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Alaina Waters March 19, 2023

Development and optimization of protocols to identify nuclear binding partners of RGS14

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Regulator of G protein signaling 14 (RGS14) is a multifunctional scaffolding protein that integrates GPCR-G, Ras/ERK, and Ca++/CaM signaling pathways in the brain linked to postsynaptic plasticity. At this point, there is significant research regarding the biochemical activity of RGS14 in the plasma membrane and cytosol of neurons, but nothing is known about the interactions of RGS14 within cell nuclei. Research has found that a subpopulation of native RGS14 regularly travels to the interior of the nuclei of neurons, which is unusual for a large scaffolding protein that is mainly involved in events occurring in the cell plasma membrane. While there is significant levels of shuttling of RGS14 in and out of the nucleus, the purpose of this is unknown as is the function of RGS14 within the nucleus

In order to determine the function of RGS14 in the nucleus, we will be identifying the nuclear-specific RGS14 interacting partners and determining what cellular processes these interacting partners are associated with. In order to do this, we will be using TurboID, a proximity labeler, to tag the proteins that RGS14 is interacting with, separating the cytosol of the cells from the nuclei, and comparing the tagged proteins. Additionally, wild-type and RGS14-L504R mutant mouse brains will undergo cellular fractionation and RGS14 immunoprecipitation where RGS14 and its binding partners will be collected.

At this time, this study has successfully optimized protocols for cellular fractionation and biotin labeling of HEK293 cells, cellular fractionation of mouse hippocampi, and RGS14 immunoprecipitation of mouse brain hemispheres. In order to accomplish the goal of the study, successful collection of fractionated HEK293 cells confirmed to undergo successful transfection and subsequent expression of the DNA vectors HA-Turbo, HA-Turbo-NLS, HA-Turbo-RGS14, and HA-Turbo-RGS14-L505R and/or fractionated whole mouse brain must occur. Once these goals are completed, samples taken will undergo mass spectrometry and proteomic analysis with help from the Emory Proteomics core, at which time the binding partners of RGS14 in both cytosol and nuclei may be identified and larger conclusions may be drawn regarding the function of the protein.

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1: INTRODUCTION

Background of RGS14 and Relevant Previous Publications

Regulator of G protein signaling 14 (RGS14) is a regulator of G protein-coupled receptors (GPCRs) and G-protein signaling events (1,2). GPCRs are activated by agonist stimulation, which activates G-proteins by triggering the exchange of GDP (inactive) for GTP (active) and decouples the heterotrimeric Gα,β,γ complex, allowing the dissociated G proteins to modulate their downstream effectors (3). RGS proteins regulate GPCRs by binding both directly to the GPCRs and G proteins and by acting as GTPase-activating proteins (GAPs) by accelerating the hydrolysis of GTP on Gα subunits to GDP (4,5). In all, there are 20 mammalian RGS proteins divided into four subfamilies. RGS14, specifically, is an approximately 62 kDa scaffolding protein and is in the D/R12 subfamily of RGS proteins (6,7).

RGS14 contains domains including the RGS domain found in all RGS proteins, dual Ras/Rap (R1/R2)-binding domains (RBD) (8), a G protein regulatory (GPR) motif and a C-terminal PDZ binding motif (DSAL) (**Figure 1.1**) (10). Gαi/o subunits, when active, bind adenylyl cyclase (AC) and inhibit its enzymatic activity. Inhibition of AC causes a decrease in intracellular cyclic adenosine monophosphate (cAMP). The RGS domain of RGS14 binds active Gαi/o-GTP subunits which accelerates GTP hydrolysis, causing deactivation of Gαi/o subunits and recoupling of the heterotrimeric G-protein complex (8). This deactivation decreases inhibition on adenylyl cyclase, which in turn increases cAMP in the cell. The R1-RBD binds to activated H-Ras-GTP and Rap2-GTP (8, 10) which is believed to decrease ERK activation in the cell as both proteins are parts of cascades that result in ERK activation (8). Finally, the GPR domain, a guanine nucleotide dissociation inhibitor, binds to Gαi1/3-GDP, preventing the exchange of GDP for GTP, and

subsequent activation of the G protein (10). A diagram of the three domains of RGS14 and its major known binding partners is shown in **Figure 1.1**. A diagram of the main functions of RGS14 at the cellular membrane is shown in **Figure 1.2**.

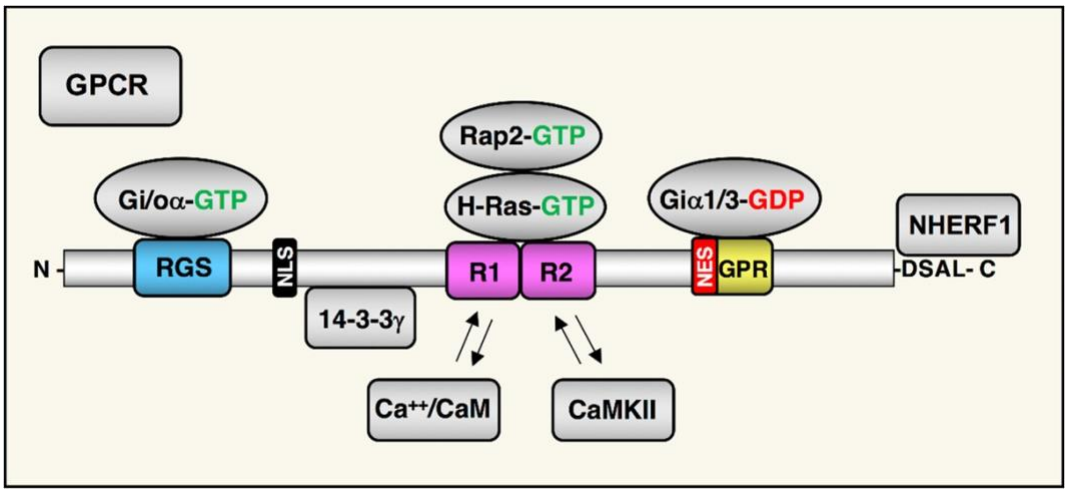


Figure 1.1: Diagram of RGS14 domains and most significant known binding partners in the cytosol and at the cellular membrane (9).

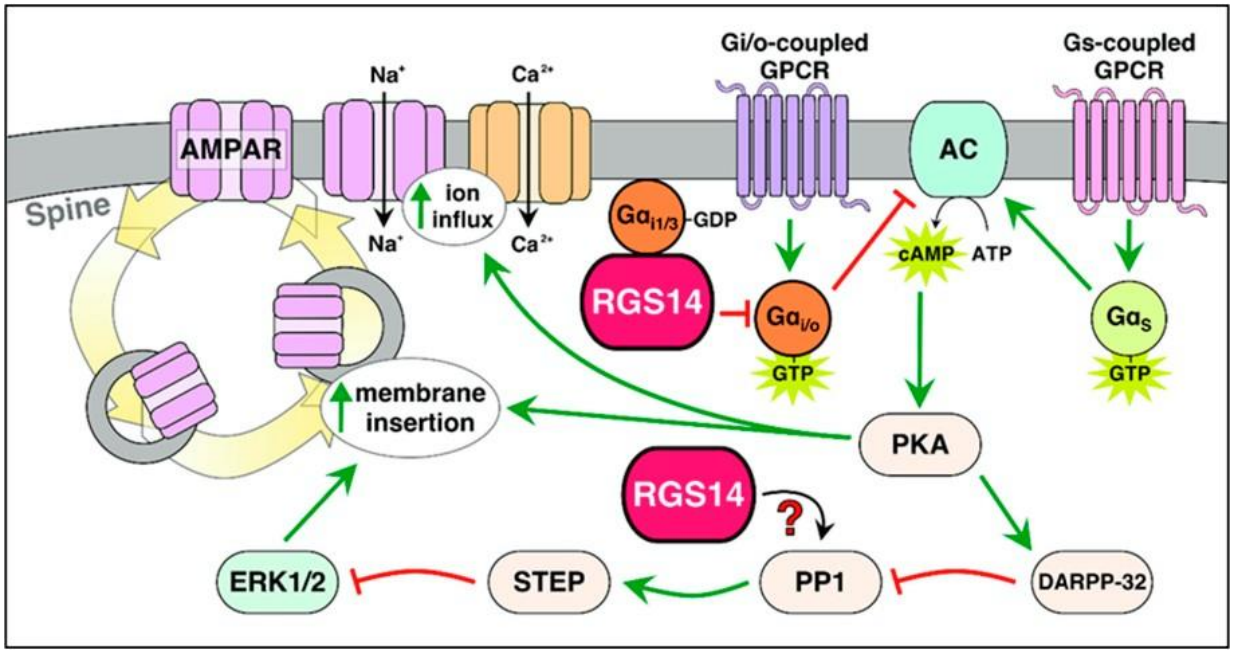


Figure 1.2: Depiction of RGS14 regulation of G protein-dependent signaling (9).

