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The impact of rare human genetic variants and disease-linked mutations of RGS14 on the RGS14-Rap2A interactions and the regulation of JNK signaling

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Neuroscience and Behavioral Biology

2021

Abstract

The impact of rare human genetic variants and disease-linked mutations of RGS14 on the RGS14-Rap2A interactions and the regulation of JNK signaling

By Gizem Terzioglu

The Regulator of G Protein Signaling 14 (RGS14) is a multifunctional signaling protein that integrates G protein, MAP kinase (MAPK), and Ca++/CaM signaling pathways in host cells. Expressed primarily in the hippocampus, RGS14 is a natural suppressor of synaptic plasticity and long-term potentiation (LTP) in area CA2 neurons and is linked to hippocampal-based learning and memory. The RGS14 domain structure consists of an RGS domain that binds active Gαi/o-GTP, a tandem (R1/R2) Ras/Rap binding domain (RBD), and a GoLoco/GPR motif that binds inactive Gαi1/3-GDP. While much is known about RGS14 regulation of H-Ras, much less is known about RGS14 regulation of Rap2A, which stimulates the TNIK-JNK activity implicated in synaptic plasticity. In order to understand how RGS14 regulates Rap2A, we identified rare human variants of RGS14 that we predicted to disrupt RGS14 interactions with Rap2A as experimental tools. Using co-immunoprecipitation and bioluminescence resonance energy transfer (BRET) assays to measure RGS14 functions, we identified two rare variants of RGS14, D310N and K334N, that block RGS14 interactions with active Rap2A. We tested the impact of wild-type and the variants of RGS14 on the regulation of Rap2A-mediated JNK MAPK signaling by analyzing phosphorylated JNK (pJNK) levels in whole-cell lysates of HEK293 cells transfected with different combinations of constitutively active Rap2A, TNIK, wild-type and the variant RGS14 via Western immunoblotting. Our results suggest that RGS14 downregulates JNK signaling by negatively regulating active Rap2A, whereas the variants fail to do so. Overall, our findings implicate RGS14 as a signaling protein that may provide neuroprotection against ischemia- and excitotoxicity-induced cell death and Alzheimer's disease pathogenesis in CA2 pyramidal neurons. This may occur by blocking AMPA receptor trafficking as a potential mechanism by which RGS14 suppresses synaptic plasticity in the area CA2. Future studies will explore the potential impacts of RGS14 genetic variants on synaptic plasticity, neuron physiology, behavior and disease states for human carriers.

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Acknowledgements

I would like to thank all members of the Hepler Lab for their continuous support and for accepting me into the Hepler Lab family as only a first-year student. Particularly, I would like to thank Carolina Montanez-Miranda for training me when I joined the lab and teaching me the co-IP, Western blotting and BRET techniques, Nicholas Harbin and Suneela Ramineni for helping me design my experiments and extensively troubleshoot every step of the way. I have been privileged to receive such rigorous scientific training, while being supported and encouraged to take risks along the way as a member of the Hepler Lab.

I would also like to thank my committee members, Drs. Mike Crutcher and Joe Manns, for their support and valuable input during my honors thesis journey. As inspiring professors, they made my undergraduate neuroscience training an incredible experience and helped me gain a solid foundation in neuroscience.

I would like to thank my dear parents, Yesim and Mustafa Terzioglu. Their constant emotional support from thousands of miles away helped me overcome some of the most challenging times of this journey.

Finally, I would like to extend my deepest gratitude to my advisor, Dr. John Hepler. With his constant support and patience, he has played a vital role in my undergraduate scientific training. This project would not have been possible without his guidance and the funding he provided.

This project was funded in part by the Undergraduate Research Programs (URP) Independent Research Grant.

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**BACKGROUND AND SIGNIFICANCE**

G protein-coupled receptors (GPCRs) mediate cellular responses to various external stimuli, such as hormones, neurotransmitters and peptides. Due to their ability to regulate important physiological processes, GPCRs have been popular targets for drug discovery (Hauser, Attwood, Rask-Andersen, Schioth, & Gloriam, 2017). GPCRs are integral membrane proteins characterized by seven transmembrane alpha-helices. When a ligand or an agonist binds to a GPCR, the GPCR activates the associated heterotrimeric G protein by promoting guanine nucleotide exchange (replacement of GDP by GTP) on the G$\alpha$ subunit. GTP-bound G$\alpha$ dissociates from the Gβγ complex, which frees G$\alpha$-GTP and Gβγ to separately activate their respective downstream effectors (Rosenbaum, Rasmussen, & Kobilka, 2009).

G$\alpha$ subunits have intrinsic GTPase activity, which allows them to hydrolyze their bound GTP to GDP and terminate G protein signaling. However, the intrinsic GTPase activity of G proteins is relatively slow and cannot account for the precise temporal regulation of signaling events. The precise regulation of G protein signaling is mediated by the Regulator of G protein Signaling (RGS) proteins, a family of GTPase-activating proteins (GAPs) which act as to accelerate the hydrolysis of Gα-bound GTP to GDP and the consequent deactivation of G protein signaling (Hollinger & Hepler, 2002; Sjogren, 2017).

RGS14 is a member of the D/R12 subfamily of RGS proteins (Snow, Antonio, Suggs, Gutstein, & Siderovski, 1997; Zheng, De Vries, & Gist Farquhar, 1999). It is a multifunctional signaling protein that integrates G protein, MAP kinase (MAPK), and $Ca^{++}$/calmodulin (CaM) signaling pathways in host cells. The RGS14 domain structure consists of the conserved RGS domain that binds active Gαi/o-GTP, a tandem (R1 and R2) Ras/Rap binding domain (RBD) that binds activated H-Ras, Rap2A, Raf kinases and $Ca^{++}$/CaM, and a GoLoco/G-protein regulatory

(GPR) motif that binds inactive Gαi1/3-GDP (**Fig. 1**) (Brown, Goswami, et al., 2015; Evans, Gerber, et al., 2018; Hollinger, Taylor, Goldman, & Hepler, 2001; Shu, Ramineni, & Hepler, 2010; Traver et al., 2000; Vellano, Brown, Blumer, & Hepler, 2013). RGS14 is also a nuclear cytoplasmic shuttling protein, and its subcellular localization and movement are regulated by a nuclear export sequence (NES) embedded within the GPR motif and functional nuclear localization sequence (NLS) located between the RGS and R1 domains (Cho, Kim, & Kehrl, 2005; Shu, Ramineni, Amyot, & Hepler, 2007; Squires et al., 2020).

RGS14 is predominantly expressed in the CA2 region of the hippocampus – a region long considered as a transition zone between the areas CA3 and CA1, but with newly appreciated roles in social memory and behavior (Tzakis & Holahan, 2019). Area CA2 has received little attention until recently, as it does not partake in the "tri-synaptic" DG-CA3-CA1 circuitry that is integral to hippocampal learning and memory. Whereas the CA3 Schaffer collateral (SC) projections onto CA1 pyramidal neurons induce long-term potentiation (LTP), which is regarded as the molecular correlate of learning and memory in the hippocampus, SC inputs onto CA2 pyramidal neurons are resistant to activity-dependent LTP (Caruana, Alexander, & Dudek, 2012; Middleton & McHugh, 2020; Zhao, Choi, Obrietan, & Dudek, 2007). This resistance is conferred, at least in part, by the differential expression of RSG14 in the CA2 pyramidal neurons (Evans, Parra-Bueno, et al., 2018). Importantly, our lab has previously found that mice that lack RGS14 (RGS14-KO) displayed LTP in the CA2 and exhibited enhanced spatial learning and object recognition memory compared with wild-type littermates (Lee et al., 2010). However, the exact mechanism(s) by which RGS14 regulates synaptic plasticity in the CA2 pyramidal neurons remain unclear.

Understanding how RGS14 regulates its binding partners may help explain its ability to regulate synaptic plasticity. Remarkably, the RGS14 binding partners H-Ras, $Ca^{2+}$/CaM and Rap2

are known as key regulators of synaptic plasticity. A previous study from our lab has found that RGS14's interaction with active H-Ras inhibits ERK signaling, which is essential for trafficking AMPA receptors (AMPA-Rs) to the dendritic spines during LTP (Patterson, Szatmari, & Yasuda, 2010; Shu et al., 2010). Another study has identified RGS14 as an effector of $Ca^{++}$/CaM, which is a central signal integrator for synaptic plasticity (Evans, Gerber, et al., 2018; Xia & Storm, 2005). However, how RGS14 regulates active Rap2A and how the RGS14-Rap2A interaction may regulate synaptic plasticity remain unexplored.

