014.

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## MATERIALS AND METHODS

### Materials

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO). QuikChange II Mutagenesis kit was purchased from Agilent (Santa Clara, CA). BL21(DE2) competent cells and Protein Thermal Shift Dye Kit was purchased from Thermo Fisher Scientific (Waltham, MA). Lumavidin Microspheres were purchased from Luminex (Austin, TX). CCG-50014 {4-[(4-fluorophenyl)methyl]-2-(4-methylphenyl)-1,2,4-thiadiazolidine-3,5-dione} was synthesized as previously described. (Blazer *et al.*, 2011)

### Protein Expression and Purification

RGS proteins were produced as previously described (Shaw *et al.*, 2018). Briefly, a his-tagged RGS domain of RGS8 in a pQE80 vector, a his-tagged RGS domain of RGS19 in a pET15b vector, and a his-tagged  $\Delta 51$  N-terminally truncated RGS4 in a pET23d vector were transformed into BL21(DE3) competent *E. coli* cells (Sigma-Aldrich). At an OD<sub>600</sub> of 2.0, protein production was induced by addition of 200  $\mu$ M IPTG, and incubation was continued at 25 °C for 16 hours. Cells were lysed and the protein was purified by nickel affinity chromatography. Mutations were induced with a QuikChange mutagenesis kit (Agilent) and verified by Sanger sequencing. All RGS proteins, including those with mutations in salt bridge-forming residues, were produced on a single-cysteine background (WT RGS19, C160A RGS8 and C74A C132A C148A RGS4). G $\alpha_o$  protein was expressed and purified as described (Lee *et al.*, 1994).

### Differential Scanning Fluorimetry

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Differential scanning fluorimetry was performed using the Protein Thermal Shift Dye Kit (ThermoFisher Scientific). Dye was added at 1X to 10  $\mu$ M protein samples in 50 mM HEPES and 100 mM NaCl buffer, pH 7.4 in a volume of 20  $\mu$ L. Fluorescence was read using a QuantStudio 7 Flex Real-Time PCR System while the temperature was ramped from 20 °C to 80 °C at a rate of 0.05 °C/s. Peak melting temperatures were defined as the point of fastest increase in fluorescence with respect to temperature. Data was analyzed using Protein Thermal Shift software v1.3 (Thermo Fisher Scientific, Waltham, MA) and GraphPad Prism 7 (GraphPad Inc, LaJolla, CA).

### **Flow Cytometry Protein Interaction Assay (FCPIA)**

FCPIA was performed as described (Blazer *et al.*, 2010) with minor modifications. RGS proteins were biotinylated by incubation at a 1:1 molar ratio with EZ-link NHS-LC-biotin (ThermoFisher Scientific, Waltham, MA) for two hours on ice, then excess biotin was removed using Amicon spin columns (catalog no. UFC501096, Millipore, Burlington, MA). RGS proteins at 50 nM were incubated with xMAP LumAvidin beads (Luminex, Austin, TX) while shaking at room temperature for 1 hour. Beads were washed and incubated with varying concentrations of CCG-50014, followed by addition of 50 nM  $G\alpha_o$  labeled with AF-532 (Blazer *et al.*, 2010). Samples were read in a Luminex 200 flow cytometer as described (Blazer *et al.*, 2010) and analysis performed in GraphPad Prism 7.

### **Hydrogen-Deuterium Exchange**

Hydrogen-deuterium exchange was performed as previously described (Chodavarapu *et al.*, 2015; Shaw *et al.*, 2018). Briefly, proteins were incubated on ice at 1.2  $\mu$ M in 90% D<sub>2</sub>O solvent with 5 mM HEPES and 100 mM NaCl, pH 7.4 for the desired time (1, 3, 10, 30, or 100 minutes).



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Exchange was quenched by 1:1 addition of ice cold 1% formic acid. A Shimadzu pump was used to load 100  $\mu$ L of each sample onto a pepsin column (Waters, Milford, MA) followed by incubation for 1 minute for digestion. Samples were then loaded to an Xbridge BEH C18 VanGuard trap column (Waters) and eluted and separated using an Ascentis Express Peptide ES-C18 column (Sigma-Aldrich) with a gradient of 0.1% formic acid to acetonitrile. All columns and solvents were maintained on ice. Peaks were detected with a Xevo G2-XS QToF mass spectrometer (Waters). Data were analyzed using MassLynx (Waters), HX-Express2 (Guttman *et al.*, 2013), and GraphPad Prism 7.

### **Molecular Dynamics (MD) Simulation**

We performed two sets of classical all-atom and explicit-solvent MD simulations for single-cysteine RGS4 and RGS4 D90L, single-cysteine RGS8 and RGS8 E84L, and WT RGS19 and RGS19 L118D (Table S1) using the NAMD software (Phillips *et al.*, 2005) on a high-performance computing cluster (Townes *et al.*, 2014) using the CHARMM force-field with the CMAP correction (MacKerell Jr *et al.*, 1998; MacKerell *et al.*, 2004). We used Visual Molecular Dynamics (VMD) for system creation and post-simulation analysis (Humphrey *et al.*, 1996). The initial coordinates were obtained from the protein data bank files with codes 1AGR (RGS4), 2DOE (RGS8), and 1CMZ (RGS19). Except for Cys95 in RGS4 and Cys89<sup>2</sup> in RGS8, all cysteines were changed to alanines. Each protein was then solvated in a simulation box of TIP3P water molecules (Jorgensen *et al.*, 1983) and charge-neutralized with NaCl. The final solvated and ionized simulation domains contained 30031 atoms (RGS4), 32257 atoms (RGS8), and 25077 atoms (RGS19). Each solvated and ionized system was energy minimized for ~500-1000 cycles via conjugate-gradient optimization, then equilibrated via 1 $\mu$ s MD simulations conducted with a time-step ( $\Delta t$ ) of 2 fs.