Beyond the binding domains and partners described above, RGS14 also contains both a nuclear localization sequence (NLS) and a nuclear export sequence (NES) (**Figure 1.1**). Abundant evidence shows that RGS14 is a cytoplasmic/nuclear shuttling protein (11). While much is known regarding the biochemical activity of RGS14 in the cytosol of neural cells, nothing is known about potential functional roles for and binding interactions of RGS14 within cell nuclei. Previous studies have shown that a subpopulation of native RGS14 can travel to the interior of the nuclei of neurons (12), which is unusual for a large scaffolding protein that is primarily involved in signaling events that occur at the cell membrane. The NLS and NES of RGS14 allow for protein shuttling in and out of the nucleus (**Figure 1.3**). While there are significant levels of shuttling of RGS14 in and out of the nucleus, the purpose of this is unknown as is any function of RGS14 within the nucleus.

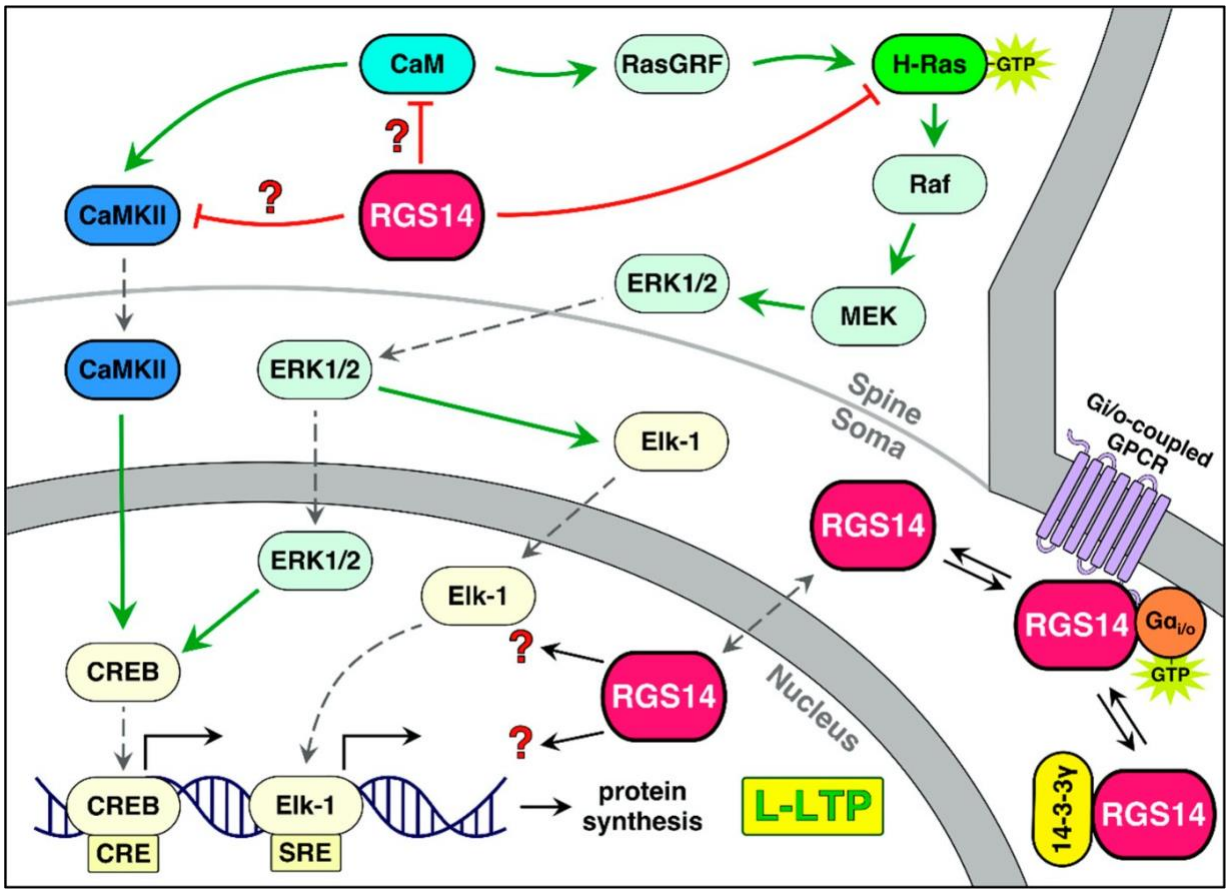


Figure 1.3: Schematic model of RGS14 subcellular localization to the nucleus (9).

The activation of long-term potentiation (LTP) is critical for memory formation and late long-term potentiation (L-LTP) is active over a period of days and is crucial for long-term memory (13). Findings from our lab show that *RGS14*-KO mice exhibit higher levels of spatial recognition compared to wild-type (WT) mice, and that this difference increases over days, indicating that RGS14 may inhibit L-LTP activation (14). A rare, but naturally occurring, human variant of RGS14, L504R (L505R in human type RGS14), includes a mutation that alters an amino acid within the NES and allows RGS14 to enter the nucleus, but prevents the protein from leaving the

nucleus after entering, eventually causing accumulation of RGS14 in the nucleus and low levels of RGS14 in the cytosol (11). When this variant was expressed in mice, the RGS14-L504R mice exhibited similar levels of spatial recognition as WT mice, contrary to the memory effects observed in RGS14-KO mice. This finding indicates that nuclear RGS14 may be sufficient for regulation of L-LTP that occurs in WT mice.

Goals of the Project and Experimental Approach

Based on these findings, the goal of my project has been to identify binding partners of RGS14 in the nucleus. Identifying nuclear-specific RGS14 interacting partners will help to determine what cellular processes these interacting partners are associated with. To accomplish this goal, I initiated the approaches to take a two-pronged approach: I 1) used TurboID, a biotin proximity labeler, to tag the proteins in near proximity with nuclear-localized RGS14, and 2) immunoprecipitated nuclear-localized RGS14-L504R from mouse brain to identify binding partners using mass spectrometry. TurboID is a biotin ligase which works by using ATP and biotin to form biotin-5'- AMP, which is a reactive intermediate that can label proteins that it comes in close contact with (15). A schematic of how TurboID can label proximal proteins with biotin is shown in **Figure 1.4**.

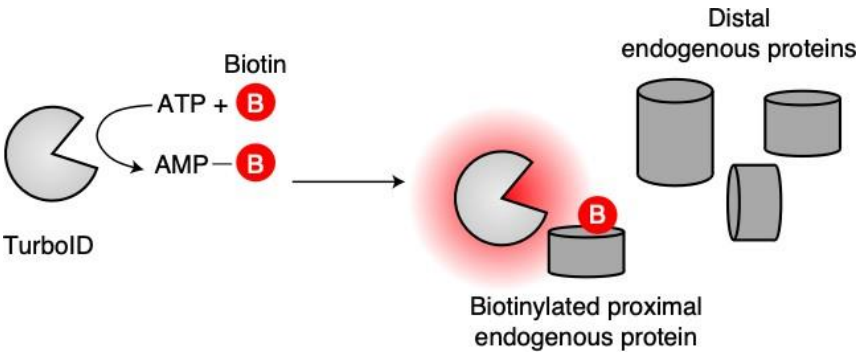


Figure 1.4: Schematic of biotin labeling through the use of TurboID (15).