Rap2A is a member of the Ras subgroup of monomeric GTPases, which are small GTP-binding proteins that serve as molecular "switches" to transduce upstream signals to downstream effectors (Takai, Sasaki, & Matozaki, 2001). Rap2A indirectly activates the c-Jun NH2-terminal kinase (JNK) MAPK signaling pathway by regulating Traf2- and Nck-interacting kinase (TNIK), which activates JNK by phosphorylation (C. A. Fu et al., 1999; Taira et al., 2004). JNK signaling pathway is implicated in synaptic plasticity, excitotoxicity-induced apoptosis and tumorigenesis. Overactivation of JNK is believed to be involved in the pathogenesis of various neurological disorders, such as epilepsy, Alzheimer's disease, Parkinson's disease and several psychiatric disorders (Coffey, 2014; Sclip et al., 2014; Zeke, Misheva, Remenyi, & Bogoyevitch, 2016; X. Zhu et al., 2001). Rap2-JNK signaling results in removal of synaptic AMPA-Rs and leads to depotentiation, the reversal of synaptic strength from a potentiated state (Chen, Ren, & Wang, 2016; Fukunaga & Miyamoto, 1998; H. H. Yang, Courtney, Martinsson, & Manahan-Vaughan, 2011; Y. Zhu et al., 2005). Consistently, constitutive activation of Rap2A is linked with depotentiation, decreased length and complexity of neurites, and loss of dendritic spines (Z. Fu et al., 2007; Y. Zhu et al., 2005). Constitutive Rap2A activation is also implicated in spatial learning impairment in mice (Ryu, Futai, Feliu, Weinberg, & Sheng, 2008).

Genetic variations account for diverse traits and individual predispositions to disease. Recent large-scale whole genome and exome sequencing efforts have identified an abundance of rare missense variation in protein-coding regions of the genome across various human populations. Many of these missense variants are predicted to impact protein function and result in disease-related phenotypes and disease pathogenesis (Fragoza et al., 2019). Our lab has previously identified genetic variants across the RGS protein family, including RGS14, that might confer changes within its functional binding domains and consequently affect RGS14's interaction with its binding partners and its cellular functions (Squires, Montanez-Miranda, Pandya, Torres, & Hepler, 2018). In fact, a recent study from our lab has identified rare variants of RGS14 within the GPR motif that disrupt RGS14's nuclear shuttling and LTP regulation by disrupting its interactions with Gαi1-GDP and the nuclear export protein Exportin 1 (XPO1) (Squires et al., 2020). Therefore, the naturally occurring genetic variants of RGS14 provide valuable experimental tools to reveal the connections between RGS14's interaction with its binding partners and cellular functions.

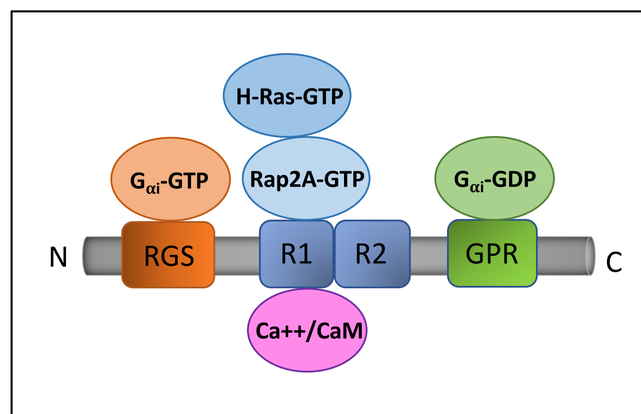


**Figure 1. Binding domains and binding partners of RGS14.** RGS: Regulator of G-protein Signaling domain, R1/R2: tandem Ras/Rap Binding domains (RBD), GPR: G-protein Regulatory Motif, N: N-terminus, C: C-terminus.

**RATIONALE**

In order to understand how RGS14 regulates active Rap2A and how this regulation affects downstream signaling mechanisms, we looked at rare human genetic variants of RGS14 that encode a missense point mutation in the R1 RBD and might disrupt RGS14's binding with active Rap2A. Among the known rare variants of RGS14, we selected six variants of interest, K334N, D310G, D310N, A363V, T323A and L315S, based on their potential impact on RBD's structure and ability to bind active Rap2A. The RBD of RGS14 shares sequence identity with RBD of Raf-1, a serine/threonine kinase that interacts with Ras proteins as well as Rap1, which shares 60% sequence identity with Rap2A (Nassar et al., 1996; Tran et al., 2021; Traver et al., 2000). Whereas the 3D model of the Raf-1 RBD based on homology modeling illustrates the structure of Raf-1's R1 and R2 RBD, the 3D model for RBD of RGS14 currently only includes R2 and not R1 (Nakanishi et al., 2011; Nassar et al., 1996). Therefore, we superimposed a homology model of the R1 domain of RGS14 referenced off the 3D model of Raf-1's R1 RBD when selecting the RGS14 variants of interest. Amino acids K334, D310 and L315 appear to be located on regions of RBD that likely interact with Rap2A (**Fig. 2**), and the point mutations K334N, D310G, D310N and L315S encode missense point mutations that might drastically alter the 3D structure of RBD. On the other hand, amino acids A363 and T323 appear to be farther away from the binding site, and hence the point mutations A363V and T323A were not expected to alter the binding of RBD with Rap2A and were selected as controls.

Most of the rare variants of RGS14 were identified as a result of large-scale sequencing projects through whole genome and exome sequencing in different human populations, while some were identified through analyses of cancerous tumors (**Table 1**) (Karczewski et al., 2020). Whereas there are currently no known phenotypes associated with the variants K334N and L315S,

the variants D310G, D310N, A363V and T323A have been linked with various somatic cancers and are estimated to be pathogenic (Hoadley, Yau, Hinoue, Wolf, Lazar, Drill, Shen, Taylor, Cherniack, Thorsson, Akbani, Bowlby, Wong, Wiznerowicz, Sanchez-Vega, Robertson, Schneider, Lawrence, Noushmehr, Malta, Cancer Genome Atlas, et al., 2018). Different bioinformatic tools, such as PolyPhen and SIFT, offer conflicting predictions for how some of these missense mutations should affect the structure and function of RGS14 RBD (**Table 1**). Therefore, the predictions by bioinformatic tools should be treated with caution, and experimentation is necessary to identify the functional impacts of these missense mutations (Castellana & Mazza, 2013).

## HYPOTHESES AND AIMS

For this study, we *hypothesized* that 1) RGS14 negatively regulates active Rap2A to passively inhibit its downstream signaling and 2) in the presence of the rare human genetic variants of RGS14 that fail to bind active Rap2A, the Rap2A-mediated JNK MAPK signaling pathway is dysregulated.

Rare missense mutations in the RBD of RGS14 have been identified across various human populations, but whether these mutations affect RGS14's binding and cellular functions is unknown (Squires et al., 2018). Therefore, the first aim of this project was to identify rare human variants that are likely to alter the structure of RBD's interaction site and to establish these variants' effect on direct binding of RGS14 with the constitutively active mutation of Rap2A(G/V) *in vitro*. Active Rap2A stimulates the JNK signaling pathway by regulating the activity of TNIK (C. A. Fu et al., 1999; Stornetta & Zhu, 2011; Taira et al., 2004). RGS14's regulation of active Rap2A might therefore serve as an additional regulation mechanism for JNK signaling. Thus, the second aim of

this project was to identify whether and how RG14-Rap2A interaction regulates JNK signaling, and whether this regulation is altered due to a variant's inability to bind active Rap2A. Based on our hypothesis that RGS14 is a negative regulator of active Rap2A, we predicted that the overexpression of wild-type RGS14 and constitutively active Rap2A, in the presence of TNIK, would decrease the levels of phosphorylated JNK in the whole cell lysate of HEK293 cells, compared with the overexpression of constitutively active Rap2A and/or TNIK. On the other hand, since Rap2A would remain active in the presence of the rare variants that are unable to bind it, we predicted that the overexpression of these variants, together with constitutively active Rap2A and TNIK, would reverse the effect observed with the overexpression of wild-type RGS14. Our results confirmed our predictions, such that the over-expression of wild-type RGS14 reduced the Rap2A- and TNIK-stimulated levels of pJNK, whereas the variants were unable to exert any effect on the pJNK levels.

## MATERIALS AND METHODS

### *Cell Culture and Transfection*

Human embryonic kidney (HEK293) cells were maintained in Gibco Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (5% after transfection), 2 mM L-glutamine (1 mM after transfection), 1% Penicillin/Streptomycin. Cells were incubated at 37°C with 5% $CO_2$ in a humidified environment.