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The NPT ensemble with a Langevin thermostat and a damping coefficient of 5 ps<sup>-1</sup> was used for temperature control and the Nosé-Hoover barostat was used for pressure control. Periodic boundary conditions were used throughout; non-bonded interactions were accounted for with a cut-off of 10 Å where smooth switching was initiated at 8 Å. Long-range electrostatic interactions were handled using the Particle Mesh Ewald (PME) method.

### Dynamic cross-correlation analysis

The dynamic cross-correlation (DCC) maps of each system were calculated based on the C<sub>α</sub> atoms of residues using the MD-TASK package (Brown *et al.*, 2017). Each cell value (C<sub>ij</sub>) in the matrix of the DCC map was calculated using the following formula:

$$C_{ij} = \frac{\langle \Delta r_i \cdot \Delta r_j \rangle}{\left( \sqrt{\langle \Delta r_i^2 \rangle} \cdot \sqrt{\langle \Delta r_j^2 \rangle} \right)}$$

With Δr<sub>i</sub> represents the displacement from the mean position of atom i, and < > denotes the time average over the whole trajectory. Positive values of C<sub>ij</sub> show correlated motion between residues i and j, moving in the same direction, whereas negative values of C<sub>ij</sub> show anti-correlated motion between residues i and j, moving in the opposite direction.

### Analysis of salt-bridge interactions

Salt-bridge interaction analysis was carried out using VMD based on a distance criterion uniformly applied to determine the existence of salt-bridges for each frame in all trajectories (Schuster *et al.*, 2019). Specifically, salt-bridge interactions were considered to be formed if the distance between any of the oxygen atoms of acidic residues and the nitrogen atoms of basic residues were within a cut-off distance of 4 Å.

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## Statistical Analysis

Statistical tests in this work are exploratory. Changes in thermal stability were analyzed by 1-way ANOVA with Sidak's multiple comparisons post-test. Differences in deuterium incorporation were analyzed using 2-way ANOVA with Sidak's multiple comparisons post-test. Error bars represent means  $\pm$  SD. Except where otherwise indicated, all experimental biochemical data was done with an n of 3 independent experiments, which was sufficient to demonstrate reproducibility. Resulting p-values are descriptive rather than hypothesis-testing. In saturation binding experiments, RGS-G $\alpha$  inhibition was determined by fitting total and nonspecific binding. In functional inhibition experiments, IC<sub>50</sub> was determined by fitting a four-parameter logistic curve. All curve fitting and statistical analysis was done using GraphPad Prism 7 (GraphPad Inc, LaJolla, CA).

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## RESULTS

Comparison of the structures for RGS19 (PDB 1CMZ) (de Alba *et al.*, 1999), RGS4 (PDB 1AGR) (Tesmer *et al.*, 1997), and RGS8 (PDB 5DO9) (Taylor *et al.*, 2016) shows that there are differing numbers of interhelical salt bridges on the exteriors of their  $\alpha_4$ - $\alpha_7$  helix bundles. Some of these may contribute to differences in stability and dynamics among the RGS isoforms.

RGS19 has only one interhelical salt bridge in this bundle, between E125 ( $\alpha_4$ ) and K138 ( $\alpha_5$ ) (Fig. 1A and B). However, this salt bridge is well conserved among all three proteins (Fig. 1A-D), so it is unlikely to contribute to observed differences in flexibility (Shaw *et al.*, 2018). A salt bridge network that connects  $\alpha_4$ , the  $\alpha_5$ - $\alpha_6$  interhelical loop, and  $\alpha_5$  is present in RGS8 (E84-R119-E111) and RGS4 (D90-K125-E117) but absent in RGS19 (Fig. 1A and B). The residues that form this network are present in 7 of the 20 RGS protein family members, all in the R4 subfamily. Between the  $\alpha_5$  and  $\alpha_6$  helices, a salt bridge is present in RGS8 (D114-R132), but absent in both RGS4 and RGS19 (Fig. 1A and C). Finally, a charged pair between the  $\alpha_6$  and  $\alpha_7$  helices is present in RGS8 (E91-K104) and RGS4 (D130-K155), but is absent in RGS19 (Fig. 1A and D).

To estimate the relevance of each of these salt bridges in maintenance of helix bundle rigidity, the time each amino acid in a charged pair spent within a 4Å of one another over the course of a long timescale (2  $\mu$ s) MD simulation (Shaw *et al.*, 2018) was measured. The  $\alpha_6$ - $\alpha_7$  salt bridge, which is present in RGS4 and RGS8 but absent in RGS19, occupied a salt bridge-forming distance for 31.5% of the simulation in RGS4 and 36.1% in RGS8. The salt bridge interaction between residues of  $\alpha_4$  and  $\alpha_5$ - $\alpha_6$  interhelical loop, also not present in RGS19, was maintained for 58.7% of time in

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RGS4 and 44.2% in RGS8 (Table S2). The charged pair that is unique to RGS8 between  $\alpha_5$  and  $\alpha_6$  helices remained in contact for 47.5% of the simulation.

We elected to make mutations that altered interhelical salt bridges to test their functional roles. There are two positions at which interhelical salt bridges are shared by RGS4 and RGS8 but are absent in RGS19:  $\alpha_4$ - $\alpha_5$  (Fig. 1B) and  $\alpha_6$ - $\alpha_7$  (Fig. 1D). In the  $\alpha_4$  helix of RGS19, L118 was mutated to an aspartate to introduce the  $\alpha_4$ - $\alpha_5$  salt bridge found in RGS4 and RGS8 (Fig. 1B). In helix  $\alpha_7$  of RGS19, Q183 was mutated to a lysine to introduce the  $\alpha_6$ - $\alpha_7$  salt bridge found in RGS4 and RGS8 (Fig. 1D). In order to eliminate confounding effects due to multiple cysteines in inhibitor potency experiments, all proteins, with and without salt-bridge mutations, used a single-cysteine protein background. Each construct has only the conserved cysteine in helix  $\alpha_4$  of the RGS domain.