We subcloned TurboID in frame with human type RGS14 (hRGS14) to create a version of RGS14, which would tag interacting proteins. We set out to create four different DNA sequences to assess: 1) HA-Turbo and 2) HA-Turbo-RGS14, which will localize to the cytosol, and 3) HA-Turbo-NLS, and 4) HA-Turbo-RGS14-L505R, which will localize to the nucleus. We expect to find that HA- Turbo and HA-Turbo-NLS are found primarily in the cytosol and nuclei, respectively, and will be used as experimental controls. HA-Turbo-RGS14 should be found primarily in the cytosol and HA-Turbo-RGS14-L505R should be found primarily in the nuclei. Validating the localization of HA-Turbo and HA-Turbo-RGS14 to the cytosol and HA-Turbo-NLS and HA- Turbo-RGS14-L505R to the nucleus must occur prior to proteomics, at which point the two experimental proteins will allow us to identify candidate proteins that RGS14 interacts with in each locale.

HEK293 cells will be used for these experiments and biotin will be added to the cell media to allow for biotin labeling of proteins proximal to the TurboID- labeled proteins. After labeling, biotinylated proteins will be immunoprecipitated from cell lysates using streptavidin beads. With

assistance from the Emory Proteomics core, biotin labeled precipitates will be subjected to mass spectrometry and proteomic analysis for identification of binding partners in each experimental condition.

To complement these studies, we will carry out a second approach. Mice carrying the RGS14-L504R mutation (LR mice) express RGS14 that is targeted to and accumulates within nuclei of neurons in mouse brain (11). Recovered brains from LR mice will be subjected to cellular fractionation to isolate nuclei. RGS14 will be immunoprecipitated from brain where RGS14 and any associated binding partners will be collected. The immunoprecipitated proteins will also be analyzed by mass spectrometry and proteomic analysis for identification of binding partners with assistance from the Emory Proteomics core. This dataset will be compared to the RGS14 interactome recovered from wild type mouse brains as we have done previously (16) This approach will allow us to compare the biotinylated binding partners that we collect from the nuclear Turbo-RGS14(LR) HEK293 cells and identify binding partners within a living mammalian organism.

After the completion of this project, we should gain insight into the types of processes that RGS14 may be involved in within cell nuclei. Future projects will focus on the more specific biochemical roles RGS14 may play within the cell and identify its specific niches in cellular activity and L-LTP.

2: METHODS

Cell Line Management

All experiments conducted in this project utilized human embryonic kidney cells (HEK293). The cells were grown in sterile, tissue culture treated plates in DMEM with 10% Fetal Bovine Serum and 1X Penicillin and Streptomycin. The cells maintained in a humidified incubator at 37 °C and 5% CO_2. All platings and incubations occurring in experiments were also under these conditions. Cells were passaged in a sterile hood at 1:10 and 1:5 ratios once they had reached 70-80% confluency. Cell lines were passaged up to 40 times at which point a new line was started.

Optimization of Cell Fractionation in HEK293 Cells

Two 10-cm well plate was plated from the maintained cell line. After 48 hours, the media was removed from the cells and replaced with trypsin. The cells were incubated at 37°C and 5% CO_2 for 3 minutes. Media was added to stop the trypsin and the cells were collected. 10% of the cell mixture was saved to be a "total" fraction. The total fractions were centrifuged at 500xg and 4°C for five minutes. The supernatant was removed, and the pellet was resuspended in 100 µL modified RIPA buffer [150 mM NaCl, 50 mM Tris, 1 mM EDTA, 2 mM DTT, 5 mM $MgCl_2$, 1X protease inhibitor (Roche, A32955) 1X Halt phosphatase inhibitor (ThermoFisher, 78428)] then placed on ice. The rest of the cell mixtures were centrifuged in a bucket centrifuge at 500xg and 4°C for five minutes then the supernatant was decanted. Each of the cell mixtures was vortexed throughout the rest of the procedure at a different speed, low or medium. The cell pellets were vortexed for five seconds, then a volume of NP-40 lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM

NaCl, 0.5% v/v NP-40 (Calbiochem 492015), 3 mM $MgCl_2$) equal to that of the cell pellet was added drop-wise down the side of the tube while vortexing to prevent clumping of the pellet. The cells were incubated for 10 minutes on ice and vortexed once every minute for five seconds. The cells were centrifuged at 1000xg and 4°C in a bucket centrifuge for five minutes, then 100 µL of the supernatant was collected to be the cytoplasmic fraction. The rest of the supernatant was decanted. The nuclear fraction was resuspended in 100 µL of RIPA buffer. 2X sample buffer with 40 mM DTT was added to each fraction, the fractions were boiled for five minutes, and stored at -20°C.

This procedure was then repeated with medium level vortexting with a 6-well plate to confirm successful nuclear fractionation after transfection. After 24 hours, 4 of the wells in each plate were transiently transfected in DMEM with 5% Fetal Bovine Serum and 1X Penicillin and Streptomycin with one of the four DNA vectors generated (HA-Turbo, HA-Turbo-NLS, HA-Turbo-RGS14, or HA-Turbo-RGS14-L505R) 24 hours after transfection, the procedure was continued as described above.

Confirmation of Construct Expression through Western Blotting

The proteins were defrosted at room temperature and subsequently separated through gel electrophoresis with one 13.5% gel. The samples were transferred to a nitrocellulose membrane and successful transfer was confirmed through staining with Ponceau solution. After stain removal with deionized water, both membranes were incubated in blocking buffer (5% non-fat milk in TBS containing 0.1% Tween-20 and 0.02% sodium azide) for 1 hour at room temperature, then washed with 3 times in TBST for 8 minutes each. Then, the membrane was incubated in anti-HA HRP conjugate antibody (Santa Cruz Biotechnologies, SC-805; 1:5000) diluted in blocking buffer

overnight at 4°C. After 3 washes in TBST, the membrane was incubated in goat anti-rabbit IgG HRP-conjugate (Invitrogen, 31460; 1:5000) diluted in TBST for 45 minutes at room temperature. The membrane was washed again 3 times in TBST, then the blots were developed through enhanced chemiluminescence. The blots were imaged using a ChemiDoc MP Imager (BioRad) and analyzed using ImageLab (BioRad) software. Band heights for the first blot were compared. against a standardized protein ladder to confirm all constructs had expressed and produced proteins of the expected lengths.

Confirmation of Cell Fractionation through Western Blotting

The protein samples for confirmation of all cell fractionation procedures were separated through gel electrophoresis and transferred to nitrocellulose membranes in the same manner as described above. After successful confirmation of transfer with Ponceau staining, the blot was cut between the 55 and 43 kDa protein standard bands to separate the nucleic and cytosolic protein markers. Both blots were incubated at room temperature in blocking buffer for 1 hour, then washed 3 times in TBST for 8 minutes each. Then, the cytosolic fraction blot was incubated in anti- GAPDH (Santa Cruz Biotechnology, 365062; 1:5000) diluted in blocking buffer and the nuclear fraction blot was incubated in anti-Lamin A/C (Cell Signaling Technology, 4777S; 1:1000) also diluted in blocking buffer, both overnight at 4°C. The next day, after 3 8-minute washes in TBST, both membranes were incubated in goat anti-mouse IgG HRP-conjugate (Jackson ImmunoResearch, 115-035-003; 1:5000) diluted in TBST for 45 minutes at room temperature. Both membranes were washed again 3 times in TBST, then the blots were developed through enhanced chemiluminescence in the manner described above.