### *RGS14 Genetic Variants and Constructs*

Human genetic variant information for RGS14 was obtained from the Genome Aggregation Database (GnomAD, version 2.1.1, https://gnomad.broadinstitute.org) (Karczewski

et al., 2020). The information for rare variants of RGS14 implicated in cancer was obtained from cBioPortal (version 3.6.3, https://www.cbioportal.org/) and pathogenicity predictions were obtained from Functional Analysis through Hidden Markov Models (FATHMM, version 2.3, http://fathmm.biocompute.org.uk) (Cerami et al., 2012; J. J. Gao et al., 2013; Shihab et al., 2014). In order to select the rare variants of RGS14 that might alter the interaction site of R1 RBD, we referenced the 3D model of Raf-1 based on homology modeling, which we retrieved from SWISS-MODEL (**Fig. 2**) (Benkert, Biasini, & Schwede, 2011; Bertoni, Kiefer, Biasini, Bordoli, & Schwede, 2017; Bienert et al., 2017; Guex, Peitsch, & Schwede, 2009; Mariani, Biasini, Barbato, & Schwede, 2013; Nassar et al., 1996; Studer et al., 2020; Studer et al., 2021; Waterhouse et al., 2018). The variants were generated by introducing point mutations to human FLAG-RGS14 using the QuickChange Lightning site-directed mutagenesis kit (Stratagene) and the oligonucleotide primers listed in Table 3.

*Co-Immunoprecipitation and Western Blotting*

Co-immunoprecipitation was used to test stable complex formations between wild-type RGS14 or RGS14 variants and constitutively active mutation of Rap2A(G12V) in cell lysates. HEK293 cells were co-transfected with FLAG-tagged wild type or variant (K334N, L315S, D310N, D301G, T323A or A363V) RGS14 and Rap2A(G12V) using polyethylenimine (PEI). The negative control was the transfection of HEK cells with Rap2A(G12V) alone. Twenty-four hours after transfection, the cells were lysed in the buffer containing 50mM Tris pH 7.05, 150mM NaCl, 1mM EDTA, 2 mM dithiothreitol, 5mM $MgCl_2$, protease inhibitor (Sigma), phosphatase inhibitor cocktail (Thermo Fisher) and 1% Triton X-100. The lysate was cleared by centrifugation at 13,000 RPM for 10 min at 4°C. Lysates were mixed with 50 µg FLAG M2 Affinity Gel (Sigma) directed

against the FLAG-tagged fusion protein (RGS14). The mixed complexes were incubated for 2 hours at 4°C with continuous rotation (Shu et al., 2010). The gel was boiled off in Laemmli buffer. The samples were loaded onto 15% acrylamide gel and the proteins were resolved via SDS-PAGE at 150V for 2 hours, then transferred onto a nitrocellulose membrane overnight. The membrane was subjected to immunoblotting using rabbit polyclonal anti-RGS14 (Proteintech) and rabbit polyclonal anti-Rap2A (Invitrogen) primary antibodies overnight at 4°C, followed by anti-rabbit HRP-conjugated secondary antibody (Sigma) for 1 hour at room temperature. The membranes were imaged using enhanced chemiluminescence (ECL) (ChemiDoc Imaging System, BioRad).

*BRET in Live Cells*

Bioluminescence resonance energy transfer (BRET) utilizes the energy transfer between bioluminescent and fluorescent protein tags attached to two proteins, respectively called the donor and acceptor proteins, in order to detect the protein-protein interactions in live cells in real time. The addition of the substrate for the bioluminescent donor results in the oxidation of the substrate, which produces luminescence. If the donor and acceptor proteins are in close proximity (<10 nm), the energy from the donor gets transferred to the fluorescent acceptor, which produces fluorescence (Brown, Blumer, & Hepler, 2015). If the two proteins do not interact, only luminescence can be detected. The detection of both luminescence and fluorescence suggests interaction between the two proteins (**Fig. 4A**).

We used BRET to confirm the variant K334N's lack of binding with active Rap2A in live cells. BRET experiments were performed as previously described in Brown et al. (2015). Briefly, HEK293 cells were seeded at 8 x $10^5$ cells per well in 6-well plates. Twenty-four hours later, the cells were transfected with a donor (Luc-RGS14 or Luc-RGS14 variant K332N, rat homolog of

the human variant K334N) and acceptor (Rap2A(G/V)-Venus) using polyethylenimine (PEI). In all of the wells, cells were transfected with a constant amount of the donor plasmid (10 ng) but increasing amounts of the acceptor plasmid (0, 10, 50, 100, 250 and 500 ng) in order to ensure that the acceptor could saturate the donor and the maximal BRET signal could be obtained (Brown, Blumer, et al., 2015). Twenty-four hours after transfection, the cells were suspended in Tyrode's solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.37 mM $NaH_2PO_4$, 24 mM $NaHCO_3$, 10 mM HEPES and 0.1% glucose(w/v) at pH 7.4. Cells were plated in triplicates onto white 96-well Optiplates. The acceptor expression was determined by measuring fluorescence using a TriStar LB 941 plate reader with 485 nm excitation and 530 nm and emission filters. After applying coelenterazine H for 2 min, BRET was measured using the 485 nm and 530 nm emission filters. The change in BRET (net BRET) was calculated by dividing the 530 nm (Venus) signal by the 485 nm (Luc) signal and subtracting the signal from the luciferase alone. The acceptor/donor ratio was calculated by dividing the initial fluorescence (530 nm) by the initial luminescence (485 nm) (Brown, Blumer, et al., 2015; Gerber, Squires, & Hepler, 2018). Data were collected with MikroWin 2000 software and analyzed using GraphPad Prism and Microsoft Excel (Vellano et al., 2013). Data are reported as the pooled mean +/- SEM of three separate experiments, each with triplicate determinations.

*Analysis of Phosphorylated JNK (pJNK)*

In order to investigate how RGS14-Rap2A interactions regulate the JNK signaling pathway and whether the variants alter this regulation, the levels of phosphorylated JNK (pJNK) were analyzed in whole cell lysates of HEK293 cells transfected with HA-TNIK or Rap2A(G/V) alone and cells co-transfected with HA-TNIK, Rap2A (G/V) and/or FLAG-RGS14 (wild type or K334N

or D310N). Whole cell lysates were prepared, and gel electrophoresis and protein transfer were carried out as described above. The membrane was subjected to immunoblotting using rabbit polyclonal anti-RGS14 (Proteintech), rabbit polyclonal anti-Rap2A (Invitrogen), monoclonal HRP-conjugated anti-HA (Sigma) and rabbit monoclonal anti-Phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling, #81E11) as primary antibodies followed by the appropriate secondary antibodies. Glyceraldehyde-3-Phosphate Dehydrogenase (GADPH) was selected as the loading control and was immunoblotted for by using mouse monoclonal anti-GADPH (Santa Cruz) primary antibody followed by HRP-conjugated anti-mouse secondary antibody. The membranes were imaged using enhanced chemiluminescence (ECL) (ChemiDoc Imaging System, BioRad). pJNK expression was quantified by densitometric analysis using the Image Lab software (BioRad). Data was analyzed using GraphPad Prism and Microsoft Excel. Briefly, the chemiluminescence signals (adjusted for background) for GAPDH on each lane were normalized by dividing the signal for each lane by the maximum signal across all lanes, which gave out the lane normalization factor. Then, the chemiluminescence signals (adjusted for background) for pJNK on each lane were divided by the lane normalization factor corresponding to their respective lanes. Finally, the pJNK signals were further normalized by dividing the pJNK signal for each condition by the maximum pJNK signal across all conditions to obtain a relative pJNK signal. Data is reported as the as the pooled mean +/- SEM of three separate experiments.

**Table 1. Functional impact and pathogenicity predictions and population statistics for the variants K334N, D310N, D310G, L315S, T323A and A363V.**

| Variant | Functional impact | | Population statistics | Pathogenicity (FATHMM Prediction) |
|---|---|---|---|---|
| | SIFT | Polyphen-2 | | |
| K334N | Tolerated | Benign | Global – 0.0004%<br>South Asian – 0.0033% | N/A |
| D310N | Deleterious | Probably_damaging | Global – 0.0370%<br>African – 0.3927%<br>East Asian – 0.0154%<br>Latino – 0.0084%<br>South Asian – 0.0032%<br>European – 0.0016% | Pathogenic |
| D310G | Deleterious | Probably_damaging | N/A | Pathogenic |
| L315S | Tolerated | Probably_damaging | Global – 0.0004%<br>European – 0.0008% | N/A |
| T323A | Tolerated | Benign | N/A | Pathogenic |
| A363V | Tolerated | Benign | Global – 0.0002%<br>Latino/Admixed American – 0.0016% | Pathogenic |

N/A: No data available.

