Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

Human Missense Mutations in Regulator of G Protein Signaling 2 Affect the Protein Function Through Multiple Mechanisms

Hoa T.N. Phan, Benita Sjögren, Richard R. Neubig

Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI 28824

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Altered functions of RGS2 missense mutations

Corresponding Author: Richard R. Neubig

Department of Pharmacology and Toxicology

Michigan State University

B423 Life Sciences building

1355 Bogue Street

East Lansing, MI 48824,

Phone: 517-353-7145

Email: rneubig@msu.edu

Number of text pages: 33

Number of tables: 1

Number of figures: 4

Number of references: 52

Number of words in abstract: 246

Number of words in introduction: 735

Number of words in discussion: 1093

Non-standard abbreviations: AT1R, angiotensin II type 1 receptor; DSF, Differential scanning fluorimetry; EDTA, Ethylenediaminetetraacetic acid; FCPIA, Flow cytometry protein interaction assay; GAP, GTPase accelerating protein; GPCR, G protein-coupled receptor; MFI, Mean fluorescent intensity; PDB, Protein data bank; RGS, Regulator of G protein signaling; SDS, Sodium dodecyl sulfate

Abstract

RGS2 plays a significant role in alleviating vascular contraction and promoting vascular relaxation due to its GTPase accelerating protein (GAP) activity toward Gαq. Mice lacking RGS2 display a hypertensive phenotype and several RGS2 missense mutations have been found predominantly in hypertensive human subjects. However, the mechanisms whereby these mutations could impact blood pressure is unknown. Here, we selected 16 rare, missense mutations in RGS2 identified in various human exome sequencing projects and evaluated their ability to inhibit intracellular calcium release mediated by the angiotensin II receptor type 1 (AT1R). Four of them had reduced function and were further investigated to elucidate underlying mechanisms. Low protein expression, protein mis-localization, and reduced G protein binding were identified as likely mechanisms of the malfunctioning mutants. The Q2L mutant had 50% lower RGS2 than wild-type (WT) protein detected by Western blot. Confocal microscopy demonstrated that R44H and D40Y had impaired plasma membrane targeting; only 46 and 35% of those proteins translocated to the plasma membrane when co-expressed with $G\alpha_q$ Q209L compared to 67% for WT RGS2. The R188H mutant had a significant reduction in $G\alpha_q$ binding affinity (10-fold increase in K_i compared to WT RGS2 in a flow cytometry competition binding assay). This study provides functional data for 16 human RGS2 missense variants on their effects on AT1 receptor-mediated calcium mobilization and provides molecular understanding of those variants with functional loss *in vitro*. These molecular behaviors can provide insight to inform antihypertensive therapeutics in individuals with variants having reduced function.

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Heart and cerebrovascular diseases have remained the major causes of death and disability in the United States (Heron, 2016; Mozaffarian et al., 2016) despite relentless efforts in cardiovascular research and drug development. Hypertension is a multifactorial disorder that places individuals at a higher risk for such diseases. Multiple genome-wide association studies (GWAS) have discovered polymorphisms in genes and loci that are associated with hypertension (Adeyemo et al., 2009; Dominiczak and Munroe, 2010; Franceschini et al., 2014; Huan et al., 2015; Levy et al., 2009; Lind and Chiu, 2013; Newton-Cheh et al., 2009; Pan et al., 2015). However, current knowledge about hypertension genetics is still far from complete. Common variants identified in GWAS only explain a small fraction of the blood pressure variance landscape (Dominiczak and Munroe, 2010). This limitation highlights the need to study rare variants that may contribute to this complex disorder (Gibson, 2012; Schork et al., 2009).

G protein coupled receptors (GPCRs) play a critical role in vascular tone regulation (Brinks and Eckhart, 2010). Several GPCRs with preferential activation of heterotrimeric G proteins of the $G\alpha_q$ family (e.g. Angiotensin II, Endothelin and α1 adrenergic receptors) mediate vasoconstrictor responses in blood vessels and many antihypertensive drugs act to counteract their effects. Regulator of G protein signaling (RGS) proteins play a crucial role in modulating GPCR signaling through their GTPase-activating protein (GAP) activity toward Gα subunits. RGS2, in particular, has been strongly implicated in cardiovascular regulation due to its selectivity toward $G\alpha_q$, resulting in diminished vasoconstrictor action (Heximer et al., 1997). Homozygous and heterozygous RGS2 knock out mice exhibit a hypertension phenotype attributed to prolonged Gαq-mediated vasoconstrictor signaling (Heximer et al., 2003) and a reduced NO/cGMP-mediated vascular relaxation response (Sun et al., 2005; Tang et al., 2003). Mice lacking RGS2 are also

prone to cardiac hypertrophy due to elevated Gαq signalling (Zhang et al., 2006). Increased sympathetic tone and altered renal mechanisms also contribute substantially to hypertension development in RGS2 knock-out mice (Gross et al., 2005; Gurley et al., 2010; Osei-Owusu et al., 2015).

The actual role of RGS2 in human hypertension is not well understood. A number of common variants in the promoter, introns and noncoding regions of RGS2 gene are associated with hypertension and suboptimal responsiveness to anti-hypertensive treatment in different ethnic groups (Freson et al., 2007; Riddle et al., 2006; Semplicini et al., 2006; Yang et al., 2005; Zhang et al., 2013). Several reported missense mutations in RGS2 were found predominantly in hypertensive subjects but with very low allele frequencies (Yang et al., 2005). Among those, the Q2L mutant allele was shown to have low protein expression due to rapid proteasomal degradation (Bodenstein et al., 2007; Park et al., 2015). The R44H mutant, on the other hand, showed less efficient plasma membrane targeting (Gu et al., 2008). Since these missense mutations of RGS2 had such low allele frequencies (less than 1%), it has been difficult to determine the functional significance of these mutations using epidemiological or informatics approaches.

The revolution in next-generation sequencing has revealed genetic information useful for prevention and clinical management (Rabbani et al., 2014). More commonly, healthcare professionals are presented with variants of uncertain significance (VUS), complicates the interpretation of genetic data (Ackerman, 2015; Gomez et al., 2016). Computational tools have been developed to predict variant effects with limited success (Schulz et al., 2015). Definitive characterization of VUS requires family segregation or functional studies. In alignment with this common theme, many missense mutations of RGS2 have been released to public databases such as NHLBI GO Exome Sequencing project (http://evs.gs.washington.edu/EVS/) and Exome

Aggregation Consortium (ExAC - http://exac.broadinstitute.org) but the functional consequences and human phenotypes of these variants is largely unknown. We hypothesized that changes in the coding sequence of RGS2 may result in protein products that have altered function. In this study, we examined 16 mutations (Table 1) found in a Japanese population (Yang et al., 2005), the NHLBI exome sequencing project (Fu et al., 2013) and the ExAC database (Lek et al., 2016) to determine whether they differ from WT RGS2 in their ability to regulate GPCR-mediated signalling. For those with reduced function, we determined the molecular characteristics contributing to those differences. Specifically, we investigated protein levels, protein localization and G protein binding activities of these mutants to further our understanding about the underlying mechanisms responsible for functional alteration of the mutants. Only a small fraction of variants showed altered function and it was due to several distinct mechanisms.

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and methods

DNA constructs. Mammalian expression vectors encoding the human, full-length, untagged or 3xHA tagged WT Angiotensin II type 1 (AT1) receptor and RGS2 in pcDNA3.1(+) were obtained from the University of Missouri-Rolla cDNA Resource Center (http://www.cdna.org). Other constructs were generated in our laboratory and the primer sequences for construct generation are available on request. RGS2 was amplified by PCR as attB1-2 fragments without a stop codon to allow subsequent cloning to an entry vector pDONR221 using Gateway® BP clonase (Invitrogen, Carlsbad, CA). RGS2-Q2L, RGS2-Q2R, RGS2-S3G, RGS2-A4V, RGS2-M5V, RGS2-K18N, RGS2-G23D, RGS2-D40Y, RGS2-R44H, RGS2-Q50K, RGS2-P55L, RGS2-Q78H, RGS2-A99G, RGS2-I110V, RGS2-R188H and RGS2-Q196R were generated in the entry vector by performing QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA). RGS2 WT and mutants were transferred into C-terminally V5 or GFP epitope-tagged expression vector (pcDNA™3.2DEST or pcDNA™DEST47, respectively) using Gateway® LR clonase (Invitrogen, Carlsbad, CA). A pMAL-C2H10T construct encoding the RH domain of human RGS2 (residues 72–203) was a kind gift from Dr. John J. Tesmer (University of Michigan). Mutagenesis for this vector was performed as described above. The open reading frame of all PCR and mutagenesis generated constructs was verified by sequencing at the RTSF Genomics Core at Michigan State University.