Cell Transfection and Biotin Labeling

Subsequently, another 6-well plate was plated from the maintained cell line. After 24 hours, three of the wells were transiently transfected in the manner described above with the HA-Turbo DNA vector and the remaining three wells were transiently transfected with the HA-Turbo-NLS DNA vector. 24 hours after transfection, the transfection media in one of the plates was replaced with 50µM biotin solution and incubated for 10 minutes to allow for detectable biotin labeling. After the biotin labeling reaction was complete, media was removed from all cells of both plates and the cells were washed 5 times with cold PBS. The cells were then collected, lysed, and stored in the same manner as above.

Confirmation of Construct Expression and Biotin Labeling through Western Blotting

The proteins were defrosted at room temperature and subsequently separated through gel electrophoresis with two 13.5% gels. All protein samples were run through both gels. The samples were transferred and prepared for incubation in the same manner as described above. One membrane was incubated in Anti-HA HRP conjugate antibody (Santa Cruz Biotechnologies, SC-805; 1:5000) and prepared for imaging as detailed above. The other membrane was incubated in 0.3 µg/mL streptavidin-HRP (Invitrogen, S21375) diluted in TBST for 30 minutes. Both membranes were washed again 3 times in TBST, then the blots were developed through enhanced chemiluminescence. The blots were imaged using a ChemiDoc MP Imager (BioRad) and analyzed using ImageLab (BioRad) software. Band heights for the first blot were compared against a

standardized protein ladder to confirm all constructs had expressed and produced proteins of the expected lengths and the second blot confirmed successful biotin labeling of proteins.

Optimization of Cell Fractionation in Mouse Brain Tissue

The hippocampi of two mouse brains were extracted. One hippocampus was homogenized in PBS and the other in NP-40 lysis buffer. Both homogenizations were performed using a Dounce tissue grinder. The homogenates were collected and 50 µL of each homogenate was taken to be a "total" sample. The total samples were spun at 800xg for 5 minutes. The supernatant was decanted and lysis buffer was added. The cells were lysed end-over end for 1 hour at 4°C. The samples were then centrifuged at 15,700xg to clear the lysates. The supernatant was collected, boiled in 2X sample buffer with 40 mM DTT for 5 minutes, and stored at -20°C. The rest of the homogenates then underwent cell fractionation as described above with medium speed vortexing, except the entire cytoplasmic fraction was collected. Once the cytosolic and nuclear fractions had been collected and RIPA buffer added to the nuclear fraction, the samples were lysed end-over end for 1 hour at 4°C. The samples were then centrifuged at 15,700xg to clear the lysates. 50-100 µL of the supernatant was collected, boiled in 2X sample buffer with 40 mM DTT for 5 minutes, and stored at -20°C. The rest of the supernatant was collected and if the remaining supernatant was less than 500 µL, RIPA buffer was added to reach a volume of 500 µL. The samples were then flash frozen in liquid nitrogen and stored at -80°C to be used in immunoprecipitation.

This procedure was repeated with two mouse brain hemispheres homogenized in NP-40 lysis buffer with NP-40 detergent added either before or after homogenization.

RGS14 Immunoprecipitation of Cytosolic and Nuclear Fractions of Mouse Brain Tissue

The lysates were thawed on ice then incubated with 5 µL of RGS14 monoclonal antibody (Neuromab 75-170) each for 2 hours at 4°C, end-over-end. 75 µL of Protein G Dynabeads were then added to new tubes, 1 tube per sample. The tubes were put on a magnetic rack, and the supernatant was decanted. The beads were then washed three times with PBS and each wash aspirated. After the samples were done incubating, the lysate-antibody conjugations were added to the beads and the samples were incubated 2 hours at 4°C, end-over-end. The beads were then washed three times with cold 0.01% Tween-20. If the samples were going to be separated via Western blot, the beads were boiled in 100 µL of 2X sample buffer with 40 mM DTT and stored at -20°C. If the samples were going to be submitted for proteomics, the beads were washed three times with cold PBS, 50 µL of PBS was added to each bead sample, and stored at -20°C.

Confirmation of RGS14 Immunoprecipitation through Western Blotting

The protein samples were separated through gel electrophoresis and transferred to nitrocellulose membranes in the same manner as described above. After successful confirmation of transfer with Ponceau staining, the blot was cut between the 55 and 43 kDa protein standard bands and the blot containing the larger proteins was incubated at room temperature in blocking buffer for 1 hour, then washed with 3 times in TBST for 8 minutes. Then, the blot was incubated in anti-RGS14 (Neuromad, 75-170; 1:500) diluted in blocking buffer overnight at 4°C. The next day, after 3 8-minute washes in TBST, both membranes were incubated in goat anti-mouse IgG HRP-conjugate (Jackson ImmunoResearch, 115-035-003; 1:5000) diluted in TBST for 45 minutes at room temperature. Both membranes were washed again 3 times in TBST, then the blots were developed through enhanced chemiluminescence in the manner described above

3. RESULTS

Optimization of Cellular Fractionation in HEK293 Cells

Before identification of RGS14 protein-protein interactions inside and outside of the nucleus can be carried out occur, a protocol with consistent successful results must be developed. The first part of the procedure to be tested was the cellular fractionation of HEK293 cells. A cellular fractionation protocol was obtained from the Corbett lab (Emory) (17). This protocol was performed on two different plates of non-transfected HEK293 cells, with each sample subjected to a different amount of vortexing (low or medium) throughout the procedure. Western blotting with GAPDH antibody (a cytosolic marker) was performed on protein samples to evaluate levels of cytosolic markers in total, cytosolic, and nuclear fractions of HEK293 cells.

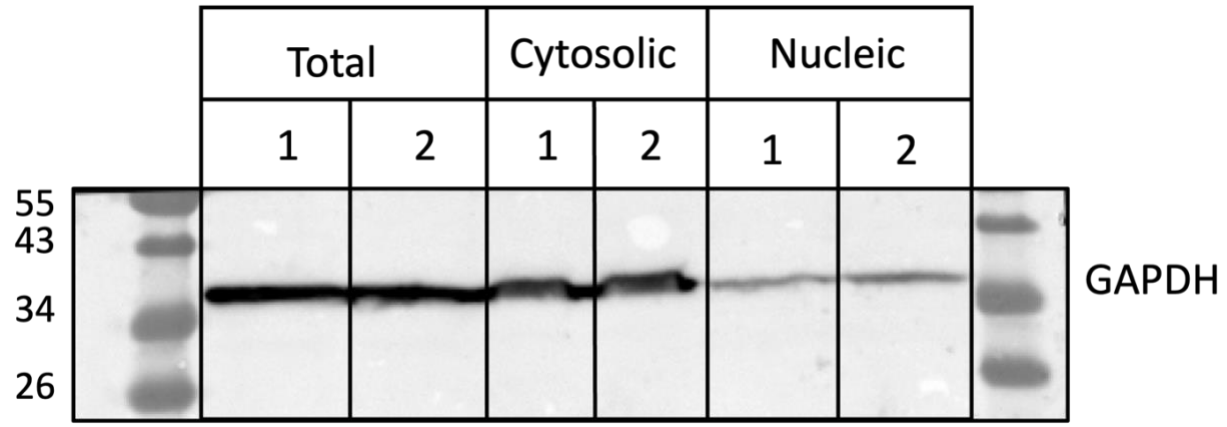
As shown in **Figure 3.1(A)**, a high intensity band between the 34 and 43 kDa protein standard bands was detected in all total and cytosolic fractions, and the same band was detected at a low level in both nuclear fractions. This band represents GAPDH, which is 35-40 kDa, and is present at high levels in cytosol, making it a reliable cytosolic marker (18). This result indicates that low and high levels of vortexing throughout the procedure both result in significant removal of cytosol from nucleic fractions.

Western blotting with Lamin A/C (a nuclear marker) antibody was performed on protein samples to evaluate levels of nuclear markers in total, cytosolic, and nuclear fractions of HEK293 cells. As shown in **Figure 3.1(B)**, in both total fractions, a band of medium intensity was detected between the 55 and 72 kD protein standard bands and a low intensity band was detected between the 72 and 95 kD protein standard bands. In the nuclear 1 fraction, both of these bands were detected at a high intensity while in the nuclear 2 fraction, the smaller band was detected at a high

intensity and the larger band was detected at a medium intensity.