**Table 2. Amino acid changes conferred by the point mutations K334N, D310N, D310G, L315S, T323A and A363V**

| Variant | Amino acid change | Change in amino acid property |
|---|---|---|
| K334N | Lysine -> Asparagine | Positively charged -> Uncharged, polar |
| D310N | Aspartic acid -> Asparagine | Negatively charged -> Uncharged, polar |
| D310G | Aspartic acid -> Glycine | Negatively charged -> Non-polar |
| L315S | Leucine -> Serine | Non-polar -> Polar |
| T323A | Threonine -> Alanine | Polar -> Non-polar |
| A363V | Alanine -> Valine | No change (both non-polar) |

**Table 3. Primers used for generating RGS14 human variants.**

| Variant | Forward | Reverse |
|---|---|---|
| K334N | CTGGCAGGGATCTGTGAGAACCG AGGCCTCTCTCTACCTGAC | GTCAGGTAGAGAGAGGCCTCGG TTCTCACGATCCCTGCCAG |
| D310G | GTGTACCTGCCCGGTGGCACAGC CTCC | GGAGGCTGTGCCACCGGGCAGG TACAC |
| D310N | TGTGTGTACCTGCCCAATGGCACA GCCTCCTTG | CAAGGAGGCTGTGCCATTGGGC AGGTACACACA |
| L315S | GATGGCACAGCCTCCTCGGCCCTG GCCAGACC | GGTCTGGCCAGGGCCGAGGAGG CTGTGCCATC |
| A363V | GACTGCACCGTGCTGGTGGATCAG GAAGTGCGG | CCGCACTTCCTGATCCACCAGCA CGGTGCAGTC |
| T323A | GCCAGACCTGGCCTCGCCATCCGA GACATGCTG | CAGCATGTCTCGGATGGCGAGGC CAGGTCTGGC |

## RESULTS

*Naturally Occurring RGS14 Variants D310N and K334N Fail to Bind Active Rap2A In Vitro*

Because RGS14 binds to active Rap2A through its R1 RBD, we sought to determine whether the naturally occurring variants of RGS14 that encode a point mutation in the R1 RBD block RGS14's interaction with Rap2A (Shu et al., 2010). We selected four rare human variants that encode point mutations predicted to be localized on the interaction site of RGS14's R1 RBD, based on where the RBD of Raf-1 appears to interact directly with Rap1 (Nassar et al., 1996; Tran et al., 2021). The point mutations encoded by the variants D310N, D310G, K334N and L315S were therefore predicted to alter RGS14's binding with active Rap2A. Variants A363V and T323A were chosen as negative controls, as the amino acids A363 and T323 appear to be localized farther away from the interactions site of RBD and were therefore not expected to alter RGS14's interaction with Rap2A (**Fig. 2**).

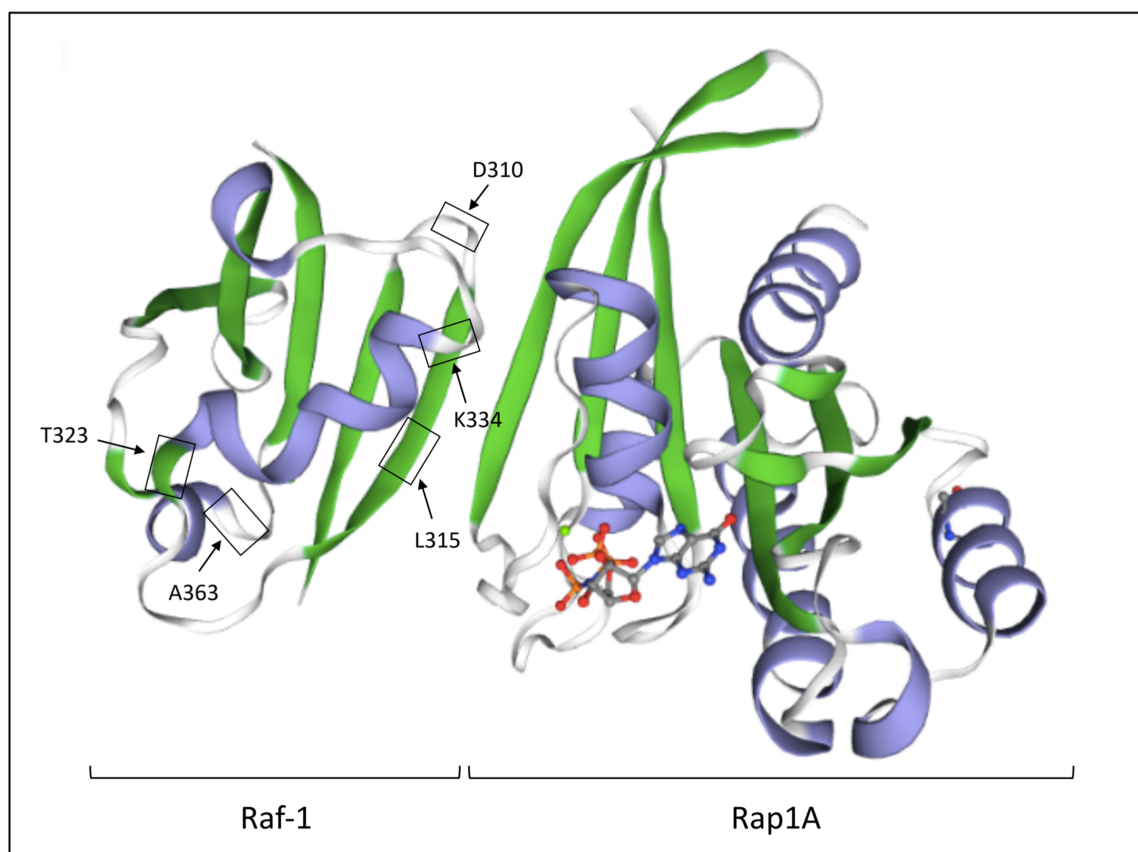


**Figure 2. Amino acids D310, L315S and K334N are likely localized on regions of RGS14 RBD that directly interact with Rap2A, whereas T323 and A363 are likely farther away from the binding site**. 3D structure of Raf-1 based on homology modeling (protein on the left) shows that amino acids of Raf-1 that correspond to K334, D310 and L315 of RGS14 are localized on the protein's interaction site for Rap1A (protein on the right). This suggests that the missense point mutations K334N, D310G, D310N and L315S might confer changes in the structure of RGS14's RBD and consequently block RGS14's interaction with activated Rap2A. Model built based on the data by Nassar et al. (1996) was acquired from SWISS-MODEL (https://swissmodel.expasy.org/templates/1gua.1)

We tested whether the variants were able to form stable complexes with the constitutively active mutation of Rap2A(G12V) by using co-immunoprecipitation followed by western blotting in HEK293 cells. We found that whereas wild-type (WT) FLAG-tagged RGS14 and the variants D310G, L315S, A363V and T323A were able to pull down Rap2A(G/V), the variants K334N and D310N disrupted RGS14's interactions with active Rap2A (**Fig. 3**).

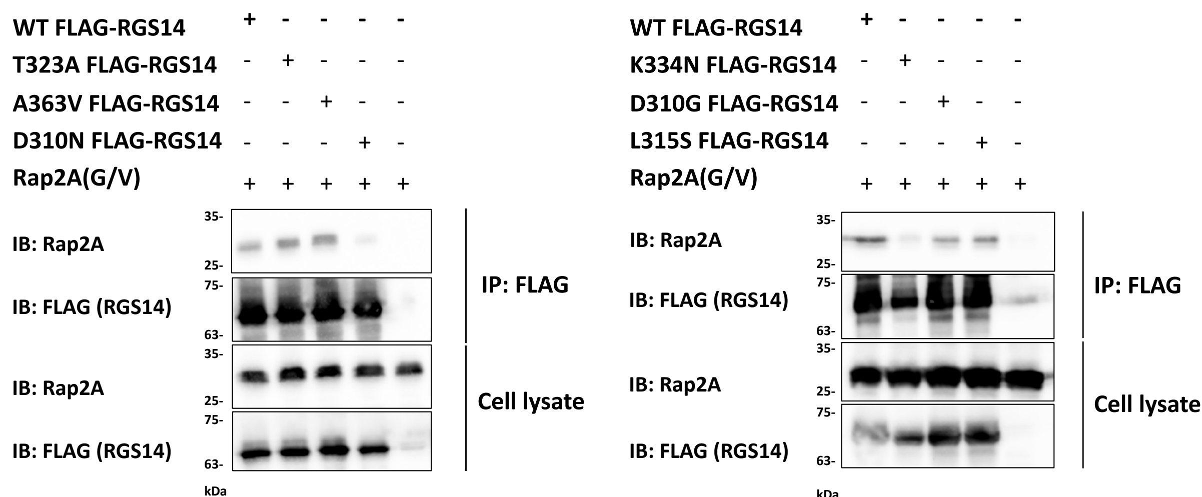


**Figure 3. Point mutations K334N and D310N hinder RGS14's binding to active Rap2A.** FLAG-RGS14 variant K334N (blot on the right) and D310N (blot on the left) failed to pull down constitutively active Rap2A(G/V) when co-immunoprecipitated. On the other hand, the variants D310G, L315S (blot on the right), A363V and T323A (blot on the left) did not alter RGS14's binding to Rap2A. HEK 293 cells were transfected with FLAG-RGS14, either as wild type or one of the variants, and Rap2A(G/V). All samples were then incubated with ANTI-FLAG M2 affinity gel to separate RGS14 from the whole cell lysate. They were then subjected to SDS page and immunoblotting. Blots are representative of five separate experiments.