Cell Culture and Transfections. All cell lines were maintained in a humidified incubator at 37°C with 5% CO2. Human embryonic kidney (HEK)-293 cells were grown to 95% confluence in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. Chinese hamster ovary (CHO-K1) cells (ATCC, Manassas, VA) were grown to 95% confluence in F-12 nutrient

mixture (GIBCO, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. HEK-293 and CHO-K1 cells were transiently transfected using, respectively, X-tremeGENE HP DNA Transfection Reagent (Roche Life Sciences) at 2 ml/mg of plasmid DNA and DNA-In® CHO (MTI-GlobalStem, Gaithersburg, MD) at 3 ml/mg of plasmid DNA according to the manufacturers' recommended protocols. Transfection mixes were prepared in Opti-MEM (GIBCO, Carlsbad, CA) and all transfections were performed under antibiotic-free conditions. Experiments were run 24-48 hours after transfection.

SDS-PAGE and Western Blot Analysis. Twenty-four hours post-transfection, cells were treated as indicated in the figure legend and harvested using modified RIPA lysis buffer containing 50mM Tris HCl pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2mM EDTA, supplemented with complete protease inhibitor (Roche Diagnostics, Indianapolis, IN). Protein concentration in the cell lysates was determined using the BCA assay (Pierce; Rockford, IL) and adjusted with an appropriate volume of Laemmli buffer (BioRad; Hercules, CA). Equal amounts of protein in each lane were resolved on a 12% SDS-PAGE gel for 1 hour at 180 V. Samples were transferred to an Immobilon-FL membrane (EMD Millipore, Darmstadt, Germany) for 1 hour at 100 V, 400mA on ice and subjected to Western immunoblot analysis. The membrane was blocked with Odyssey blocking buffer (PBS) (Licor Biosciences, Lincoln, NE) for 30 minutes at room temperature on an orbital shaker. The membrane was probed overnight at 4°C with primary antibody diluted in blocking buffer. Mouse anti-V5 antibody (Invitrogen, Cat. 46-0705, Lot # 1620500), rabbit anti-HA (Santa Cruz, Dallas, TX; Cat. sc-805, Lot # D0413) and rabbit anti-actin (Santa Cruz, Dallas, TX; Cat. sc-1615-R, Lot # C2609) were used at a dilution of 1:5000, 1:1000 and 1:1000, respectively. The membrane was washed with PBS buffer supplemented with 0.1% Tween 20 (PBS-T) four times and probed for 1 hour at room temperature with IRDye–conjugated

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

secondary antibody diluted in blocking buffer. IRDye 680RD donkey anti-mouse (Cat. 926-68072, Lot # C31216-02) and IRDye 800CW donkey anti-rabbit antibody (Cat. 926-32213, Lot # C40130-03) (1:10.000) were from LiCor. After four washes in blocking buffer, the protein bands were visualized using LiCor Odyssey FC scanner and images were scanned and analyzed using the Image Studio™ Lite (Licor Biosciences, Lincoln, NE).

Confocal microscopy. HEK293 cells were seeded into collagen-coated 35 mm glass-bottom Petri dishes (MatTek Corporation, Ashland, MA) and transfected with 0.5 μg of plasmid DNA encoding RGS2-GFP with or without 0.5 μg of plasmid encoding $G\alpha_q^{Q209L}$. Confocal microscopy was performed on live cells 24 hours after transfection using an Olympus FluoView 1000 laser scanning confocal microscope. Images represent single planes on the basal side of the cell obtained with a 60x oil objective using 488 nm excitation and 505–530 nm emission wavelengths for GFP. Shown are pictures representative of at least 100 live cells from 3 independent experiments. Densitometric quantitation of protein expression was performed in a blinded manner using the line scan analysis function of the ImageJ software package.

Ca^{2+} mobilization assays. Twenty-four hours after transfection, CHO cells were split into black, flat, clear bottom 384-well plates (Greiner Bio-one, Kremsmünster, Austria) and allowed to attach overnight. The medium was aspirated and cells loaded with 1X fluo-4 NW (Molecular Probes, Eugene, OR) in a loading buffer consisting of Hank's basal saline solution supplemented with 20 mM HEPES, pH 7.4 and 2.5 μM probenecid following manufacturer's protocol. The cells were incubated for 30 min at 37°C, then 30 min at room temperature. Dye mix was removed and loading buffer was added to the cell plate. A concentration gradient of angiotensin II (Sigma, Cat. A9525) at 2x the final concentration was freshly prepared in loading buffer supplemented with 0.1% BSA for automated injection into the wells by a FDSS/μCell kinetic fluorescence plate reader

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

(Hamamatsu Photonics, Japan). Assay plates were then placed onto the plate reader for measuring the changes of intracellular free calcium in response to the receptor activation.

Protein expression and purification. The RGS domain of WT and mutant RGS2 was expresses as a C-terminal fusion on bacterial maltose binding protein (MBP). Protein was purified as described previously (Shankaranarayanan et al., 2008). All proteins had similar purity and concentration (Supplemental Fig. 1). Biotinylated ΔN-Gαq encoding residues 35-359 of murine $G\alpha_q$ was a gift from Dr. John J. Tesmer (University of Michigan).

Flow Cytometry Protein-Interaction Assays (FCPIA). The binding between $G\alpha_q$ with MBP-RGS2 and its point mutants were determined by a flow cytometry based assay (Nance et al., 2013; Roman et al., 2007). MBP-$RGS2^{72-203}$ was fluorescently labelled with amine reactive Alexa Fluor (AF) 532 NHS ester (Molecular Probes, Eugene, OR). Biotinylated murine $G\alpha_q$ was attached to xMAP LumAvidin beads (Luminex, Austin, TX). The indicated concentration of unlabelled MBP-RGS2 WT and mutants were used to compete with 80 nM of the Alexa Fluor (AF) 532 labelled WT MBP-RGS2. Binding was performed as described (Nance et al., 2013) in a full skirt 96 well plate (Bioexpress). Samples were analysed on a Luminex 200 flow cytometer, collecting at least 100 events per well. Data from at least 4 independent experiments of duplicate samples were fit by nonlinear regression using GraphPad Prism 7. K_i values were calculated using the Cheng-Prusoff equation.

Differential Scanning Fluorimetry Analysis. MBP-RGS2 proteins were cleaved using TEV protease (Promega, Madison, WI) and the RGS2 WT and mutant RGS domains were purified using Ni resin (Supplemental Fig. 1). Differential scanning fluorimetry analysis was performed in triplicate in 96 well-plates in final volume of 20 μl/well. Protein (7 μM final) was prepared in 20mM HEPES pH 8.0, 500 mM NaCl, 2mM DTT, 1x protein thermoshift dye (Applied

Biosystems, Cat. 4461146) according to manufacturer's protocol. Samples were heated from 25 °C to 99 °C with ramp rate of 1°C in continuous mode in an ABI 7500-Fast Real time PCR (Applied Biosystems). Data from melting curves were analysed in GraphPad Prism 7 using Boltzmann sigmoidal equation to obtain melting temperature.

Structure modelling. The structure of human RGS2 in complex with murine $G\alpha_q$ R183C (PDB - 4EKD) was modified to mimic the R188H mutant using the Pymol mutagenesis tool. The rotamer of the modified amino acid with least steric interference was chosen on the basis of least overlap of van der Waals radii.

Data Analysis and Statistics. All data were analyzed using GraphPad Prism 7.0 (GraphPad; LaJolla, CA). Dose response curves were fit using nonlinear regression (log(agonist) vs. response - Variable slope (four parameters)). Datasets with three or more groups were analyzed with one-way analysis of variance with Bonferroni's post hoc test for multiple comparisons, unless indicated in figure legend. Data are presented as mean ± S.E.M, and a P value less than 0.05 was considered significant.