To determine how disruption or addition of a salt bridge may alter protein structure or dynamics, thermal stability was measured by differential scanning fluorimetry. Addition of a salt bridge in RGS19 by the L118D mutation caused a 7 °C increase in thermal stability compared to WT (Fig. 2A). In contrast, the Q183K mutation in RGS19 did not alter thermal stability or inhibitor potency (Supplemental Figure 1). Removal of a salt bridge in RGS8 by the E84L mutation caused an 8 °C decrease in thermal stability (Fig 2B). Unexpectedly, RGS4 showed a more complex pattern in which the D90L mutation resulted in a biphasic melt curve and a 5 °C increase in melting temperature rather than a decrease (Fig 2C).

To probe the molecular details of changes in structural flexibility in the mutant proteins, we conducted microsecond timescale classical MD simulations in explicit-solvent for RGS19 L118D,

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RGS8 E84L, and RGS4 D90L. Root-mean-square deviations (RMSDs) of these simulations are shown in Supplemental Figure 2. To understand the effect of the mutations on the protein structures, particularly in helices in the vicinity of the mutated site, we computed the root-mean-square fluctuation (RMSF) per residue from two independent MD simulations of mutated and WT RGS19, RGS8, and RGS4. The calculated change in RMSF per residue of the mutant RGS19 L118D from wild-type RGS19 reveals a strong stabilization and decrease in fluctuations of residues located in helices  $\alpha_4$ - $\alpha_7$  and in the interhelical loops between these helices. There is a particularly pronounced decrease in motion in the  $\alpha_5$ - $\alpha_6$  interhelical loop (Fig. 3A). We find a modest increase in fluctuation of residues in mutant RGS8 E84L vs. the wild-type structure (Fig. 3B). These changes are in the loop region connecting helices  $\alpha_5$  and  $\alpha_6$ , the  $\alpha_6$  helix, and the loop connecting helices  $\alpha_6$  and  $\alpha_7$ . Similar changes but of lesser extent were found in the mutant RGS4 D90L (Fig 3C). Additionally, small decreases were observed in the RMSF values of residues in helices  $\alpha_3$  and  $\alpha_8$  of the mutated RGS19 (Fig. 3A), but not in the mutated RGS8 and RGS4 (Fig. 3B and C).

To further investigate whether salt bridge-modifying mutations in RGS4, RGS8, and RGS19 affect residue-residue interactions, we calculated dynamic cross-correlation matrices for the  $C_\alpha$  atoms in all MD trajectories. For WT RGS19, RGS8, and RGS4, there is a modest positive correlation between the motions of residues of the  $\alpha_4$  helix and the residues of the  $\alpha_5$  helix (Fig. 4A-C). For the RGS19 L118D mutant, we find higher residue-residue correlations between helices  $\alpha_4$  and  $\alpha_5$  in comparison to unmutated RGS19 (see arrows, Fig. 4A). For wild-type RGS8, we find that the motions of residues in the  $\alpha_4$  helix (aa 79-93) and the  $\alpha_5$  helix (aa 97-113) are marginally positively correlated (see arrows, Fig. 4B). This positive correlation between the  $\alpha_4$  and  $\alpha_5$  helices remains

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in the RGS8 E84L mutant, but shows a modest shift in areas of correlation away from the loop connecting  $\alpha_4$ - $\alpha_5$  to mid-regions of the  $\alpha_4$  and  $\alpha_5$  helices (see arrows, Fig. 4B). There was no appreciable change between WT and mutant RGS4 (Fig. 4C).

In order to experimentally determine which regions in WT and mutant proteins were affected by the salt bridge mutations, hydrogen-deuterium exchange studies were performed. After exposure to solvent containing 90% D<sub>2</sub>O, proteins were digested with pepsin and deuterium incorporation (DI) was measured by mass spectrometry as previously reported (Shaw *et al.*, 2018). In RGS19, mutation of L118 to a salt bridge-forming residue, aspartic acid, caused significant decreases in DI in both  $\alpha_4$  helical fragments, aa 116-119 and aa 120-125. In the 116-119 fragment, WT RGS19 had reached 43.1% DI by 10 minutes, while the RGS19 L118D mutant showed less than half as much DI (18.7%). In fragment 120-125, WT RGS19 reached 18.5% DI at 10 minutes, while the RGS19 L118D mutant reached only 6.2%. Unlike RGS4 and RGS8, the RGS19 L118D mutant's changes in DI were more restricted to fragments from helices neighboring the mutation site, and were most pronounced in the early (1 to 10 minute) timescale (Fig. 5A). In RGS8, removal of the salt-bridge forming residue by the E84L mutation did not cause a significant change in DI in either of the fragments of the  $\alpha_4$  helix but trended toward a global increase in DI throughout the protein (Fig. 5B). In RGS4, the fragment surrounding the salt-bridge mutation site (aa 88-91) took up deuterium very slowly in both the WT and D90L mutant constructs, reaching 8.1% and 6.7% DI, respectively. However, the D90L mutation led to a substantial increase in deuterium exchange in the 92-97 fragment surrounding Cys95, from 17.5% to 37.0% DI. The RGS4 D90L mutant also trended toward increased DI across all protein fragments compared to WT RGS4, especially at higher timepoints (Fig. 5C).