These bands represent Lamin A and C. Lamin A is ~74 kDa and Lamin C is ~65 kDa. Lamin A and C are located proximal to the internal nuclear membrane which makes them reliable nuclear markers (19). The lack of Lamin A and C detection in the cytosolic samples indicates that both protocols result in excellent separation of nuclei from cytosol. Slightly more intense bands in the nuclear fraction that underwent medium strength vortexing indicate that medium strength vortexing may result in slightly better concentration of nuclear fractions, so medium strength vortexing was used in all following cell fractionation protocols.

A.



B.

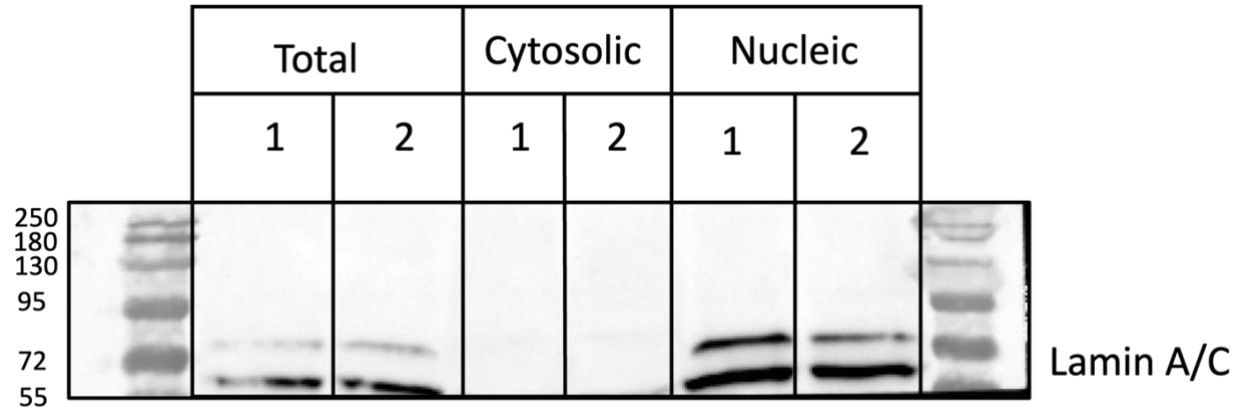


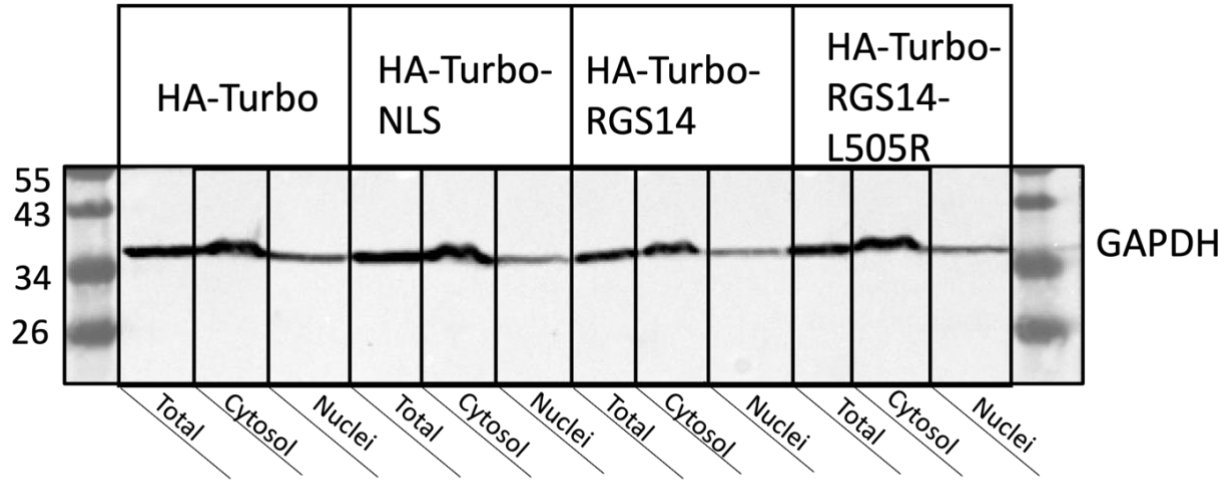
Figure 3.1: Cell fractionation of HEK293 cells into isolated cytosol and nuclei is slightly more effective when the samples are consistently vortexed at a medium speed than a low speed. In both (A, top) and (B, bottom), gray bands on either side of the figure indicate the standard protein ladder used to determine the size (kDa) of the proteins in the experimental samples. (A) shows GAPDH staining, while (B) shows Lamin A/C staining. Column titles "Total," "Cytosolic," or "Nucleic" indicate the expected cellular contents of the sample in that lane of the blot. Column titles "1," or "2" indicate that the sample in that column subjected to medium or low level vortexing, respectively. (A) "GAPDH" and (B) "Lamin A/C" indicate the name of the protein detected in the gray or black bands between the protein ladders.

Once the cell fractionation protocol had been optimized, it was performed on HEK293 cells that had been transfected with one of four DNA vectors 24 hours after plating. Western blotting with GAPDH antibody was performed on protein samples to evaluate levels of cytosolic markers in total, cytosolic, and nuclear fractions of HEK293 cells. As shown in **Figure 3.2(A)**, an intense band between the 34 and 43 kD protein standard bands was detected in all total and cytosolic fractions, and the same band was detected at a low intensity in all nuclear fractions. As detailed above, this band represents the cytosolic marker GAPDH and these results indicate that some, but not all cytosol was removed from the nucleic fractions.

Western blotting with anti-Lamin A/C antibody was performed on protein samples to evaluate levels of nuclear markers in total, cytosolic, and nuclear fractions of HEK293 cells. As shown in **Figure 3.2(B)**, a medium intensity band was detected between the 55 and 72 kDa protein standard bands and a low intensity band was detected just above the previous band in all total fractions. In the HA-Turbo-NLS and HA-Turbo-RGS14-L505R nucleic fractions, both of these bands were detected at a high intensity while in the nuclear HA-Turbo and HA-Turbo-RGS14 fractions, the smaller band was detected at a high intensity and the larger band was detected at a medium intensity. These bands were not detected in any cytosolic fractions except at a low level in the HA-Turbo cytosolic fraction.

As detailed above, these bands represent the nucleic markers Lamin A and C. The extremely low detection of Lamin A and C in all cytosolic fractions and high levels in all nuclear fractions indicates highly successful separation of nuclei from cytosol.

A.



B.