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Differential effects of human RGS2 mutants to inhibit AT1 receptor-mediated intracellular calcium release

In the current study, we investigated the biochemical properties of 16 SNPs in the coding region of RGS2 previously identified in humans (Figure 1A). As an initial screen to determine the function of RGS2 mutants, CHO cells were co-transfected with cDNAs encoding AT1R (1 μg) and WT or mutant RGS2 (0.75μg). At this ratio, the suppression of AT1R-stimulated Ca^{2+} release by RGS2 was sub-maximal, enabling the identification of both gain- and loss-of function mutations (Supplemental Fig. 2). In the absence of RGS2, AT1R stimulation by angiotensin II caused a rapid and transient peak of intracellular calcium $[Ca^{2+}]_i$ (Fig. 1 B, C). Maximal $[Ca^{2+}]_i$ obtained from the dose response curves in cells expressing receptor without RGS2 (Fig. 1 D, E) was set as 100%. WT RGS2 protein co-expression inhibits peak $[Ca^{2+}]_i$ by 48 ± 2% (Fig. 1 B, C, F) and increases the EC_{50} from 4.2±0.3 nM to 7.9±0.7 nM (Supplemental Table 1) without changing receptor protein level (Supplemental Fig. 3). Four mutants including the Q2L, R188H, R44H and D40Y showed reduced function (Fig. 1 C, E, F). The 12 remaining mutants had comparable inhibitory effects to RGS2-WT. Therefore, they are considered to have normal function in this screen (Fig. 1 B, D, F). There was no significant change in EC_{50} mediated by RGS2 mutants compared to RGS2 WT (Supplemental table 1).

Transient protein expression levels of C-terminal V5 tagged RGS2

Differential protein expression could explain the different effects of these mutations. Specifically, the Q2L mutant has been shown to have low protein levels in HEK-293T (Bodenstein et al., 2007) and HeLa cells (Park et al., 2015) due to enhanced proteasomal degradation. In this study, we expressed C-terminal V5 tagged RGS2 WT and mutant constructs in CHO cells and total cell

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

lysates were probed with V5 tag antibody using Western blot. Consistent with previous work, the Q2L protein levels were also low in CHO cells (60% reduction, $p < 0.05$, Fig. 2) due to rapid protein turnover ($t_{1/2}$ Q2L: 6 ± 1 min vs RGS2 WT: 17 ± 4 min) (Supplemental Fig. 4). None of the other 15 mutants showed low protein expression as compared to RGS2 WT (Fig. 2).

D40Y and R44H mutants are mis-localized

Two of the mutants with reduced function, D40Y and R44H, had disrupted plasma membrane (PM) localization (Fig. 3) This had previously been demonstrated for the R44H mutant (Gu et al., 2008). In HEK-293 cells transfected with C-terminal GFP-tagged RGS2, nearly 100% localized in the nucleus when it was expressed alone (Fig. 3 A, B, C, I). Co-transfection with $G\alpha_q$ Q209L induced 67% of WT RGS2 to translocate to the PM (Fig. 3 E, J, K). The Q2L mutant had a similar translocation level compared to RGS2 WT (Fig. 3 K). The R44H mutant, as expected, had less protein localizing to the PM (21 ± 2% less than WT RGS2, $p < 0.0001$, Fig. 3 G, J, K). The D40Y mutant was also less efficiently translocated with only 35% protein in the PM (32 ± 2% less than RGS2 WT, $p<0.0001$, Fig. 3 F, J, K). A fraction of total R188H-GFP protein formed multiple aggregates throughout the cytoplasm either with or without $G\alpha_q$ Q209L co-expression (white arrow – Fig. 3 D, H).

Biochemical characteristics of RGS2 mutants: RGS2-$G\alpha_q$ binding and thermal stability

Among the 16 RGS2 mutants investigated, there are 4 mutations located in the RGS domain of RGS2: A99G, I110V, R188H and Q196R. Only the R188H mutant was identified as having reduced function. We hypothesized that that mutation could interfere with the binding of RGS2 and $G\alpha_q$, thereby reducing the GAP activity of RGS2 toward $G\alpha_q$. Binding of fluorescently labelled MBP-RGS2 WT to biotinylated $G\alpha_q$ was measured by Flow cytometry protein-interaction assay (FCPIA). The K_d in saturation binding studies was 83 ± 5 nM. Competition binding

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

measurements were performed, in which various concentrations of unlabeled MBP-RGS2 WT or mutants were mixed with 80 nM fluorescently labelled MBP-RGS2 WT then incubated with AlF_4^- activated $G\alpha_q$ bound to microspheres. Bound, labelled RGS2 was analyzed by flow cytometry. All MBP-RGS2 variants were further purified by gel filtration to ensure similar purity and then concentrated to approximately a 90 μM stock. K_i values of each mutant were calculated from IC_{50} values derived from the curves, using the Cheng-Prussoff equation in GraphPad Prism. Most RGS2 proteins (WT, Q196R, Q78H and A99G) had similar K_i values, 19, 20, 18, 15 nM, respectively. The R188H mutant, on the other hand, had a much lower affinity (K_i 233 nM, Fig. 4A).

We also measured the thermal stability of the R188H and Q196R mutants using differential scanning fluorimetry. Melting temperatures (T_m) of WT RGS2 and Q196R were similar, 46.8 and 49°C, respectively. R188H had a significantly lower T_m at 40 °C which reflects markedly lower protein stability ($p < 0.001$, Fig. 4B). R188H also had significantly higher basal fluorescence, indicating a less stable protein (Supplemental Fig. 5). This measure of protein stability, however, did not correlate with protein stability in cells. The proteasome inhibitor MG-132 increased protein levels of R188H with a similar magnitude as that of RGS2 WT (Supplemental Fig.6).

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Over 70 rare non-synonymous RGS2 mutations have been identified in humans through multiple exome sequencing projects and these may contribute to a propensity for hypertension. Of these, 32 mutations are reported in at least 2 individuals, with the Q50K mutant having the highest allele frequency of 0.08% of overall population. Data are publicly available through The Exome Aggregation Consortium. In this study, we focused on 16 mutations (nonsynonymous polymorphisms) that were found in at least 2 individuals in both the ExAC and the NHLBI GO Exome Sequencing project databases. We identified 4 mutations that result in RGS2 proteins that display a functional deficit in inhibiting AT1R-mediated increases in intracellular calcium in CHO cells. Of these 4, the results with the D40Y and R188H mutants are novel while the Q2L and R44H mutants have been investigated in the past. Lower basal protein expression, impaired plasma membrane targeting, and G protein binding deficiency were identified as the mechanisms most likely responsible for the reduced function of these mutants.

Multiple $G\alpha_q$ coupled receptors have been used to probe RGS2 function *in vitro* such as M1 or M3 muscarinic receptors (Bodenstein et al., 2007; Gu et al., 2007; Gu et al., 2008) or the vasopressin receptor (Osei-Owusu et al., 2007). In this study, we chose the angiotensin II receptor because of its relevance in systematic regulation of vascular function and blood pressure (Cameron et al., 2016; de Kloet et al., 2015; Li and Zhuo, 2016). Moreover, RGS2 has been proposed to serve as a selective and potent regulator of AT1R, due to interaction through its amino terminal domains (Hercule et al., 2007; Heximer et al., 2003; Matsuzaki et al., 2011). As 12 of the RGS2 mutations included in the study are located in the RGS2 amino terminal region (Fig. 1 A), a functional screen against AT1R could reveal mutations that selectively disrupt RGS2 protein activity by blocking RGS2-receptor coupling. However, we did not find such mutants in our screen (Fig. 1 F). We also

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

did not identify any gain-of-function mutations, despite using conditions that would enable such identification (Supplemental Fig. 2).

Low expression of RGS2 protein resulting in prolonged GPCR signaling has been proposed for hypertension in the case of the rare Q2L missense mutation and the common C1114G polymorphism in the 3'-UTR of the RGS2 gene (Park et al., 2015; Semplicini et al., 2006). We further confirmed that the Q2L mutant RGS2 has low protein expression (Fig. 2) due to a rapid turnover rate (Supplemental Fig. 4 A, C). No other RGS2 mutants that we investigated had low cellular protein levels in our system (Fig. 2).

The amphipathic α helix at the amino terminus of RGS2 promotes plasma membrane association and affects protein function (Gu et al., 2007). The R44H mutation has been shown to impair RGS2 membrane targeting by interference with lipid bilayer association (Gu et al., 2008). In this study, we confirmed that effect of the R44H mutation (Fig. 3 C, G, J, K). We also identified another mutation in this α helix, D40Y, that exhibited reduced plasma membrane localization (Fig. 3 B, F, J, K) and function (Fig. 1 C, E, F). Unlike the R44H mutant in which both residues have a positive charge, the amino acid substitution in D40Y changes from the negatively charged aspartic acid to the hydrophobic tyrosine residue. This more dramatic alteration could explain the somewhat larger effect on $G\alpha_q$-dependent membrane localization (R44H: 46% of control vs D40Y: 35%, Fig. 3 K).