We used BRET to quantitatively measure the interaction of constitutively active Rap2A(G/V)-Venus with wild-type Luc-RGS14 and RGS14 K332N (the rat homolog of the human variant K334N) to confirm the co-immunoprecipitation findings. Transfection of HEK293 cells with increasing amounts of Venus-tagged Rap2A(G/V) plasmid and a fixed amount (10 ng) of Luc-RGS14 plasmid showed a robust BRET signal, whereas the BRET signal obtained from the HEK293 cells transfected with variant K334N (10 ng) instead of wild-type RGS14 was drastically lower (**Fig. 4**). This suggests that RGS14 variant K332N blocked RGS14's binding to Rap2A, confirming that the variant K334N confers a loss-of-function mutation in the R1 RBD.

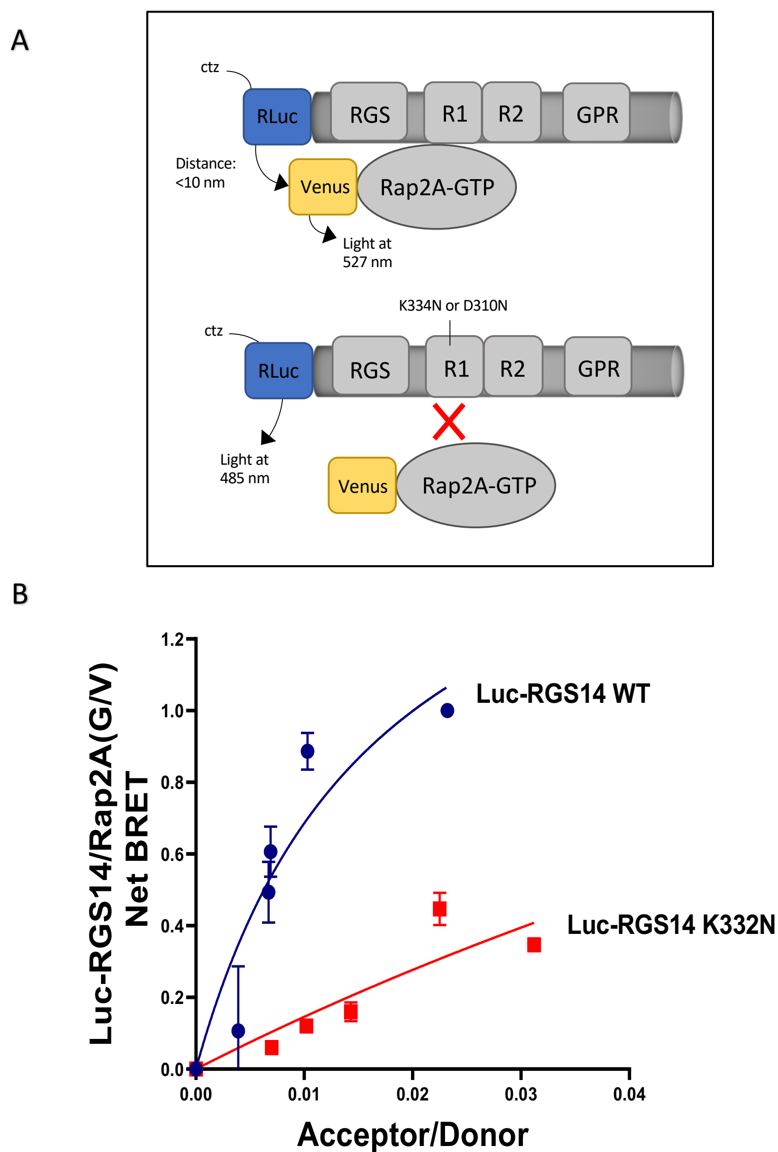


**Figure 4. RGS14 variant K334N's inability to bind active Rap2A is confirmed in live cells. A)** BRET signal depends on the distance between the donor (Luc-RGS14) and the acceptor (Rap2A-Venus). When the physical distance between the luminescent tag of the donor and the fluorescent tag of the acceptor is less than 10 nm, the energy from the donor, produced as a result of the oxidation of coelenterazine H (ctz) by luciferase, gets transferred to the acceptor, which produces light at 527 nm (top). If the two proteins are farther than 10 nm apart, only luminescence is detected at 485 nm (bottom). **B)** RGS14 variant K332N (the rat homologue of the human variant K334N) does not bind Rap2A(G/V) in live cells. HEK 293 were transfected with 10 ng of either Luc-RGS14 WT or Luc-RGS14-K332N plasmid along with 0, 25, 50, 100, 250 or 500 ng of Venus-Rap2A(G/V). BRET ratios were recorded and net BRET was calculated by subtracting the BRET signal from the luciferase alone. Wild-type RGS14 shows a robust BRET signal with Rap2A(G/V) (blue line), whereas the variant K332N displays a drastically reduced BRET signal (red line), which indicates a lack of binding. Data shown are the pooled mean +/- SEM of three independent experiments, each with triplicate determinations.

*RGS14 Suppresses Active Rap2A and Downregulates the Phosphorylation of JNK, the Variants D310N and K334N Disrupt This Regulation*

RGS14 has been known to be an effector of active Rap2A for the past two decades, and yet how RGS14 regulates active Rap2A has remained unknown (Traver et al., 2000). We hypothesized that since RGS14 binds active H-Ras and likely sequesters it away from its downstream effectors, RGS14 might negatively regulate active Rap2A to inhibit its downstream signaling pathways in a similar way (Vellano et al., 2013). Because the rare variants D310N and K334N are unable to bind Rap2A, we hypothesized that in the presence of these variants, Rap2 would remain active. Active Rap2A indirectly activates the JNK MAPK signaling pathway by regulating TNIK, which can phosphorylate JNK (C. A. Fu et al., 1999; Stornetta & Zhu, 2011; Taira et al., 2004). Therefore, we hypothesized that RGS14 might downregulate JNK signaling by negatively regulating active Rap2, while the variants D310N and K334N would disrupt the regulation of JNK signaling. We tested these hypotheses by investigating the levels of phosphorylated JNK (pJNK) in HEK293 cells that overexpress constitutively active Rap2A(G/V), TNIK, and wild-type or the variants of RGS14. We first confirmed that HA-tagged TNIK, Rap2A(G/V) and FLAG-tagged RGS14 were successfully transfected into and expressed by HEK293 cells by Western blotting **(Fig. 5A)**. We then used Western blotting to identify the levels of pJNK expressed in the whole cell lysates of HEK293 cells, which we normalized against the levels of GAPDH, a housekeeping gene stably expressed in HEK293 cells, in each sample (**Fig. 5B**) (Adeola, 2018). We found that the co-overexpression of Rap2A(G/V) and TNIK results in the highest levels of pJNK compared to the overexpression of Rap2A(G/V) and TNIK individually. Overexpression of TNIK stimulated the phosphorylation of pJNK to a higher extent than the overexpression of Rap2A(G/V) alone did. These findings are consistent with the idea that

Rap2A(G/V) acts through TNIK to activate the JNK signaling pathway. Furthermore, the overexpression of wild-type (WT) RGS14 alongside Rap2A(G/V) and TNIK resulted in lower levels of pJNK than that observed with the co-overexpression of Rap2A(G/V) and TNIK. On the other hand, the overexpression of the variants D310N or K334N alongside Rap2A(G/V) and TNIK resulted in levels of pJNK similar to that observed with the co-overexpression of Rap2A(G/V) and TNIK. These results suggest that wild-type RGS14 downregulates pJNK by negatively regulating active Rap2A(G/V), whereas in the presence of the variants D310N and K334N, Rap2A remains active and stimulates the phosphorylation of pJNK through TNIK (**Fig. 5C**).