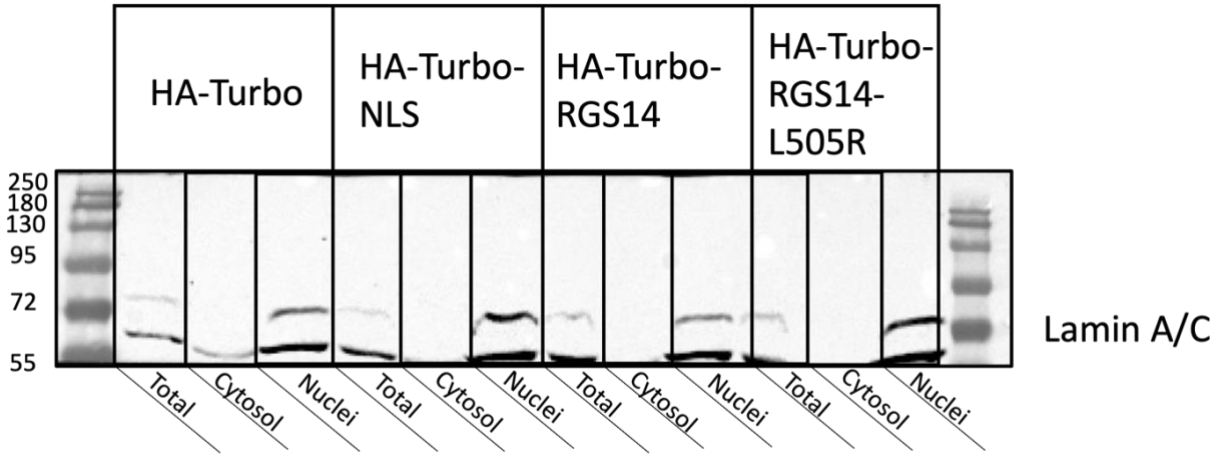


Figure 3.2: Cell fractionation of HEK293 cells into isolated cytosol and nuclei can be successfully completed after transfection with DNA vectors. For both (A, top) and (B, bottom), gray bands on either side of the figure indicate the standard protein ladder used to determine the size of the proteins (kDa) in the experimental samples. (A) shows Lamin A/C staining while (B) shows GAPDH staining. Column titles "Total," "Cytosolic," or "Nucleic" indicate the expected cellular contents of the sample in that lane of the blot. Column titles "HA-Turbo," "HA-Turbo-NLS," "HA-Turbo-RGS14," or "HA-Turbo-RGS14L505R" indicate which DNA vector those samples were transfected with 24 hours before cell fractionation occurred. The labels "Lamin A/C" (A) and "GAPDH" (B) indicate the name of the protein detected in the gray or black bands between the protein ladders.

Confirmation of Recombinant Turbo and Turbo-RGS14 Construct Expression after Cellular Fractionation

After successful cellular fractionation was confirmed, the samples were tested to determine whether the DNA vectors had been properly transfected, and the appropriate proteins expressed. Western blotting with HA antibody was performed on protein samples to confirm or deny successful expression of HA-Turbo, HA-Turbo-NLS, HA-Turbo-RGS14, and HA-Turbo-RGS14L505R proteins in total, cytosolic, and nuclear fractions of HEK293 cells.

As shown in **Figure 3.3(A)**, an intense band of ~43 kDa was detected in the samples that had been transfected with either the HA-Turbo or HA-Turbo-NLS DNA vectors. A weak intensity band of approximately the same size was also detected in the samples that had been transfected with the HA-Turbo- RGS14 and HA-Turbo-RGS14-L505R DNA. This band represents proteins with an HA- tag, which here is HA-Turbo or HA-Turbo-NLS, which are each ~40 kDa (14). This result demonstrates that both HA-Turbo and HA-Turbo-NLS were both successfully transfected and expressed.

As shown in **Figure 3.3(B)**, a low intensity band between 90 and 130 kDa was detected in the sample that had been transfected with the HA-Turbo-RGS14 DNA, with a band of a slightly higher intensity present in the input fraction. Streaked bands were detected in the samples that had been transfected with the HA-Turbo and HA-Turbo-NLS DNA vectors, with high intensity streaks in these nucleic fractions. The weak intensity bands detected in the samples that had been transfected with the HA-Turbo-RGS14 DNA represent the protein HA-Turbo-RGS14 which is ~97 kDa. No expression was detected in cells that had been transfected with HA-Turbo-RGS14-L505R.

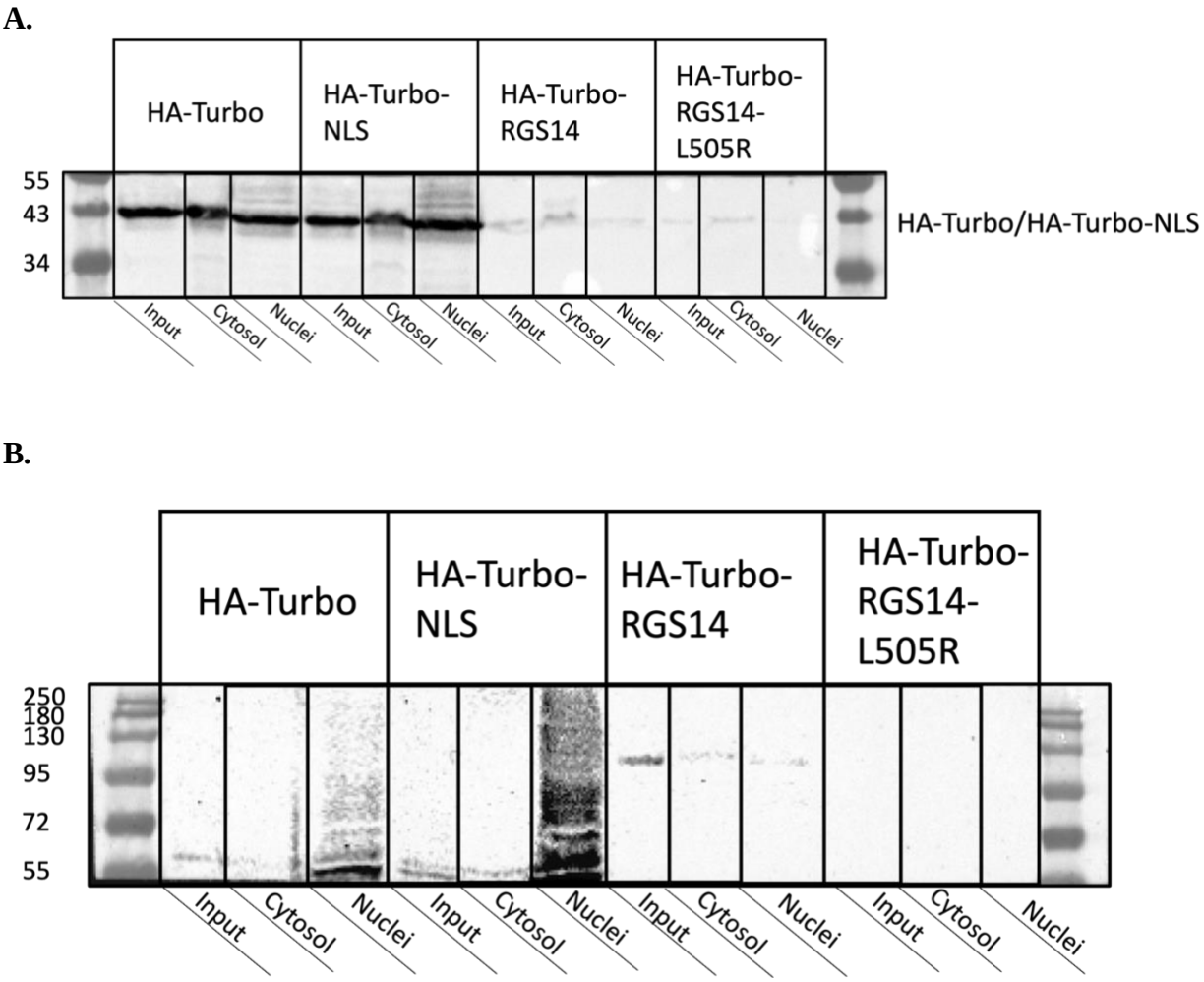


Figure 3.3: **Successful HA-Turbo and HA-Turbo-NLS protein expression and unsuccessful HA-Turbo-RGS14 and HA-Turbo-RGS14-L505R protein expression in fractionated HEK293K cells.** For both (A, top) and (B, bottom), gray bands on either side of the figure indicate the standard protein ladder used to determine the size of the proteins (kDa) in the experimental samples. (A) shows HA-Turbo staining while (B) shows HA-RGS14 staining. Column titles "Total," "Cytosolic," or "Nucleic" indicate the expected cellular contents of the sample in that lane of the blot. Column titles "HA-Turbo," "HA-Turbo-NLS," "HA-Turbo-RGS14," or "HA-Turbo-RGS14L505R" indicate which DNA vector those samples were transfected with 24 hours before cell fractionation occurred. The label "HA-Turbo/HA-Turbo-NLS" (A) indicates the name of the protein detected in the gray or black bands between the protein ladders.