While protein expression levels and membrane localization indirectly affect the negative regulatory effects of RGS2 on $G\alpha_q$-coupled receptor signaling, the interaction between RGS2 and $G\alpha_q$ protein is absolutely critical for RGS2 function. The published crystal structure of the RGS2 domain/$G\alpha_q$ protein complex shows that the arginine residue at position 188 is close to the binding interface between two proteins (Nance et al., 2013). It may also form networked salt bridges

(Donald et al., 2011) with the glutamate residue at position 104 to stabilize protein tertiary structure (Fig. 4C). When this arginine is mutated to a histidine residue, the histidine rotamer with the least steric hindrance may not be sufficiently close to E104 to form a salt bridge, potentially rendering RGS2 more flexible and less structurally stable (Fig. 4D). Besides the lower melting temperature (Fig. 4B), the initial fluorescence of the R188H mutant with the thermostability dye was noticeably higher than that of the WT and Q196R proteins (Supplemental Fig. 5). This suggests a disruption of basal protein folding. This misfolding could also explain the aggregation of the R188H-GFP in cells in the localization study. Disruption of the binding interface and structural instability properties of the R188H may explain the reduced binding affinity to $G\alpha_q$. Further investigation demonstrated that this mutant has slightly shorter half-life than WT RGS2 (16 ± 1.5 min vs 22 ± 1.3 min, N.S., Supplemental Fig. 3 B, D) and is also subjected to proteasomal degradation (Supplemental Fig. 6).

The regulatory function of RGS2 in cardiovascular homeostasis is not limited to its inhibitory effect toward $G\alpha_q$ signaling. RGS2 can also control protein synthesis through interaction with the eukaryotic initiation factor, eIF2B which has been implicated in protection against cardiac hypertrophy (Chidiac et al., 2014; Nguyen et al., 2009). RGS2 may also interact with $G\alpha_s$ and several adenylate cyclase subtypes to decrease cAMP production in cell-based assays (Roy et al., 2006; Salim et al., 2003). Overall, our study mainly focused on the canonical RGS2 effect to reduce $G\alpha_q$ signaling. How RGS2 mutants function with respect to these other mechanisms has not been tested so the 12 mutants with “normal” function here may be perturbed in other ways.

Some common polymorphisms in regulatory regions of RGS2 gene such as in-del mutations in the 3’ UTR have been shown to contribute to cardiovascular diseases such as

hypertension and responsiveness to anti-hypertensive treatment (He et al., 2015; Kvehaugen et al., 2014; Riddle et al., 2006; Semplicini et al., 2006; Zhang et al., 2013). In this study, we demonstrate that rare mutations in the protein coding region of RGS2 can affect protein function at least in 3 different ways (protein stability, localization, and G protein binding), and result in signaling deregulation. How the function of RGS2 mutants *in vitro* correlates with their activities in vascular tissue or in human physiology will need to be determined. The results from this study could guide the development RGS2 transgenic animal models to derive further knowledge about the activity of rare RGS2 mutations *in vivo*. It will also provide a strategy to selectively target these mechanisms with directed repurposed or novel cardiovascular therapeutics.

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

The authors would like to thank the Exome Aggregation Consortium and the groups that provided exome variant data for comparison. A full list of contributing groups can be found at http://exac.broadinstitute.org/about. We also thank Dr. John Tesmer (University of Michigan) for kindly providing MBP-RGS2 plasmid and biotinylated $G\alpha_q$ protein, Dr. Thomas Dexheimer and Dr. Matthew Bernard (Michigan State University) for their technical assistance in using the Hamamatsu plate reader and the Luminex flow cytometry system. Vincent Shaw assisted with protein purification and structural modelling.

Conflict of interest

The authors declare no conflict of interest related to the contents of this article.

Authorship contribution

Participated in research design: Neubig, Sjögren, Phan

Conducted experiments: Phan

Performed data analysis: Phan, Sjögren, Neubig

Wrote or contributed to the writing of the manuscript: Phan, Sjögren, Neubig

References

Ackerman MJ (2015) Genetic purgatory and the cardiac channelopathies: Exposing the variants of uncertain/unknown significance issue. *Heart Rhythm* **12**(11): 2325-2331.

Adeyemo A, Gerry N, Chen G, Herbert A, Doumatey A, Huang H, Zhou J, Lashley K, Chen Y, Christman M and Rotimi C (2009) A genome-wide association study of hypertension and blood pressure in African Americans. *PLoS Genet* **5**(7): e1000564.

Bodenstein J, Sunahara RK and Neubig RR (2007) N-terminal residues control proteasomal degradation of RGS2, RGS4, and RGS5 in human embryonic kidney 293 cells. *Mol Pharmacol* **71**(4): 1040-1050.

Brinks HL and Eckhart AD (2010) Regulation of GPCR signaling in hypertension. *Biochim Biophys Acta* **1802**(12): 1268-1275.

Cameron AC, Lang NN and Touyz RM (2016) Drug Treatment of Hypertension: Focus on Vascular Health. *Drugs* **76**(16): 1529-1550.

Chidiac P, Sobiesiak AJ, Lee KN, Gros R and Nguyen CH (2014) The eIF2B-interacting domain of RGS2 protects against GPCR agonist-induced hypertrophy in neonatal rat cardiomyocytes. *Cell Signal* **26**(6): 1226-1234.

de Kloet AD, Liu M, Rodriguez V, Krause EG and Sumners C (2015) Role of neurons and glia in the CNS actions of the renin-angiotensin system in cardiovascular control. *Am J Physiol Regul Integr Comp Physiol* **309**(5): R444-458.

Dominiczak AF and Munroe PB (2010) Genome-wide association studies will unlock the genetic basis of hypertension: pro side of the argument. *Hypertension* **56**(6): 1017-1020; discussion 1025.

Donald JE, Kulp DW and DeGrado WF (2011) Salt bridges: geometrically specific, designable interactions. *Proteins* **79**(3): 898-915.

Franceschini N, Chasman DI, Cooper-DeHoff RM and Arnett DK (2014) Genetics, ancestry, and hypertension: implications for targeted antihypertensive therapies. *Curr Hypertens Rep* **16**(8): 461.

Freson K, Stolarz K, Aerts R, Brand E, Brand-Herrmann SM, Kawecka-Jaszcz K, Kuznetsova T, Tikhonoff V, Thijs L, Vermylen J, Staessen JA, Van Geet C and European Project on Genes in Hypertension I (2007) -391 C to G substitution in the regulator of G-protein signalling-2 promoter increases susceptibility to the metabolic syndrome in white European men: consistency between molecular and epidemiological studies. *J Hypertens* **25**(1): 117-125.

Fu W, O'Connor TD, Jun G, Kang HM, Abecasis G, Leal SM, Gabriel S, Rieder MJ, Altshuler D, Shendure J, Nickerson DA, Bamshad MJ, Project NES and Akey JM (2013) Analysis of 6,515 exomes reveals the recent origin of most human protein-coding variants. *Nature* **493**(7431): 216-220.

Gibson G (2012) Rare and common variants: twenty arguments. *Nat Rev Genet* **13**(2): 135-145.

Gomez J, Reguero JR and Coto E (2016) The Ups and Downs of Genetic Diagnosis of Hypertrophic Cardiomyopathy. *Rev Esp Cardiol (Engl Ed)* **69**(1): 61-68.

Gross V, Tank J, Obst M, Plehm R, Blumer KJ, Diedrich A, Jordan J and Luft FC (2005) Autonomic nervous system and blood pressure regulation in RGS2-deficient mice. *Am J Physiol Regul Integr Comp Physiol* **288**(5): R1134-1142.

Gu S, He J, Ho WT, Ramineni S, Thal DM, Natesh R, Tesmer JJ, Hepler JR and Heximer SP (2007) Unique hydrophobic extension of the RGS2 amphipathic helix domain imparts

increased plasma membrane binding and function relative to other RGS R4/B subfamily members. *J Biol Chem* **282**(45): 33064-33075.

Gu S, Tirgari S and Heximer SP (2008) The RGS2 gene product from a candidate hypertension allele shows decreased plasma membrane association and inhibition of Gq. *Mol Pharmacol* **73**(4): 1037-1043.

Gurley SB, Griffiths RC, Mendelsohn ME, Karas RH and Coffman TM (2010) Renal actions of RGS2 control blood pressure. *J Am Soc Nephrol* **21**(11): 1847-1851.

He F, Luo J, Zhang Z, Luo Z, Fan L, He Y, Wen J, Zhu D, Gao J, Wang Y, Qian Y, Zhou H, Chen X and Zhang W (2015) The RGS2 (-391, C>G) genetic variation correlates to antihypertensive drug responses in Chinese patients with essential hypertension. *PLoS One* **10**(4): e0121483.