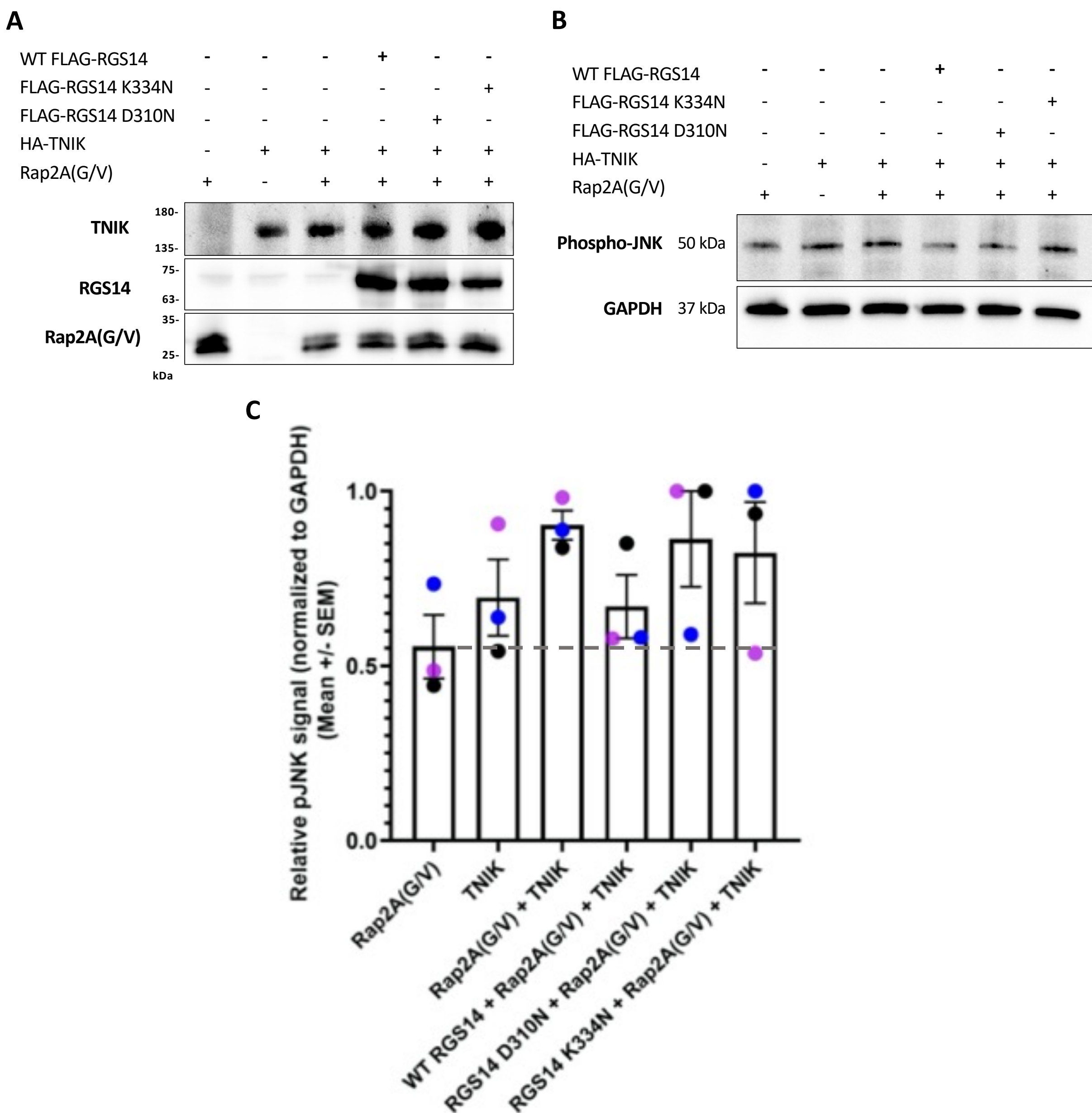


**Figure 5. Wild-type RGS14 downregulates the Rap2A- and TNIK-stimulated levels of pJNK, which is disrupted by the rare variants D310N and K334N. A)** RGS14, Rap2A(G/V) and TNIK are successfully overexpressed and detected by Western blotting when transfected into HEK293 cells. **B-C)** Levels of phopsho-JNK are identified in whole-cell lysates of HEK293 cells transfected with different combinations of Rap2A(G/V), HA-TNIK, wild-type FLAG-RGS14 and FLAG-RGS14 variant D310N or K334N via Western blotting. The blots in A-B are representative of three independent experiments. pJNK signals were quantified using densitometric analysis and normalized against the GAPDH signal in the respective lanes. The data points from independent experiments are represented by different colors, and the bars represent the average of the three independent experiments. The error bars represent standard error of the mean (SEM). Dashed line represents the relative mean pJNK signal for the Rap2A(G/V) only condition for comparison.

**DISCUSSION**

In the present study, we investigated the impacts of the naturally occurring variation in RGS14 on RGS14's binding dynamics and regulation of the downstream JNK signaling pathway. We identified four rare human variants, D310N, D310G, K334N and L315S, which encode point mutations that we estimated to be located on the interaction site of RGS14's R1 subunit of the Ras/Rap binding domain (RBD), based on the homology-based 3D model of Raf-1's R1 RBD. Because all of the amino acid substitutions conferred by the four point mutations result in a change in amino acid property (**Table 2**), we expected these point mutations to alter the structure of the interaction site of RGS14's R1 RBD, and consequently block its interaction with active Rap2A. We also selected two rare variants, A363V and T323A, as negative controls, since the amino acids A363 and T323 appear to be localized farther away from the interaction site of the RBD. Therefore, we did not expect these point mutations to alter the structure of the interaction site and disrupt RGS14's binding with active Rap2A (**Fig. 2**). We found that two of the point mutations, K334N and D310N, blocked RGS14's capacity to bind constitutively active Rap2A(G/V), whereas the variants D310G, L315S, T323A and A363V did not alter RGS14-Rap2A binding.

The variants T323A and A363V's ability to bind active Rap2A is consistent with our expectation and supports our prediction that the amnio acids T323 and A363 are localized farther away from RBD's interaction site. On the other hand, we offer two possible explanations for why the point mutations D310G and L315S did not alter RGS14's binding to active Rap2A, contrary to our expectations. First, the amino acid changes conferred by the point mutations D310G and L315S might not have drastically altered the 3D structure of R1 RBD. Second, the structure of the R1 RBD of RGS14 might be different than that of Raf-1, the structure on which we based our predictions. We find the first explanation less likely, since L315S appears to be localized in the

middle region of a $\beta$-strand **(Fig. 2)**, and hydrophobic amino acids such as leucine are more preferred at the middle region of $\beta$-strands than polar amino acids such as serine (Bhattacharjee & Biswas, 2010). However, the first explanation is more likely to be the case for why the variant D310G did not alter RGS14's binding to active Rap2A, while the variant D310N did. Glycine is strongly favored in $\beta$-turns due to its flexibility, and therefore it is possible that the point mutation D310G might have further stabilized the structure of RBD's interaction site instead of altering it (Vanooyen & Vanpelt, 1994). The second explanation is overall highly likely, since the R1 RBD of RGS14 has only 31% sequence similarity with that of Raf-1. Though these results bring us closer to predicting the structure of RGS14's R1 RBD and the location of its binding site, nuclear magnetic resonance or mass spectrometry analyses are needed to fully characterize the structure of RGS14's R1 RBD and identify its interaction site.

We next aimed to establish how RGS14 regulates active Rap2A and the JNK signaling pathway and identify how the RGS14 variants D310N and K334N might impact this regulation. Active Rap2A mediates the removal of synaptic AMPA-Rs during depotentiation by stimulating JNK signaling through its interaction with TNIK (Stornetta & Zhu, 2011). We reasoned that if RGS14 negatively regulates active Rap2A by either sequestering it away from its downstream effectors, such as TNIK, or by acting in another unknown way, then the RGS14-Rap2A interaction might serve to downregulate JNK signaling. By investigating the levels of phosphorylated JNK (pJNK) in HEK293 cells overexpressing Rap2A(G/V), TNIK and/or RGS14, we found that the overexpression of wild-type RGS14 alongside Rap2A(G/V) and TNIK resulted in lower levels of pJNK compared to the Rap2A- and TNIK-stimulated pJNK levels. Furthermore, the average levels of pJNK in cells overexpressing wild-type RGS14, Rap2A(G/V) and TNIK was similar to the average levels of pJNK in cells overexpressing TNIK only. On the other hand, the overexpression

of the RGS14 variants D310N or K334N, alongside Rap2A(G/V) and TNIK, resulted in pJNK levels similar to the Rap2A- and TNIK-stimulated levels, reversing the effect of wild-type RGS14. Together, these findings support a model in which RGS14 binds and sequesters active Rap2A, which is no longer able to stimulate the TNIK-mediated phosphorylation of JNK, resulting in the downregulation of JNK signaling. In the presence of the RGS14 variants D310N and K334N, Rap2A remains in the active state and continues to stimulate the TNIK-mediated phosphorylation of JNK, resulting in enhanced JNK signaling (**Fig. 6**).