Confirmation of Construct Expression and Biotin Labeling

Because expression could not be confirmed for the HA-Turbo-RGS14 and HA-Turbo-RGS14-L505R, the next portion of the protocol to be optimized, biotin labeling, was performed with only HA-Turbo and HA-Turbo-NLS. To test for optimal biotin labeling times, cells were incubated with the biotin labeling solution for 0, 5, or 10 minutes. Western blotting with anti-HA antibody was performed on protein samples to confirm expression of HA-Turbo and HA-Turbo-NLS proteins in HEK293 cells that were subjected to 0, 5, or 10 minutes of biotin labeling. As shown in **Figure 3.4**, high intensity bands of ~43 kDa were detected in every sample. As described above, these bands represent HA-Turbo or HA- Turbo-NLS protein, which indicates that there was successful protein expression in all samples analyzed.

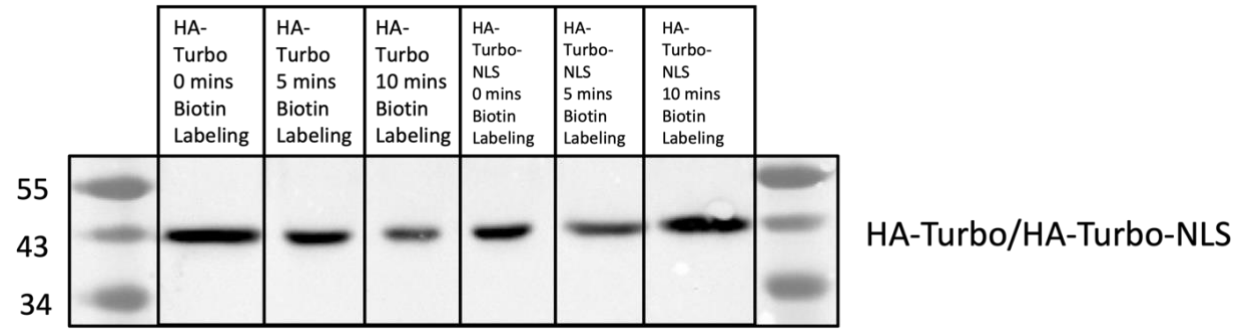


Figure 3.4: HA-Turbo and HA-Turbo-NLS proteins expressed in HEK293K cells after varying lengths of time of biotin labeling. This figure is paired with **Figure 3.5.** below. Gray bands on either side of the figure indicate the standard protein ladder used to determine the size (kDa) of the proteins in the experimental samples. Column titles indicate which DNA vector those samples were transfected with 24 hours before biotin labeling occurred and for how long the samples were incubated in biotin labeling solution. The label "HA-Turbo/HA-Turbo-NLS" indicates the name of the protein detected in the gray or black bands between the protein ladders.

Western blotting with streptavidin-HRP antibody was performed on protein samples to confirm successful biotin labeling in HEK293 cells that were biotin labeled for 0, 5, or 10 minutes. As shown in **Figure 3.5**, some background was detected in every sample. These bands represent proteins of all sizes that had been labeled by biotin. The intensity of these bands increased with length of biotin labeling. This indicates that there was successful biotin labeling in all samples, and the density of biotin labeling increased with increased time of biotin labeling. Some background was detected even in samples not incubated in biotin labeling solution because proteins in these samples were labeled by biotin that was naturally produced by the cell.

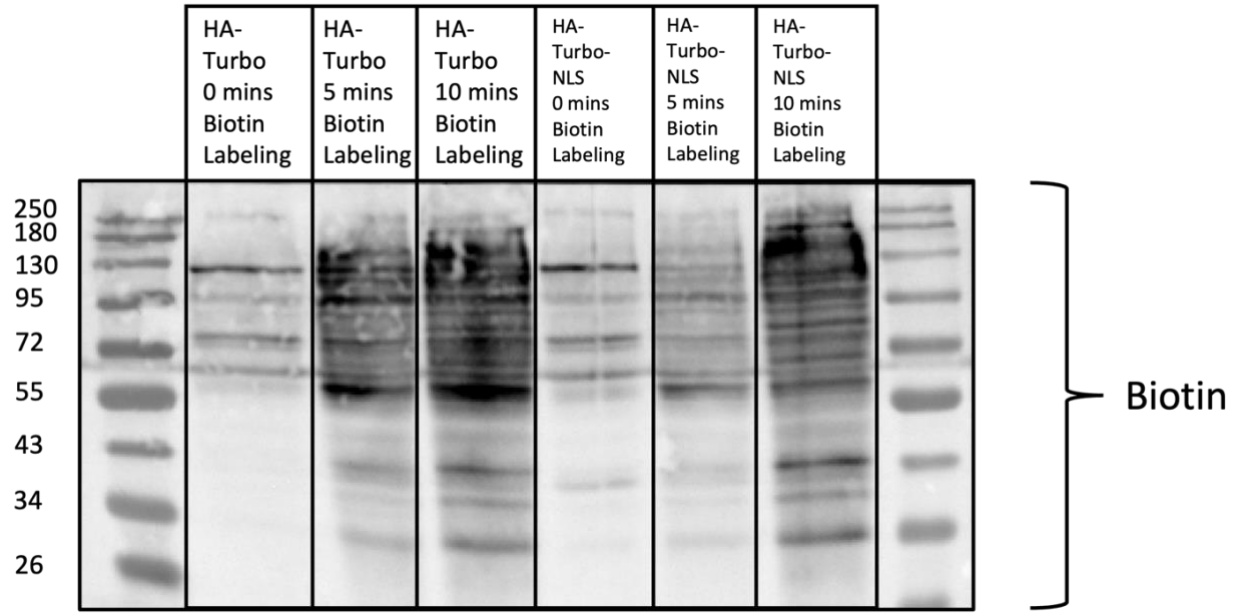


Figure 3.5: Confirmation of successful biotin labeling in HEK293K cells after varying lengths of time of biotin labeling. Gray bands on either side of the figure indicate the standard protein ladder used to determine the size (kDa) of the proteins in the experimental samples. Column titles indicate which DNA vector those samples were transfected with 24 hours before biotin labeling occurred and for how long the samples were incubated in biotin labeling solution. The label "Biotin" indicates the name of the protein detected in the gray or black bands between the protein ladders.

Optimization of Cell Fractionation in Mouse Brain Tissue

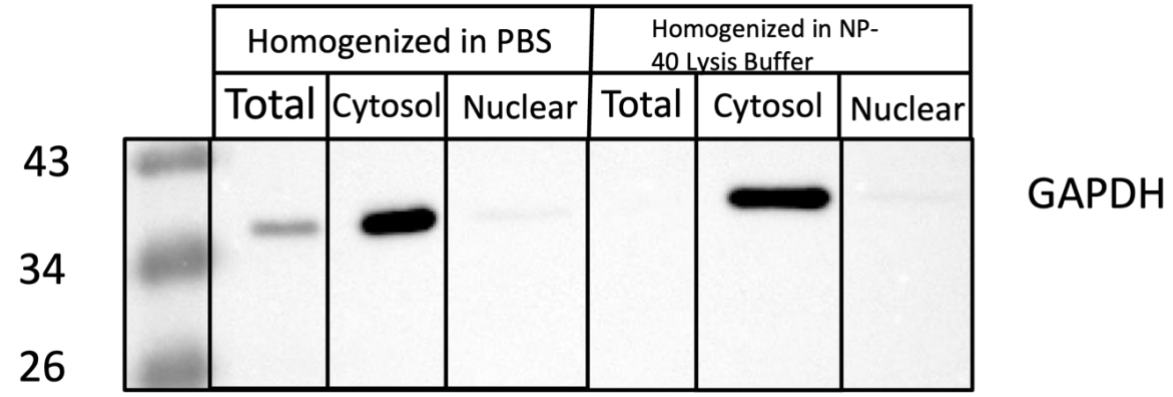
The unexpectedly poor expression of the HA-Turbo-RGS14 and HA-Turbo-RGS14-L505R DNA constructs and apparently degradation of protein detected with HA antibody could not be resolved in the time frame of the project. Trouble-shooting experiments are continuing to resolve the issue. However, in parallel, we planned to isolate native RGS14(LR) and protein binding partners from the CRISPR LR knock-in mouse hippocampi and brain hemispheres. Any identified RGS14(LR) binding partners would be collected from the endogenous environment with nuclear-localized RGS14(LR).