Hercule HC, Tank J, Plehm R, Wellner M, da Costa Goncalves AC, Gollasch M, Diedrich A, Jordan J, Luft FC and Gross V (2007) Regulator of G protein signalling 2 ameliorates angiotensin II-induced hypertension in mice. *Exp Physiol* **92**(6): 1014-1022.

Heron M (2016) Deaths: Leading Causes for 2014, National vital statistics reports. **65**.

Heximer SP, Knutsen RH, Sun X, Kaltenbronn KM, Rhee MH, Peng N, Oliveira-dos-Santos A, Penninger JM, Muslin AJ, Steinberg TH, Wyss JM, Mecham RP and Blumer KJ (2003) Hypertension and prolonged vasoconstrictor signaling in RGS2-deficient mice. *The Journal of clinical investigation* **111**(4): 445-452.

Heximer SP, Watson N, Linder ME, Blumer KJ and Hepler JR (1997) RGS2/G0S8 is a selective inhibitor of Gqalpha function. *Proc Natl Acad Sci U S A* **94**(26): 14389-14393.

Huan T, Meng Q, Saleh MA, Norlander AE, Joehanes R, Zhu J, Chen BH, Zhang B, Johnson AD, Ying S, Courchesne P, Raghavachari N, Wang R, Liu P, International Consortium

for Blood Pressure G, O'Donnell CJ, Vasan R, Munson PJ, Madhur MS, Harrison DG, Yang X and Levy D (2015) Integrative network analysis reveals molecular mechanisms of blood pressure regulation. *Mol Syst Biol* **11**(1): 799.

Kvehaugen AS, Melien O, Holmen OL, Laivuori H, Dechend R and Staff AC (2014) Hypertension after preeclampsia and relation to the C1114G polymorphism (rs4606) in RGS2: data from the Norwegian HUNT2 study. *BMC Med Genet* **15**: 28.

Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, O'Donnell-Luria AH, Ware JS, Hill AJ, Cummings BB, Tukiainen T, Birnbaum DP, Kosmicki JA, Duncan LE, Estrada K, Zhao F, Zou J, Pierce-Hoffman E, Berghout J, Cooper DN, Deflaux N, DePristo M, Do R, Flannick J, Fromer M, Gauthier L, Goldstein J, Gupta N, Howrigan D, Kiezun A, Kurki MI, Moonshine AL, Natarajan P, Orozco L, Peloso GM, Poplin R, Rivas MA, Ruano-Rubio V, Rose SA, Ruderfer DM, Shakir K, Stenson PD, Stevens C, Thomas BP, Tiao G, Tusie-Luna MT, Weisburd B, Won HH, Yu D, Altshuler DM, Ardissino D, Boehnke M, Danesh J, Donnelly S, Elosua R, Florez JC, Gabriel SB, Getz G, Glatt SJ, Hultman CM, Kathiresan S, Laakso M, McCarroll S, McCarthy MI, McGovern D, McPherson R, Neale BM, Palotie A, Purcell SM, Saleheen D, Scharf JM, Sklar P, Sullivan PF, Tuomilehto J, Tsuang MT, Watkins HC, Wilson JG, Daly MJ, MacArthur DG and Exome Aggregation C (2016) Analysis of protein-coding genetic variation in 60,706 humans. *Nature* **536**(7616): 285-291.

Levy D, Ehret GB, Rice K, Verwoert GC, Launer LJ, Dehghan A, Glazer NL, Morrison AC, Johnson AD, Aspelund T, Aulchenko Y, Lumley T, Kottgen A, Vasan RS, Rivadeneira F, Eiriksdottir G, Guo X, Arking DE, Mitchell GF, Mattace-Raso FU, Smith AV, Taylor K, Scharpf RB, Hwang SJ, Sijbrands EJ, Bis J, Harris TB, Ganesh SK, O'Donnell CJ,

Hofman A, Rotter JI, Coresh J, Benjamin EJ, Uitterlinden AG, Heiss G, Fox CS, Witteman JC, Boerwinkle E, Wang TJ, Gudnason V, Larson MG, Chakravarti A, Psaty BM and van Duijn CM (2009) Genome-wide association study of blood pressure and hypertension. *Nat Genet* **41**(6): 677-687.

Li XC and Zhuo JL (2016) Recent Updates on the Proximal Tubule Renin-Angiotensin System in Angiotensin II-Dependent Hypertension. *Curr Hypertens Rep* **18**(8): 63.

Lind JM and Chiu CL (2013) Genetic discoveries in hypertension: steps on the road to therapeutic translation. *Heart* **99**(22): 1645-1651.

Matsuzaki N, Nishiyama M, Song D, Moroi K and Kimura S (2011) Potent and selective inhibition of angiotensin AT1 receptor signaling by RGS2: roles of its N-terminal domain. *Cell Signal* **23**(6): 1041-1049.

Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jimenez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW, Turner MB, American Heart Association Statistics C and Stroke Statistics S (2016) Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. *Circulation* **133**(4): e38-360.

Nance MR, Kreutz B, Tesmer VM, Sterne-Marr R, Kozasa T and Tesmer JJ (2013) Structural and functional analysis of the regulator of G protein signaling 2-galphaq complex. *Structure* **21**(3): 438-448.

Newton-Cheh C, Johnson T, Gateva V, Tobin MD, Bochud M, Coin L, Najjar SS, Zhao JH, Heath SC, Eyheramendy S, Papadakis K, Voight BF, Scott LJ, Zhang F, Farrall M, Tanaka T, Wallace C, Chambers JC, Khaw KT, Nilsson P, van der Harst P, Polidoro S, Grobbee DE, Onland-Moret NC, Bots ML, Wain LV, Elliott KS, Teumer A, Luan J, Lucas G, Kuusisto J, Burton PR, Hadley D, McArdle WL, Wellcome Trust Case Control C, Brown M, Dominiczak A, Newhouse SJ, Samani NJ, Webster J, Zeggini E, Beckmann JS, Bergmann S, Lim N, Song K, Vollenweider P, Waeber G, Waterworth DM, Yuan X, Groop L, Orho-Melander M, Allione A, Di Gregorio A, Guarrera S, Panico S, Ricceri F, Romanazzi V, Sacerdote C, Vineis P, Barroso I, Sandhu MS, Luben RN, Crawford GJ, Jousilahti P, Perola M, Boehnke M, Bonnycastle LL, Collins FS, Jackson AU, Mohlke KL, Stringham HM, Valle TT, Willer CJ, Bergman RN, Morken MA, Doring A, Gieger C, Illig T, Meitinger T, Org E, Pfeufer A, Wichmann HE, Kathiresan S, Marrugat J, O'Donnell CJ, Schwartz SM, Siscovick DS, Subirana I, Freimer NB, Hartikainen AL, McCarthy MI, O'Reilly PF, Peltonen L, Pouta A, de Jong PE, Snieder H, van Gilst WH, Clarke R, Goel A, Hamsten A, Peden JF, Seedorf U, Syvanen AC, Tognoni G, Lakatta EG, Sanna S, Scheet P, Schlessinger D, Scuteri A, Dorr M, Ernst F, Felix SB, Homuth G, Lorbeer R, Reffelmann T, Rettig R, Volker U, Galan P, Gut IG, Hercberg S, Lathrop GM, Zelenika D, Deloukas P, Soranzo N, Williams FM, Zhai G, Salomaa V, Laakso M, Elosua R, Forouhi NG, Volzke H, Uiterwaal CS, van der Schouw YT, Numans ME, Matullo G, Navis G, Berglund G, Bingham SA, Kooner JS, Connell JM, Bandinelli S, Ferrucci L, Watkins H, Spector TD, Tuomilehto J, Altshuler D, Strachan DP, Laan M, Meneton P, Wareham NJ, Uda M, Jarvelin MR, Mooser V, Melander O, Loos RJ, Elliott

P, Abecasis GR, Caulfield M and Munroe PB (2009) Genome-wide association study identifies eight loci associated with blood pressure. *Nat Genet* **41**(6): 666-676.

Nguyen CH, Ming H, Zhao P, Hugendubler L, Gros R, Kimball SR and Chidiac P (2009) Translational control by RGS2. *The Journal of cell biology* **186**(5): 755-765.

Osei-Owusu P, Owens EA, Jie L, Reis JS, Forrester SJ, Kawai T, Eguchi S, Singh H and Blumer KJ (2015) Regulation of Renal Hemodynamics and Function by RGS2. *PLoS One* **10**(7): e0132594.