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Finally, to assess the functional relevance of the  $\alpha_4$  salt-bridge forming residues, we used a flow-cytometry based protein-protein interaction assay (FCPIA) (Roman *et al.*, 2007; Blazer *et al.*, 2010) to measure the binding of RGS proteins to  $G\alpha_o$  and the potency of inhibition by CCG-50014. The L118D mutation in RGS19 induced an increase in  $pIC_{50}$  from  $-5.96 \pm 0.23 \log(M)$  (WT) to  $-5.08 \pm 0.25 \log(M)$  (L118D) (Fig. 6A). Conversely, removal of this charged  $\alpha_4$  residue in RGS4 and RGS8 induced a decrease in  $IC_{50}$  (Fig. 6B and C). CCG-50014 inhibited the RGS- $G\alpha$  interaction with an  $pIC_{50}$  of  $-5.08 \pm 0.16 \log(M)$  for WT RGS4 and  $-5.63 \pm 0.19 \log(M)$  for the RGS4 D90L mutant. It showed a potency of  $-5.09 \pm 0.69 \log(M)$  for WT RGS8 and  $-5.29 \pm 0.41 \log(M)$  for the RGS8 E84L mutant. None of the mutations to salt bridge-forming residues on the  $\alpha_4$  helix caused notable changes in affinity between  $G\alpha_o$  and RGS proteins. The L118D mutation in RGS19 shifted the  $K_d$  of the  $G\alpha_o$  interaction from  $20.5 \pm 6.3$  nM to  $23.9 \pm 5.3$  nM, the E84L mutation in RGS8 shifted the  $K_d$  from  $3.9 \pm 1.8$  nM to  $4.8 \pm 0.3$  nM, and the D90L mutation in RGS4 shifted the  $K_d$  from  $8.8 \pm 3.1$  nM to  $6.7 \pm 2.6$  nM (Table S3).



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## DISCUSSION

A comparison of the crystal structures of the three RGS proteins studied here revealed several differences in charged residue contacts among the proteins. We first observed that RGS19 has fewer interhelical salt bridges in its  $\alpha_4$ - $\alpha_7$  helical bundle than RGS4 or RGS8. This may be responsible for the high flexibility previously observed in WT RGS19 (Shaw *et al.*, 2018). RGS8 has four distinct interhelical salt bridges within the helical bundle, while RGS4 has three and RGS19 has one (Fig 1A), correlating with previously observed flexibility differences. RGS19 is most flexible, followed by RGS4 and RGS8 (Shaw *et al.*, 2018). This further supports a role of salt bridges in RGS protein flexibility.

The changes in thermal stability in response to mutations in the  $\alpha_4$  helix salt bridge-forming residues suggest that this location may be responsible for differences in stability and dynamics among the isoforms. This is supported by the increase in thermal stability in response to the L118D mutation in RGS19, and destabilization in RGS8 response to the E84L mutation. While the D90L mutation altered thermal stability in RGS4, it stabilized rather than destabilized the protein. The biphasic melt curves in D90L RGS4 make the thermal stability data difficult to interpret. HDX clarifies the effect of the D90L mutation in RGS4 by showing localized increases flexibility of the protein. The lack of effect on thermal stability with the Q183K mutation in RGS19 correlates with the observation that the  $\alpha_6$ - $\alpha_7$  salt bridges in RGS4 and RGS8 were less stably maintained in simulations than were the  $\alpha_4$ - $\alpha_5$  salt bridges. In light of these results, we found it unlikely that the difference between Q183 in  $\alpha_6$  of RGS19 and the lysines found in RGS4 and RGS8 (K155 and K149 respectively) play a major role in the flexibility differences between these proteins. Rather, the salt bridge-forming residue on  $\alpha_4$  is a stronger driver of differences in protein flexibility.

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To determine the effects of mutations in salt bridge-forming residues on protein dynamics, both an *in silico* approach (all-atom MD simulations) and an experimental approach (hydrogen-deuterium exchange) were employed. In simulations, the increase in positive correlation between residues in the  $\alpha_4$  and  $\alpha_5$  helices in the RGS19 L118D mutant likely results from the introduced interhelical salt-bridge. The decrease in DI in the  $\alpha_4$  helix of RGS19 in the HDX studies is consistent with reduced solvent exposure. This is of particular interest given that the Cys123 target of the TDZD compounds is located in that helix. Conversely, mutations that eliminated salt bridges in RGS4 and RGS8 increased DI in some fragments from their  $\alpha_4$  helices (Fig. 5A and B), suggesting that this results in increased solvent exposure and greater compound accessibility at the buried cysteine. Surprisingly, the RGS4 D90L mutant did not have increased DI in the fragment spanning the mutation site (Fig. 5C). In addition, the  $\mu$ s timescale MD simulations captured positive residue-residue ( $C_\alpha$ - $C_\alpha$ ) correlations between the  $\alpha_4$  and  $\alpha_5$  helices of that were similar in WT and mutated RGS4 D90L. This fits with the thermal stability data and suggests that the effect of the D90L mutation in RGS4 is more complex than simple disruption of an ionic contact.

In MD simulations, the RGS4 D90L and RGS8 E84L mutations did not have as large an effect on the magnitude of residue fluctuations as did the L118D mutation in RGS19 (Fig. 3A and B). This may be because differences become apparent on shorter timescales in RGS19 than in RGS4 and RGS8, so simulations on  $\mu$ s timescales may not have captured all of the differences in dynamics caused by mutations in RGS4 D90L and RGS8 E84L. Indeed, in HDX studies, stronger differences in DI were observed between RGS19 and RGS19 L118D at shorter timepoints (1 and 3 minutes) than in RGS4 D90L and RGS8 E84L (Fig 5A-C).

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Finally, to determine how changes in protein flexibility affected the potency of inhibition by an RGS inhibitor, we used FCPIA to evaluate the inhibition of  $G\alpha$  binding by CCG-50014. Importantly, manipulation of RGS protein flexibility induced the expected changes in the potency of inhibition by TDZD covalent modifiers. Thus, enhancing flexibility by removal of salt bridge-forming residues increased the potency of inhibition by CCG-50014 while reducing protein flexibility reduced potency of inhibition by CCG-50014. These results support a causal relationship between RGS protein flexibility and potency of inhibition.

In conclusion, differences in flexibility among RGS isoforms appear to drive differences in the potency of a covalent inhibitor, CCG-50014. The differences in isoform flexibility in turn are strongly influenced by the presence or absence of an  $\alpha_4$ - $\alpha_5$  salt bridge and manipulation of this salt bridge is sufficient to induce changes in inhibitor potency among single-cysteine RGS proteins. Developing a deeper understanding of these differences in flexibility may enable the development of a new generation of RGS inhibitors with novel specificities.