*Potential Implications of RGS14 Regulation of JNK Signaling*

The JNK signaling pathway contributes to the regulation of various important cellular processes, such as gene expression, cell proliferation, differentiation, programmed cell death, cell survival and stress response. Humans express three JNKs: JNK1 and JNK2, which are ubiquitously expressed, and JNK3, whose expression is largely restricted to the brain. More than 90% of the hippocampal pyramidal neurons express JNK3, while JNK1 expression is restricted to the hippocampal areas CA3 and CA4, also known as the hilus region of the dentate gyrus (DG) (Lee, Park, Lee, Lee, & Han, 1999). Knockout studies have revealed important roles for the different JNKs in the brain, such as programmed cell death during early development, neuronal pathfinding and migration, neurite formation and maintenance during late development, and excitotoxicity- and ischemia-induced apoptosis and synaptic plasticity in the adult brain (reviewed in Yamasaki, Kawasaki, & Nishina, 2012 and Coffey, 2014).

Neuronal insults such as excitotoxicity, the main molecular mechanism underlying epilepsy, and ischemia/hypoxia, which occur during stroke, activate JNK signaling, leading to neuronal death (Barker-Haliski & White, 2015; Sun, 1999). Previous studies have found that

disruption of the gene encoding Jnk3 as well as the pharmacological inhibition of JNK by the peptide inhibitor D-JNKI-1 eliminate excitotoxicity- and/or ischemia-induced apoptosis (Borsello et al., 2003; Centeno et al., 2007; Spigolon, Veronesi, Bonny, & Vercelli, 2010; D. D. Yang et al., 1997). Interestingly, the hippocampal area CA2 neurons are naturally resilient to such neuronal insults. Several studies found no cell death in the pyramidal neurons of CA2 following ischemia, in contrast with the other regions of the hippocampus that underwent large cell loss (Kirino, 1982; Sadowski et al., 1999). CA2 neurons survive after epileptic seizures as well, which was shown in both human epileptic patients and in animal models of epilepsy (Haussler, Rinas, Kilias, Egert, & Haas, 2016; Sloviter, 1983; Williamson & Spencer, 1994). Our lab previously proposed that RGS14, whose expression in the hippocampus is restricted to the area CA2, might be one of the signaling proteins that confer area CA2's resistance to ischemia- or excitotoxicity-induced cell death (Evans, Dudek, & Hepler, 2015). The findings of the present study provide evidence to support this hypothesis. By downregulating JNK signaling, RGS14 might provide neuroprotection against excitotoxicity or ischemia-induced neuronal death in the CA2 neurons. This hypothesis could be directly tested in RGS14 variant D311N or K335N (mouse homologs of the human variants D310N and K334N, respectively) knock-in mice by investigating whether neuronal loss occurs in the area CA2 in response to seizures or hypoxia/ischemia.

JNK's regulation of physiological responses to stress signals, such as inducing apoptosis, positions JNK as one of the key signaling pathways involved in the pathogenesis of neurodegenerative disorders, one of them being Alzheimer's disease (AD). AD is a progressive neurodegenerative disorder characterized by extracellular deposits of amyloid-β (Aβ) that lead to neuritic plaques and intracellular aggregation of hyperphosphorylated tau protein into neurofibrillary tangles (Long & Holtzman, 2019). High levels pJNK expression was identified in

post-mortem brains of AD patients, where pJNK was found to be localized in association with Aβ plaques and neurofibrillary tangles (Ferrer, Blanco, Carmona, & Puig, 2001; Gourmaud et al., 2015; X. Zhu et al., 2001). While a causative relationship between JNK activation and AD pathogenesis has not yet been established, a bidirectional relationship is possible. JNK activation has been found to be required for Aβ plaque formation, and Aβ has been found to induce JNK activation (Mazzitelli, Xu, Ferrer, Davis, & Tournier, 2011; Morishima et al., 2001; Yoon et al., 2012). JNK also contributes to hyperphosphorylation of tau protein and phosphorylation of amyloid precursor protein (APP) at threonine 668, a critical step in amyloidogenic processing that eventually leads to Aβ generation (Mazzitelli et al., 2011; Reynolds, Utton, Gibb, Yates, & Anderton, 1997; T. Zhang, Chen, & Lee, 2019) reviewed in (Coffey, 2014; Yarza, Vela, Solas, & Ramirez, 2015). The hippocampus is one of the first brain regions to be affected during the early stages of AD pathogenesis, which makes memory loss one of the hallmark symptoms of AD. In particular, areas CA1 and CA3 undergo substantial cell loss during the course of the disease, whereas area CA2 neurons are relatively spared. Area CA2 neurons are also resilient to neurofibrillary tangle formation, except in rare cases of certain human tauopathies (Ishizawa et al., 2002; Padurariu, Ciobica, Mavroudis, Fotiou, & Baloyannis, 2012). Therefore, RGS14's selective expression in the CA2 neurons and ability to downregulate JNK signaling make RGS14 a candidate signaling protein that might confer resilience against AD-related pathological changes in CA2 neurons.

*RGS14's Regulation of Synaptic Plasticity*

RGS14 is a natural suppressor of synaptic plasticity in the CA2 neurons, but the exact mechanisms by which RGS14 suppresses synaptic plasticity remain to be identified.

Understanding how RGS14 regulates its binding partners, particularly the ones that bind to RBD, might elucidate some of these mechanisms. For example, RGS14's interaction with active H-Ras inhibits ERK MAPK signaling (Shu et al., 2010). H-Ras-ERK signaling mediates the insertion of AMPA-R with long cytoplasmic termini (AMPA-Rs containing GluA1 and GluA2) to the postsynaptic membrane, which is critical for the induction of long-term potentiation (LTP) (Kim, Dunah, Wang, & Sheng, 2005; Patterson et al., 2010; Peng, Zhang, Zhang, Wang, & Ren, 2010; J. J. Zhu, Qin, Zhao, Van Aelst, & Malinow, 2002). RGS14's regulation of active H-Ras and ERK signaling is therefore a possible mechanism by which RGS14 blocks LTP in the CA2 neurons.

In the present study, we found that RGS14 negatively regulates active Rap2 and JNK signaling. Rap2-TNIK-JNK signaling is known to induce removal of AMPA-Rs with long cytoplasmic termini from the postsynaptic membrane during depotentiation of synaptic transmission, which is the reversal of LTP at the synapse (Chen et al., 2016; L. Zhang et al., 2018; J. J. Zhu et al., 2002; Y. Zhu et al., 2005). Therefore, by negatively regulating both active H-Ras and active Rap2A, RGS14 might altogether impede the trafficking of GluA1- and GluA2-containing AMPA-Rs, which would be consistent with RGS14's role in suppressing activity-induced changes in synaptic transmission. RGS14 binds to both active H-Ras and Rap2 through R1 RBD, though it is unknown whether RGS14 can bind both proteins simultaneously or whether Rap2A and H-Ras binding to RGS14 are mutually exclusive, which is as a topic for further study. Moreover, RGS14 variants that cannot bind active Rap2A are also unable to bind active H-Ras. We have shown this to be the case for the variant K334N – the variant K334N blocked RGS14's binding to active H-Ras in live cells. (**Supplemental Fig. 1**). Future studies will be needed to test whether there are synaptic plasticity-related changes at SC synapses of CA2 neurons and whether

Rap2A-mediated JNK and H-Ras-mediated ERK signaling are altered in the presence of the variants D310N and K334N.

*Rare Human Genetic Variants of RGS14: Human Health and Disease*

In the present study, we discovered that RGS14 rare variants D310N and K334N block RGS14's binding with active Rap2A. Variants D310N and K334N are both identified in a small percentage of various human populations (**Table 2**). Whereas there have been no phenotypes associated with the variant K334N, variant D310N was found in a living 70-year old, white male patient with lung squamous cell carcinoma (Bhandari et al., 2019; Bonneville et al., 2017; Ding et al., 2018; Ellrott et al., 2018; Q. Gao et al., 2018; Hoadley, Yau, Hinoue, Wolf, Lazar, Drill, Shen, Taylor, Cherniack, Thorsson, Akbani, Bowlby, Wong, Wiznerowicz, Sanchez-Vega, Robertson, Schneider, Lawrence, Noushmehr, Malta, Stuart, et al., 2018; Liu et al., 2018; Poore et al., 2020; Sanchez-Vega et al., 2018; Taylor et al., 2018). However, the lack of association of a phenotype with these rare variants does not necessarily mean that these variants do not result in a phenotype. The RGS14 variants' inability to bind Rap2A and regulate JNK signaling might have significant implications for the physiology of CA2 neurons of the carriers of these variants, including response to neuronal insults such as ischemia and excitotoxity, AD pathogenesis and synaptic plasticity. Further studies on mouse models will be needed to elucidate any phenotypes at the cellular and/or behavioral level associated with the variants D310N and K334N.