Osei-Owusu P, Sun X, Drenan RM, Steinberg TH and Blumer KJ (2007) Regulation of RGS2 and second messenger signaling in vascular smooth muscle cells by cGMP-dependent protein kinase. *J Biol Chem* **282**(43): 31656-31665.

Pan S, Naruse H and Nakayama T (2015) Progress and issues of the genome-wide association study for hypertension. *Curr Med Chem* **22**(8): 1016-1029.

Park SE, Kim JM, Seok OH, Cho H, Wadas B, Kim SY, Varshavsky A and Hwang CS (2015) Control of mammalian G protein signaling by N-terminal acetylation and the N-end rule pathway. *Science* **347**(6227): 1249-1252.

Rabbani B, Tekin M and Mahdieh N (2014) The promise of whole-exome sequencing in medical genetics. *J Hum Genet* **59**(1): 5-15.

Riddle EL, Rana BK, Murthy KK, Rao F, Eskin E, O'Connor DT and Insel PA (2006) Polymorphisms and haplotypes of the regulator of G protein signaling-2 gene in normotensives and hypertensives. *Hypertension* **47**(3): 415-420.

Roman DL, Talbot JN, Roof RA, Sunahara RK, Traynor JR and Neubig RR (2007) Identification of small-molecule inhibitors of RGS4 using a high-throughput flow cytometry protein interaction assay. *Mol Pharmacol* **71**(1): 169-175.

Roy AA, Baragli A, Bernstein LS, Hepler JR, Hebert TE and Chidiac P (2006) RGS2 interacts with Gs and adenylyl cyclase in living cells. *Cell Signal* **18**(3): 336-348.

Salim S, Sinnarajah S, Kehrl JH and Dessauer CW (2003) Identification of RGS2 and type V adenylyl cyclase interaction sites. *J Biol Chem* **278**(18): 15842-15849.

Schork NJ, Murray SS, Frazer KA and Topol EJ (2009) Common vs. rare allele hypotheses for complex diseases. *Curr Opin Genet Dev* **19**(3): 212-219.

Schulz WL, Tormey CA and Torres R (2015) Computational Approach to Annotating Variants of Unknown Significance in Clinical Next Generation Sequencing. *Lab Med* **46**(4): 285-289.

Semplicini A, Lenzini L, Sartori M, Papparella I, Calo LA, Pagnin E, Strapazzon G, Benna C, Costa R, Avogaro A, Ceolotto G and Pessina AC (2006) Reduced expression of regulator of G-protein signaling 2 (RGS2) in hypertensive patients increases calcium mobilization and ERK1/2 phosphorylation induced by angiotensin II. *J Hypertens* **24**(6): 1115-1124.

Shankaranarayanan A, Thal DM, Tesmer VM, Roman DL, Neubig RR, Kozasa T and Tesmer JJ (2008) Assembly of high order G alpha q-effector complexes with RGS proteins. *J Biol Chem* **283**(50): 34923-34934.

Sun X, Kaltenbronn KM, Steinberg TH and Blumer KJ (2005) RGS2 is a mediator of nitric oxide action on blood pressure and vasoconstrictor signaling. *Mol Pharmacol* **67**(3): 631-639.

Tang KM, Wang GR, Lu P, Karas RH, Aronovitz M, Heximer SP, Kaltenbronn KM, Blumer KJ, Siderovski DP, Zhu Y and Mendelsohn ME (2003) Regulator of G-protein signaling-2 mediates vascular smooth muscle relaxation and blood pressure. *Nat Med* **9**(12): 1506-1512.

Yang J, Kamide K, Kokubo Y, Takiuchi S, Tanaka C, Banno M, Miwa Y, Yoshii M, Horio T, Okayama A, Tomoike H, Kawano Y and Miyata T (2005) Genetic variations of regulator of G-protein signaling 2 in hypertensive patients and in the general population. *J Hypertens* **23**(8): 1497-1505.

Zhang C, Wang L, Liao Q, Zhang L, Xu L, Chen C, Ye H, Xu X, Ye M and Duan S (2013) Genetic associations with hypertension: meta-analyses of six candidate genetic variants. *Genet Test Mol Biomarkers* **17**(10): 736-742.

Zhang W, Anger T, Su J, Hao J, Xu X, Zhu M, Gach A, Cui L, Liao R and Mende U (2006) Selective loss of fine tuning of Gq/11 signaling by RGS2 protein exacerbates cardiomyocyte hypertrophy. *J Biol Chem* **281**(9): 5811-5820.

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This work was supported by the American Heart Association pre-doctoral fellowship [15PRE24680004] to H.T.N.P). The MSU College of Veterinary Medicine George Ward Endowed Research Fund and the MSU AgBioResearch provided financial support for the Hamamatsu plate reader.

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Figure 1. Impact of RGS2 mutations on AT1 receptor-mediated intracellular calcium release. A. RGS2 mutation map. Needle plot showing the 2D positions of 16 missense mutations on the human RGS2 protein. The plot was visualized by inputting amino acid changes of RGS2 protein to MutationMapper tool (cBioPortal). CHO cells transiently expressing AT1R receptor with or without RGS2 WT or RGS2 mutants were used for measurement of intracellular Ca^{2+}. **B, C.** Representative fluorescence traces (AngII 1μM) from cells expressing AT1R alone (black), cells co-expressing AT1R and RGS2 WT, K18N (normal function), or D40Y (decreased function). **D, E.** Concentration response curves. K18N inhibits AT1R-mediated Ca^{2+} as equally as RGS2 WT while D40Y showed reduced inhibition as compared to WT. **F.** Bar graph of maximal percentage inhibition mediated by RGS2 WT or mutants. Reduced function mutants are shown in dark grey (Q2L, R188H, R44H, D40Y) and mutants that did not show a significant difference from WT (uncorrected $p > 0.05$) are shown in light grey. **** $p < 0.0001$ compared to AT1R + Ctrl, # $p < 0.05$ compared to AT1R+RGS2 WT (n=6-8, Student t-test).

Figure 2. Protein expression of RGS2 WT and mutants. CHO cells were transiently transfected with the V5-tagged (RGS2-V5) constructs; RGS2 protein levels were analysed by anti-V5 Western blot. **A.** Representative Western blot of 10 independent experiments showing results with total cell lysates of CHO cells expressing RGS2 WT and RGS2 mutant proteins. **B.** Quantification of band intensities is normalized to the RGS2 WT protein level. Data are presented as mean ± SEM. * $p < 0.05$ (one-way ANOVA with Bonferroni post-test).

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3. Effects of RGS2 missense mutations on RGS2-GFP localization. A-C. When overexpressed in HEK293 cells, RGS2-GFP is localized to the nucleus. **D, H.** Unlike the other mutants, the R188H-GFP mutant showed punctate intracellular localization. **E.** Co-expression of WT-RGS2-GFP with constitutively active Gαq results in translocation of the RGS2-GFP to the plasma membrane. **F, G.** This translocation is impaired with D40Y and R44H; these 2 mutants remained in the nucleus when co-expressed with $G\alpha_q$ Q209L. **I.** Representative line scans across cells expressing WT, D40Y, R44H RGS2-GFP without $G\alpha_q$. **J.** Representative line scans across cells expressing WT + $G\alpha_q$, D40Y + $G\alpha_q$ or R44H + $G\alpha_q$. **K.** Localization of RGS2 in at least 100 cells determined by a blinded observer shows impaired membrane localization of D40Y and R44H. **** $p < 0.0001$, One-way ANOVA with Bonferroni post-test (scale bar: 20 μm).

Figure 4. Impaired $G\alpha_q$ binding by the R188H mutant. A. Binding affinities of RGS2 proteins with $G\alpha_q$ measured in a bead-based flow cytometry competition binding assay. As described in methods, $G\alpha_q$ was immobilized on beads and mutant proteins were used to compete for binding of AF532 labelled WT-RGS2. The R188H mutant exhibited a reduction in $G\alpha_q$ binding affinity comparing to RGS2 WT protein, demonstrated by the right ward shift in competition binding curve. The Q78H, A99G and Q196R had comparable binding affinities. **B.** Thermoshift analysis of the RGS spanning domains from RGS2 WT, R188H and Q196R. The bar graph shows the melting temperature of these proteins as mean ± SEM from 3 independent experiments. * $p<0.05$, ****$p<0.0001$ (One-way ANOVA with Bonferroni post-test). **C, D.** Proposed structural mechanism of the impaired $G\alpha_q$ binding and thermal instability of the R188H mutant RGS2. Structure of the RGS2 domain (violet) – $G\alpha_q$ complex (cyan) (Nance et al., 2013) is shown. **C.**

The WT arginine at position 188 forms salt bridges with the glutamate residue at position 104. **D.** Histidine substitution at position 188 does not favor salt bridge formation.