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## ACKNOWLEDGEMENTS

The authors would like to acknowledge the RTSF Mass Spectrometry and Metabolomics Core Facility (Michigan State University). We are grateful for computational support in part from Trillian, a Cray XE6m-200 supercomputer supported by the NSF MRI program under Grant PHY-1229408, and the NSF-supported (ACI-1053575) Extreme Science and Engineering Discovery Environment (XSEDE) Comet resource at San Diego Supercomputer Center (SDSC) under Grant TG-MCB160183 (H.V.).

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## **AUTHORSHIP CONTRIBUTIONS**

Participated in research design: Shaw, Mohammadi, Vashisth, and Neubig

Conducted experiments: Shaw, Mohammadi, and Quinn

Performed data analysis: Shaw, Mohammadi, and Quinn

Wrote or contributed to the writing of the manuscript: Shaw, Mohammadi, Vashisth, and Neubig

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## FOOTNOTES

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This work was supported by the National Science Foundation [Grants 1507588 (R.R.N.), 1508595 (H.V.)] and the National Institutes of Health Training Grant [Grant T32 GM092715 (V.S.S., R.R.N.)]. This work was also supported in part by a National Institutes of Health Centers of Biomedical Research Excellence (COBRE) Center of Integrated Biomedical and Bioengineering Research (CIBBR) [Grant P20 GM113131] through an Institutional Development Award (IDeA; to H.V.) from the National Institute of General Medical Sciences.

<sup>1</sup> Authors contributed equally.

<sup>2</sup> Note, amino acid numbering follows that for RGS8, Isoform 2, NCBI RefSeq: NP\_001095920.1

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## LEGENDS FOR FIGURES

**Figure 1.** (A) Alignment of RGS19, RGS4, and RGS8 sequences in  $\alpha_4$ - $\alpha_7$  helix bundle. Charged residues that make interhelical contacts are indicated in red and blue. RGS19 has 1, RGS4 has 3, and RGS8 has 4 salt bridges. Structural alignments of  $\alpha_4$ - $\alpha_5$  (B),  $\alpha_5$ - $\alpha_6$  (C), and  $\alpha_6$ - $\alpha_7$  (D) helix pairs are shown, with highlighted residues in panel a rendered as sticks. RGS19 (PDB 1CMZ) is in green, RGS4 (PDB 1AGR) is in yellow, and RGS8 (PDB 5DO9) is in cyan. Black brackets in panel A indicate residues depicted in panels B, C, and D. Arrows show which panels depict each set of bracketed residues.

**Figure 2.** Thermal stability was determined by differential scanning fluorimetry. (A) The L118D mutation in RGS19 increased melting temperature by 7 °C compared to WT. (B) The E84L mutation in RGS8 decreased melting temperature by 8 °C. (C) The RGS4 D90L mutation introduced a biphasic melt curve and increased melting temperature by 5 °C. For each pair, the three replicate derivative melt curves are shown on the left and average melt temperatures are shown on the right. Error bars represent SD. n=3. Analyzed by 1-way ANOVA with Sidak's Multiple Comparisons test. \*\*\*\* $p < 0.0001$

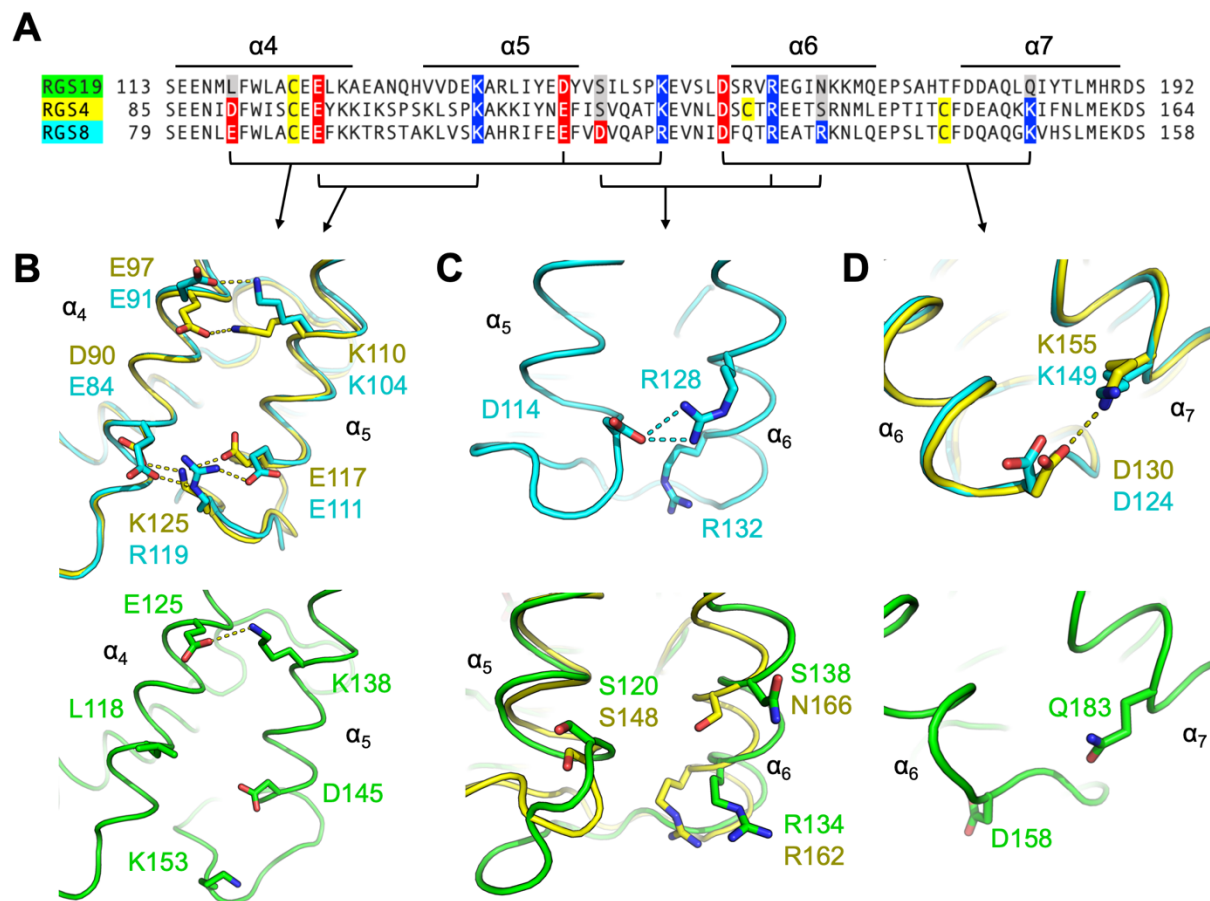
**Figure 3.** Change in RMSF per residue ( $\Delta$ RMSF) between wild-type RGS proteins and RGS proteins with mutation in the  $\alpha_4$ - $\alpha_5$  salt bridge forming residue. (A) L118D in RGS19 (B) E84L in RGS8 and (C) D90L in RGS4. Data represent differences in RMSF from two independent MD simulations of the mutated forms of RGS proteins.