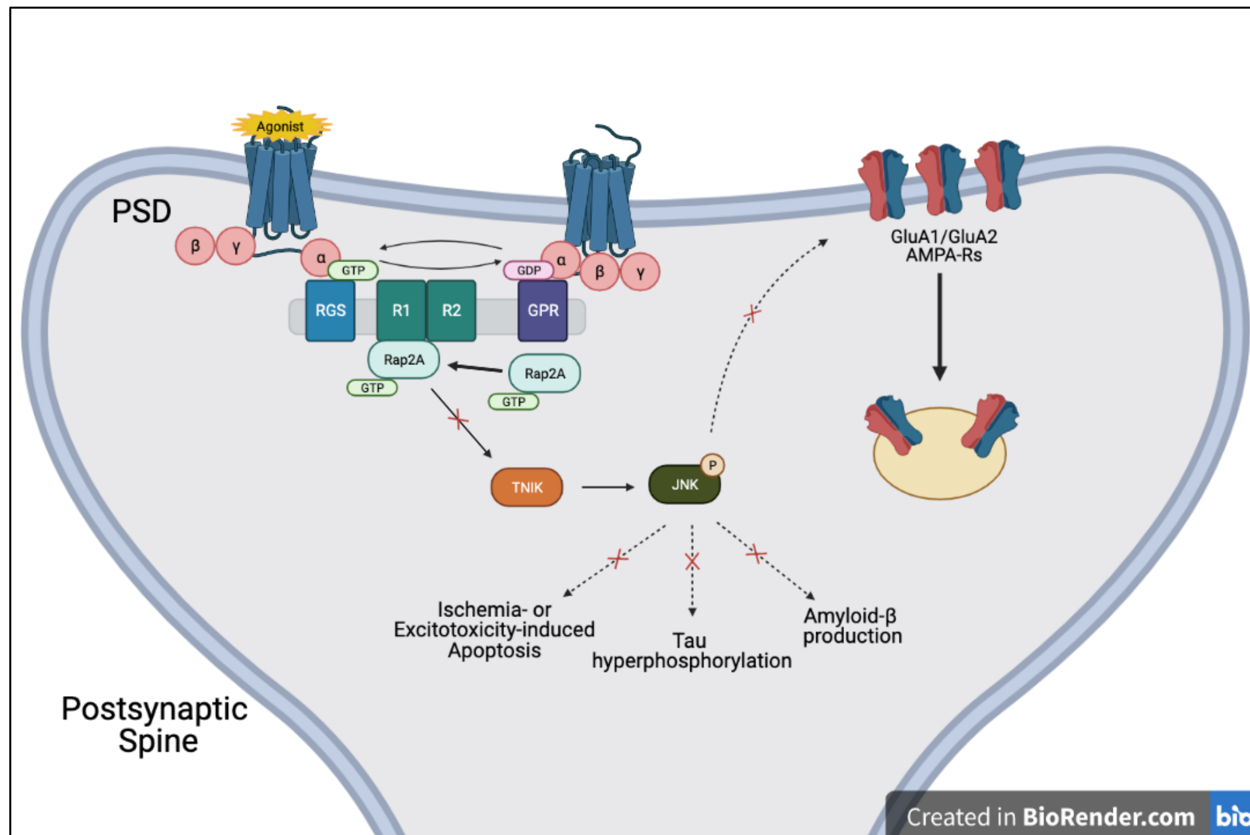


**Figure 6. Proposed model for RGS14's regulation of active Rap2A and JNK signaling in CA2 neurons.** In the postsynaptic dendrite of CA2 neurons, agonist binding to a $G_{i/o}$-coupled G protein-coupled receptor (GPCR) activates the $\alpha$ subunit of the heterotrimeric G protein by exchanging the GDP bound $\alpha$ subunit for a GTP. Activated $G\alpha_i$-GTP recruits RGS14 from the cytosol to the postsynaptic density (PSD) and binds to the RGS domain of RGS14. There, RGS14 acts as a GTPase-activating protein (GAP) to catalyze the hydrolysis of $G\alpha_i$-GTP to $G\alpha_i$-GDP, thereby deactivating the G protein signaling. RGS14 is then able to bind to the newly formed or another surrounding $G\alpha_i$-GDP via its GPR motif. This way, RGS14 becomes captured at the plasma membrane. The resulting RGS14:$G\alpha_i$-GDP complex is positioned to recruit more RGS14:$G\alpha_I$ complexes to the PSD, which can collectively form a signaling node that can intercept signaling proteins/pathways involved in promoting synaptic plasticity, such as H-Ras, $Ca^{2+}$/calmodulin (CaM) and Rap2A (Brown, Goswami, et al., 2015; Evans et al., 2015). We propose that RGS14 intercepts Rap2A-mediated JNK signaling by sequestering active Rap2A away from TNIK. Sequestered Rap2A can no longer regulate TNIK, which phosphorylates JNK to stimulate JNK signaling. Therefore, RGS14's binding to active Rap2A results in the downregulation of JNK signaling, which might consequently prevent ischemia- or excitotoxicity-induced apoptosis, Alzheimer's disease pathogenesis and the internalization of GluA1- and GluA2-containing AMPA receptors in CA2 neurons. Illustration created with BioRender.com.

**CONCLUSION**

In this study, we identified two rare human genetic variants of RGS14, variants K334N and D310N, which block RGS14's binding with active Rap2A. We showed that wild-type RGS14 negatively regulates active Rap2A and downregulates JNK signaling, whereas the rare variants D310N and K334N fail to do so. The JNK signaling pathway is involved in various important cellular processes and is implicated in the pathogenesis of certain neurodegenerative and psychiatric diseases. We propose that by negatively regulating active Rap2A and consequently downregulating JNK signaling, RGS14 regulates synaptic plasticity while serving a neuroprotective function in the CA2 neurons. Particularly, we believe that RGS14 might confer resistance to excitotoxicity- or ischemia-induced cell death, neurofibrillary tangle formation and amyloid-β production in the CA2 neurons. RGS14 has been established to be a natural suppressor of synaptic plasticity in the hippocampal area CA2. We propose that one of the mechanisms by which RGS14 suppresses synaptic plasticity is impeding the trafficking of AMPA receptors to and from the postsynaptic membrane by sequestering active H-Ras and Rap2A and downregulating the ERK and JNK signaling pathways mediated by H-Ras and Rap2A, respectively. Our findings provide further evidence that RGS14 is a complex multifunctional signaling protein with various important cellular functions and highlight the potential impacts of naturally occurring genetic variation on protein structure and function, which has become increasingly associated with individual predispositions to disease. Future studies will be aimed at testing the impacts of the rare RGS14 variants on neuron physiology, behavior and disease states, identifying potential neuroprotective roles of RGS14 and the establishing the exact mechanisms by which RGS14 suppresses synaptic plasticity in the CA2 neurons.

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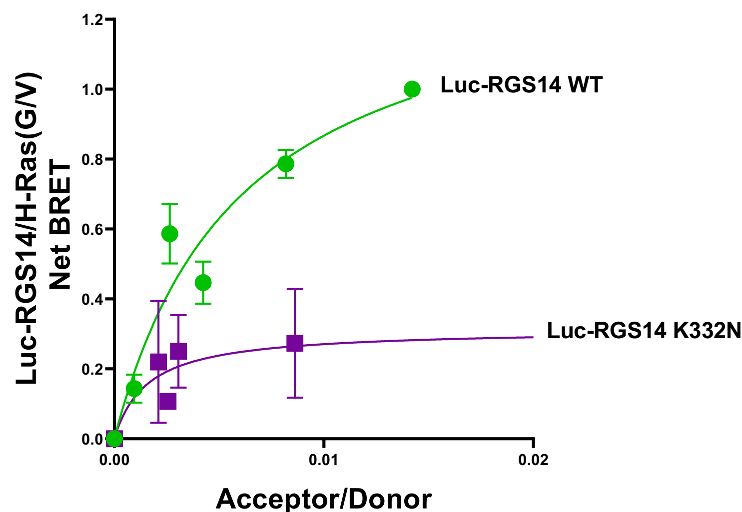


**Supplementary Figure 1. RGS14 variant K334N hinder RGS14's binding to active H-Ras.** RGS14 variant K332N (the rat homologue of the human variant K334N) does not bind constitutively active H-Ras(G/V) in live cells. HEK 293 were transfected with 10 ng of either Luc-RGS14 WT or Luc-RGS14-K332N plasmid along with 0, 25, 50, 100, 250 or 500 ng of Venus-H-Ras(G/V). BRET ratios were recorded and net BRET was calculated by subtracting the BRET signal from the luciferase alone. Wild-type RGS14 shows a robust BRET signal with H-Ras(G/V) (green line), whereas the variant K332N displays a drastically reduced BRET signal (purple line), which indicates a lack of binding. Data shown are the pooled mean +/- SEM of three independent experiments, each with triplicate determinations.