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

Tables

Table 1. Missense mutations in the coding region of RGS2 used in this study

Mutation ID	Missense variant	Allele count (ExAC)	Codon change	Reference
N/A	Q2L*	2/121304	CAA>CTA	(Lek et al., 2016; Yang et al., 2005)
rs141030117	Q2R	65/121312	CAA>CGA	(Lek et al., 2016; Yang et al., 2005)
rs145125159	S3G	32/121316	AGT>AGC	(Lek et al., 2016; Yang et al., 2005)
rs142499684	A4V	4/121338	GCT>GTT	(Lek et al., 2016)
rs193051407	M5V	82/121346	ATG>GTG	(Lek et al., 2016; Yang et al., 2005)
rs74466425	K18N	34/121370	AAG>AAC	(Lek et al., 2016)
rs148489044	G23D	66/121358	GGC>GAC	(Lek et al., 2016)
rs201233692	D40Y	62/118360	GAT>TAT	(Lek et al., 2016)
rs200339834	R44H*	12/119308	CGT>CAT	(Lek et al., 2016; Yang et al., 2005)
rs80221024	Q50K	103/119910	CAA>AAA	(Lek et al., 2016)
rs140811638	P55L	6/120456	CCT>CTT	(Lek et al., 2016)
N/A	Q78H	3/2055	CAG>CAC	(Yang et al., 2005)
rs139237239	A99G	2/121332	GCT>GGT	(Lek et al., 2016)
rs146862218	I110V	53/121334	ATT>GTT	(Lek et al., 2016)
rs369752935	R188H	4/121388	CGT>CAT	(Lek et al., 2016)
rs112707798	Q196R	18/121352	CAG>CGG	(Lek et al., 2016)

ExAC: The Exome Aggregation Consortium - http://exac.broadinstitute.org

* RGS2 mutations were found in disease cohort (Yang et al., 2005)