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**Figure 4.** Dynamic cross correlation matrix calculated for the C $\alpha$  atoms of (A) RGS19/RGS19 L118D, (B) RGS8/RGS8 E84L, (C) RGS4/RGS4 D90L. Horizontal dotted lines indicate the regions of the  $\alpha_4$  helix, while vertical solid lines indicate the regions of the  $\alpha_5$  helix for each protein. The color scheme ranges from anticorrelation (-1.0, blue), no correlation (0, green), and positive correlation (+1.0, red). Values are the average for the two independent simulation runs.

**Figure 5.** Difference in % deuterium incorporation ( $\Delta\%$ DI) between mutated and unmutated proteins in RGS19 L118D (A), RGS8 E84L (B), and RGS4 D90L (C) fragments, as measured by HDX. Red arrows indicate fragments containing mutated residue, and black arrows indicate fragments containing conserved  $\alpha_4$  cysteine. Kinetics of deuterium incorporation in these fragments for individual constructs are shown below. n=3. Error bars represent SD. Analyzed by 2-way ANOVA with Sidak's multiple comparisons test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ .

**Figure 6.** Potency of inhibition of CCG-50014 against  $\alpha_4$  is altered in salt bridge mutants of RGS proteins. (A) RGS19 pIC $_{50}$ :  $-5.96 \pm 0.23 \log(\text{M})$ , RGS19 L118D pIC $_{50}$ :  $-5.08 \pm 0.25 \log(\text{M})$ . (B) RGS8 pIC $_{50}$ :  $-5.09 \pm 0.69 \log(\text{M})$ , RGS8 E84L pIC $_{50}$ :  $-5.29 \pm 0.41 \log(\text{M})$ . (C) RGS4 pIC $_{50}$ :  $-5.08 \pm 0.16 \log(\text{M})$ , RGS4 D90L pIC $_{50}$ :  $-5.63 \pm 0.19 \log(\text{M})$ . n=3.