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

Figures

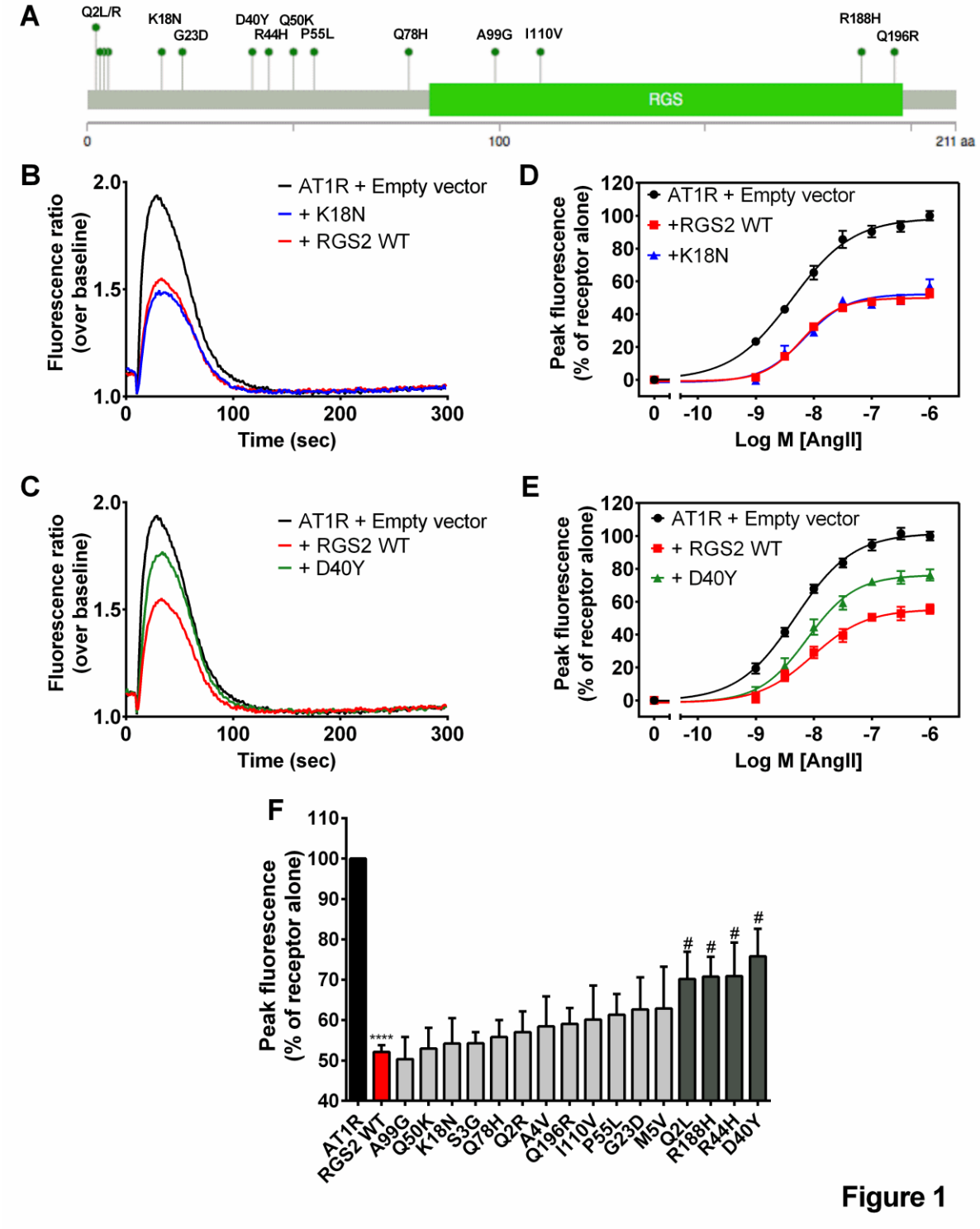

Figure 1

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

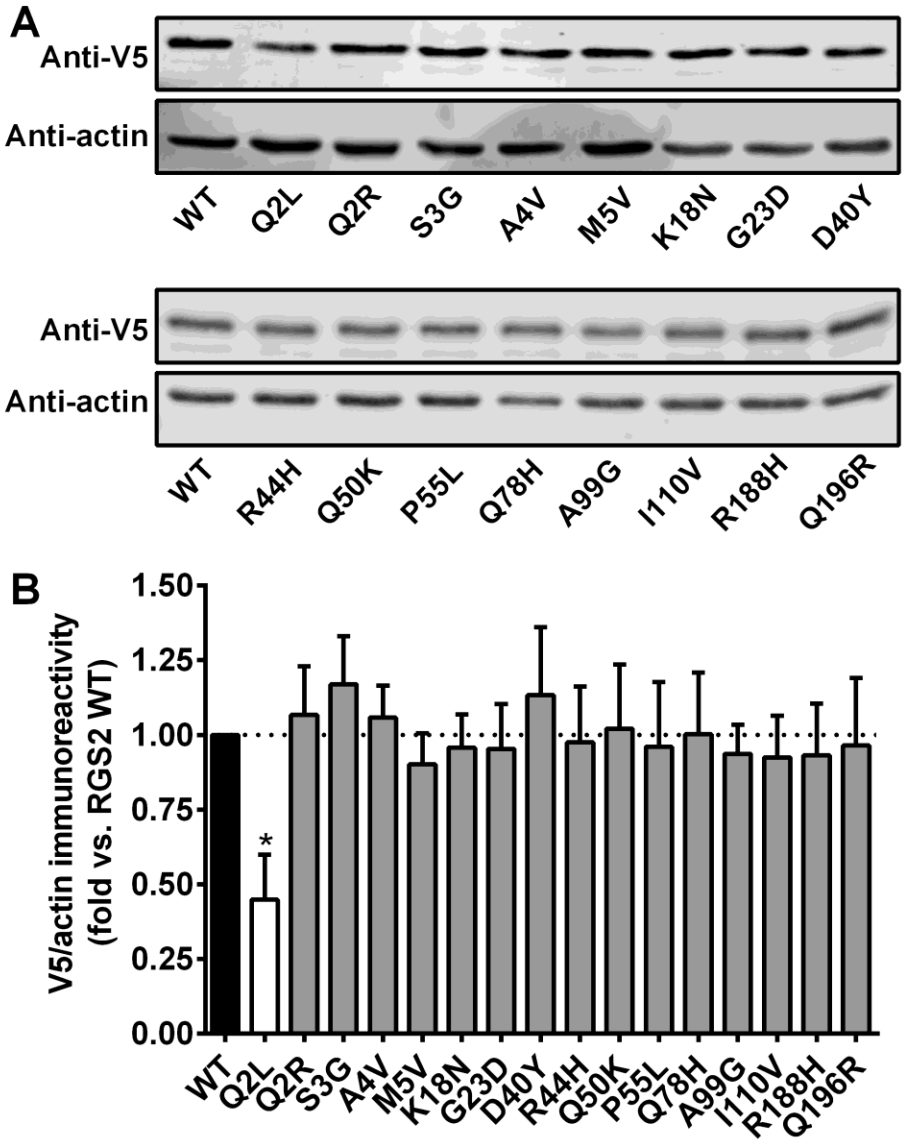

Figure 2

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

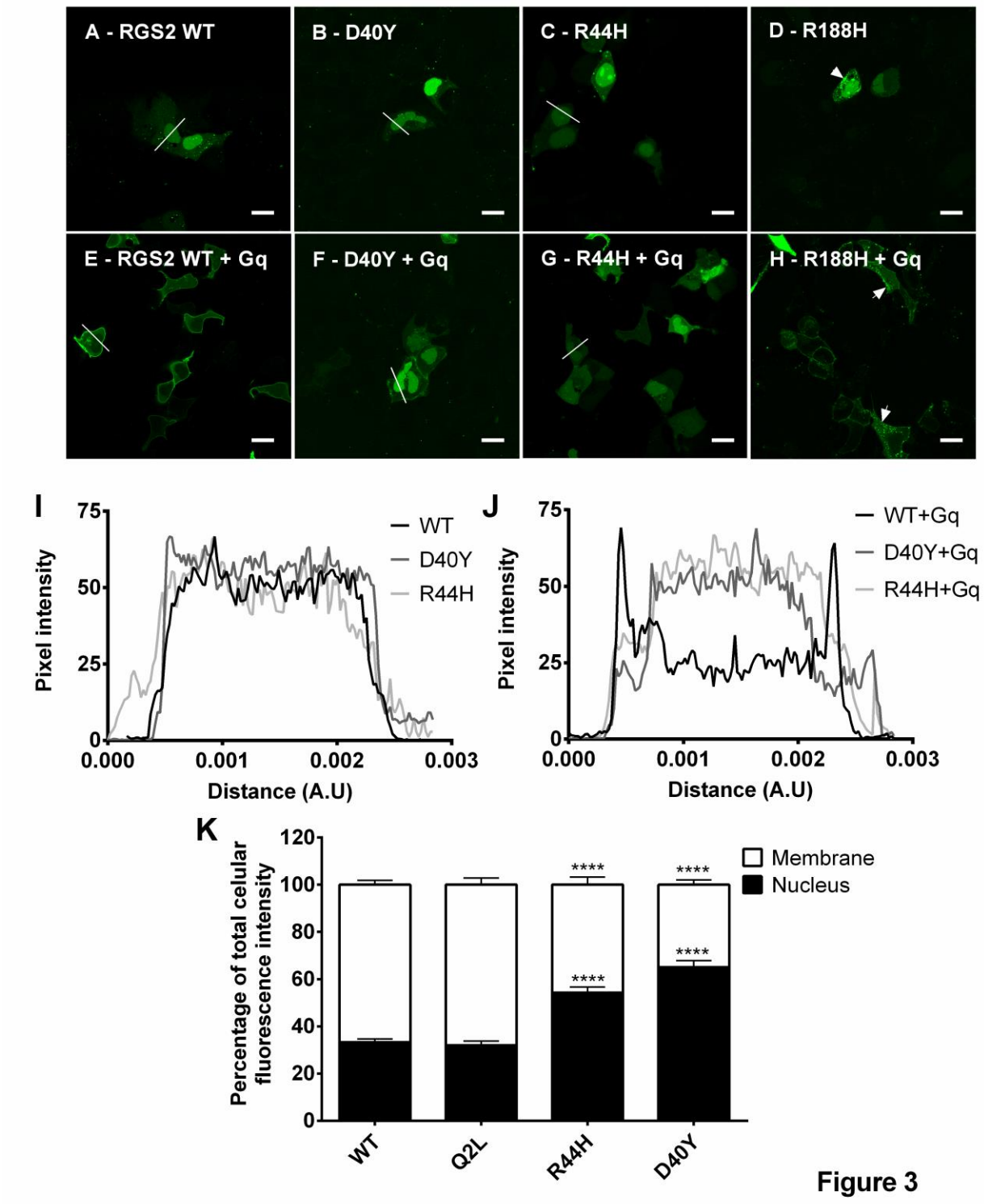

Figure 3

Molecular Pharmacology Fast Forward. Published on August 7, 2017 as DOI: 10.1124/mol.117.109215
This article has not been copyedited and formatted. The final version may differ from this version.

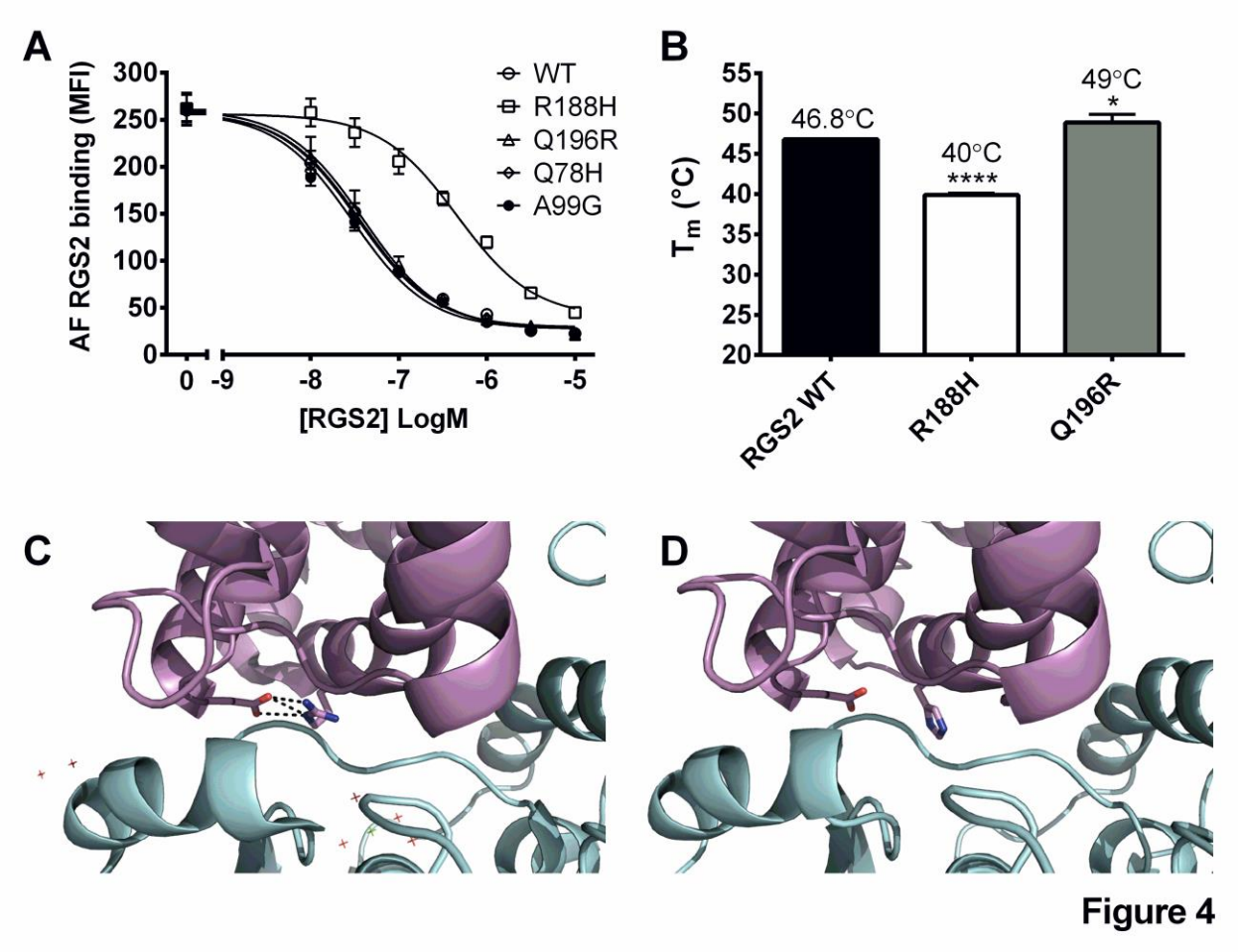

Figure 4