**Figure 1**

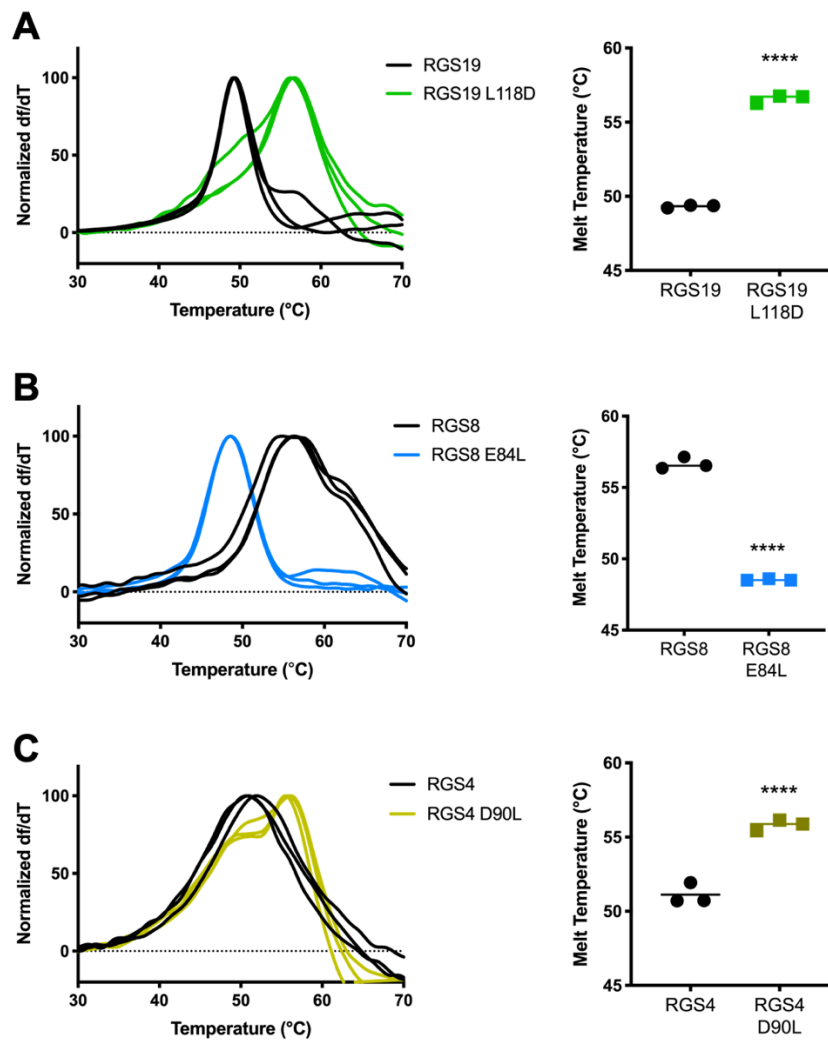


Figure 2

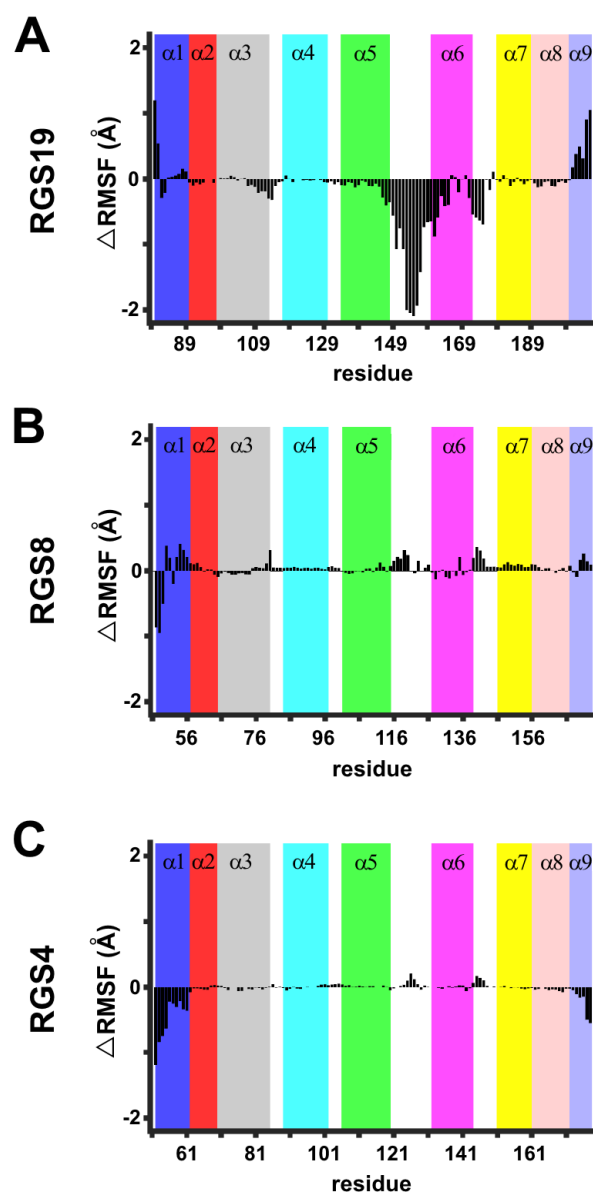


Figure 3



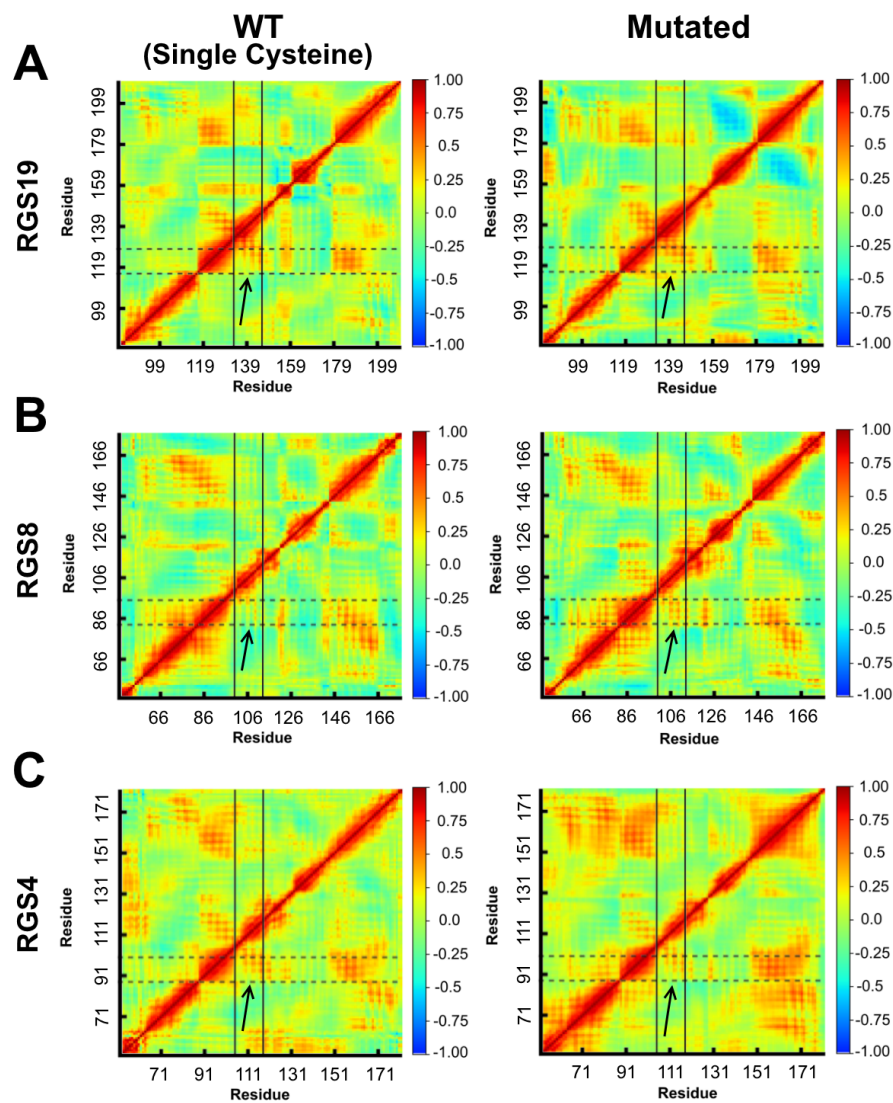
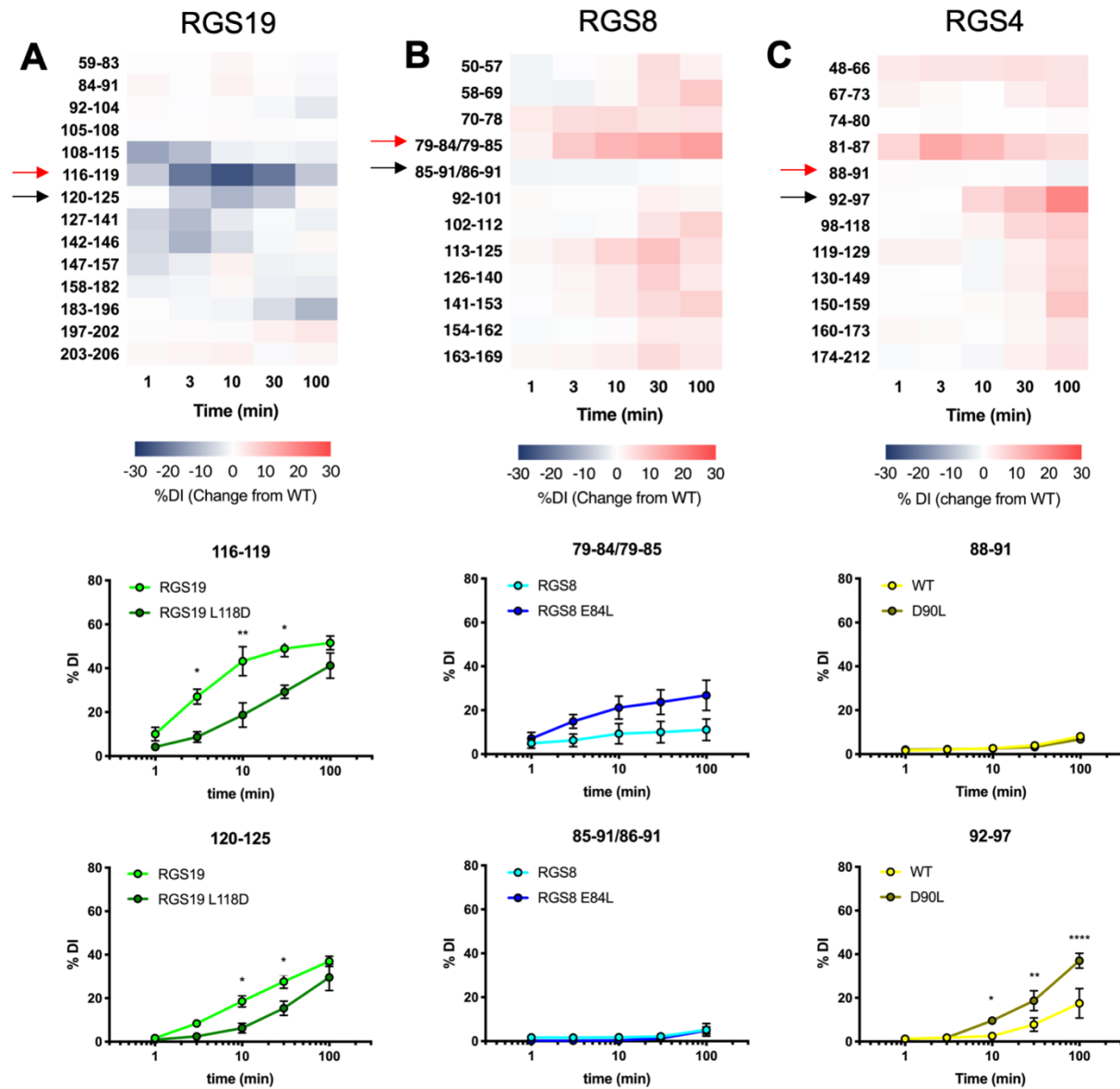
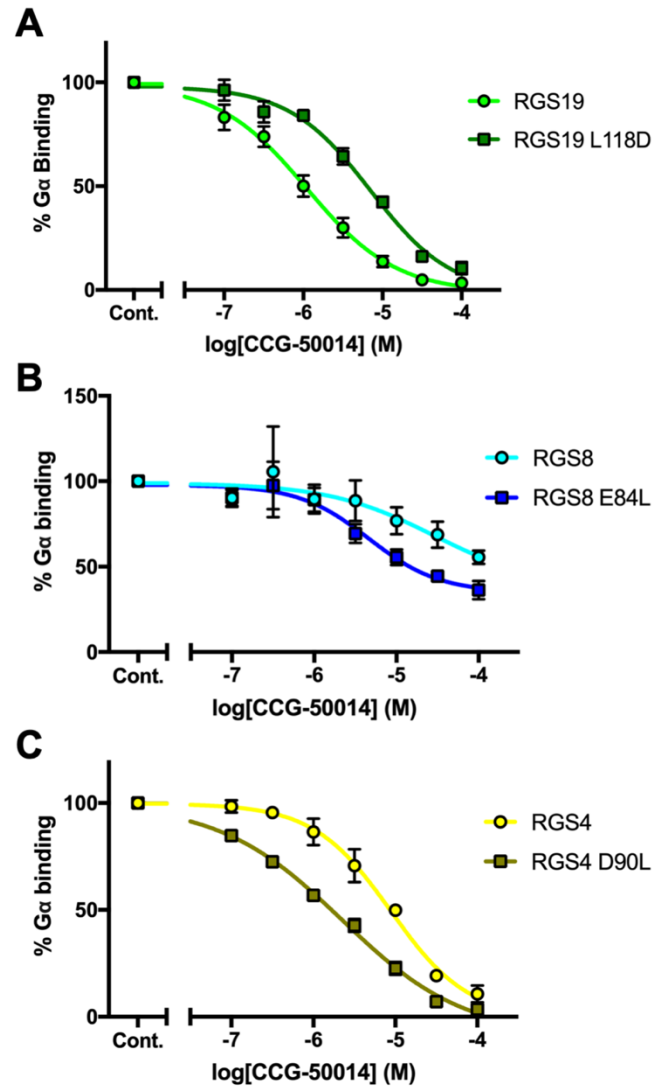


Figure 4



**Figure 5**



**Figure 6**