RGS14 is most highly expressed in mouse hippocampus (14). Therefore, to optimize the cellular fractionation of mouse brains, two wild type mouse hippocampi were collected and one was homogenized in PBS while the other was homogenized in NP-40 lysis buffer. Western blotting with GAPDH antibody was performed on protein samples to evaluate levels of cytosolic markers in total, cytosolic, and nuclear fractions of mouse hippocampus tissue homogenized in PBS or NP-40 lysis buffer. As shown in **Figure 3.6(A)**, a high intensity band between 34 and 43 kDa consistent with GAPDH was detected in both cytosolic fractions. Bands of the same size were detected at a medium intensity in the total fraction homogenized in PBS and at a very low level in the nuclear fraction homogenized in PBS and the total and nuclear fractions homogenized in NP-40 lysis buffer. As detailed above, these bands represent the cytosolic marker GAPDH and these results indicate that nearly all cytosol was removed from the nucleic fractions.

Western blotting with Lamin A/C antibody was performed on protein samples to evaluate levels of nuclear markers in total, cytosolic, and nuclear fractions of mouse hippocampus tissue homogenized in PBS or NP-40 lysis buffer. As shown in **Figure 3.6 (B)**, two high intensity bands were detected between the 72 and 55 kDa protein standard bands in the nuclear fraction

homogenized in NP-40 lysis buffer. One band at this same size was detected at a medium intensity in both total fractions and the nuclear fraction homogenized in PBS, and not at all in both cytosolic fractions. As detailed above, these bands represent the nucleic markers Lamin A and C. The extremely low level of Lamin A and C detected in the cytosolic fractions and medium to strong detection in the nucleic fractions indicates successful separation of nuclei from cytosol. The much higher intensity bands and detection of an additional band in the nuclear fraction of the sample that had been homogenized in lysis buffer compared to the band of the sample that had been homogenized in PBS indicated that homogenization in lysis buffer may allow for better concentration of nuclei during cell fractionation. Therefore, all future cellular fractionation of brain tissue was conducted with homogenization in NP-40 lysis buffer.

A.



B.

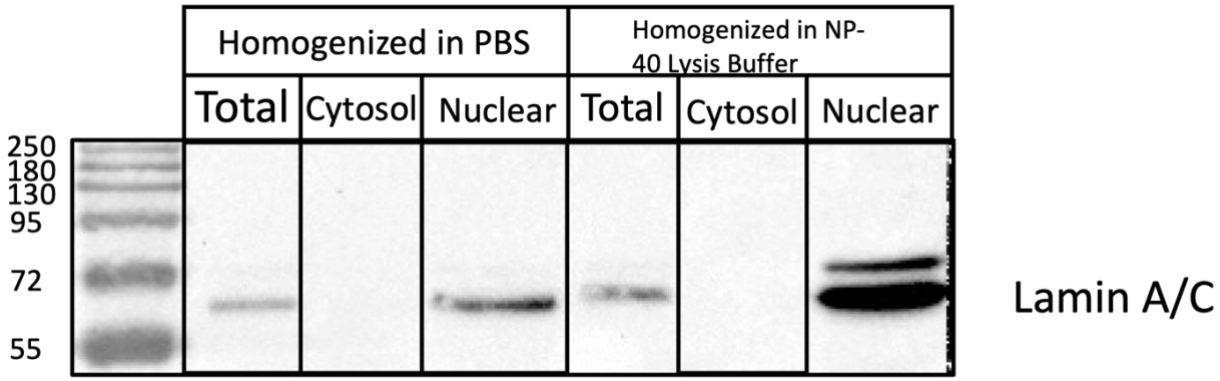


Figure 3.6: Cell fractionation of mouse brain hippocampi is more effective when the brain tissue is homogenized in NP-40 lysis buffer than PBS. For both (A) and (B), gray bands on either side of the figure indicate the standard protein ladder used to determine the size (kDa) of the proteins in the experimental samples. (A) shows staining of GAPDH and (B) shows staining of Lamin A/C in hippocampal brain fractions. Column titles "Total," "Cytosolic," or "Nucleic" indicate the expected cellular contents of the sample in that lane of the blot. Column titles "Homogenized in PBS" or Homogenized in NP-40 Lysis Buffer" indicate which solution was used to homogenize the hippocampal tissue. "GAPDH" and "Lamin A/C" indicate the name of the protein detected in the gray or black bands between the protein ladders.

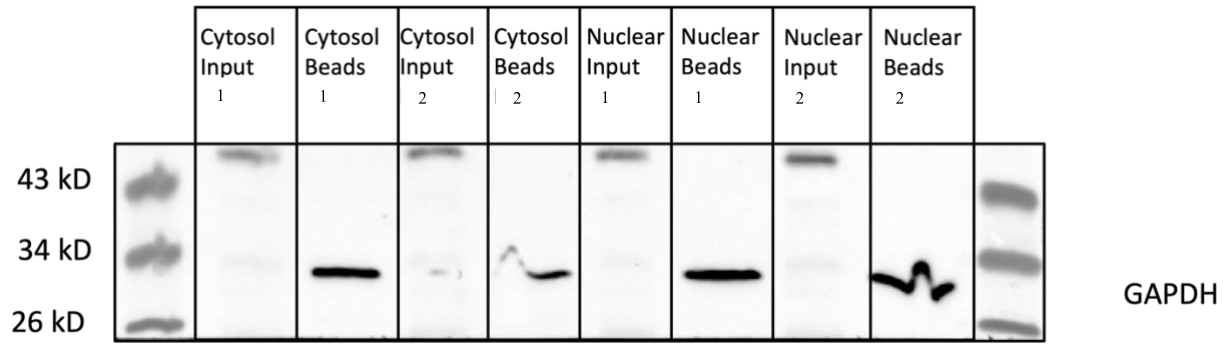
RGS14 is also expressed outside of the hippocampus in the striatum, and using only hippocampi limits total protein needed for LC-MS/MS. Therefore, the same procedure was then performed on two mouse brain hemispheres in order to gather higher levels of RGS14 and total protein for proteomics. One hemisphere was homogenized in lysis buffer not containing the detergent NP-40 (the detergent was added immediately after homogenization), and the other hemisphere was homogenized in complete NP-40 lysis buffer. The samples also underwent immunoprecipitation to increase concentrations of RGS14.

Western blotting with anti-GAPDH or anti-Lamin A/C antibody was performed on protein samples to evaluate levels of cytosolic or nuclear markers, respectively, in cytosolic and nuclear fractions of mouse hemisphere tissue homogenized in lysis buffer with NP-40 detergent or without NP-40 detergent.

As shown in **Figure 3.7 (A)**, weak bands were detected just above the 43 kDa protein standard band in all input fractions and strong bands were detected between the 26 and 34 kDa protein standard bands in all fractions that had undergone immunoprecipitation. These bands represent the cytosolic marker GAPDH, though it is unclear why it appears to be smaller after immunoprecipitation. As can be seen in **Figure 3.7 (B)**, strong bands were detected around 55 kDa in all samples that underwent immunoprecipitation, and no bands were detected in any input samples. These bands represent the nucleic markers Lamin A and C. The nucleic marker may be undetected in the input samples because the concentration is so high in the samples that have been immunoprecipitated that the imager could not expose the blot long enough to detect the input samples without overexposing the immunoprecipitated samples.

The cytosolic marker being present in all samples and the nuclear marker being present in all immunoprecipitated samples indicated that nuclear fractionation was unsuccessful.

A.



B.

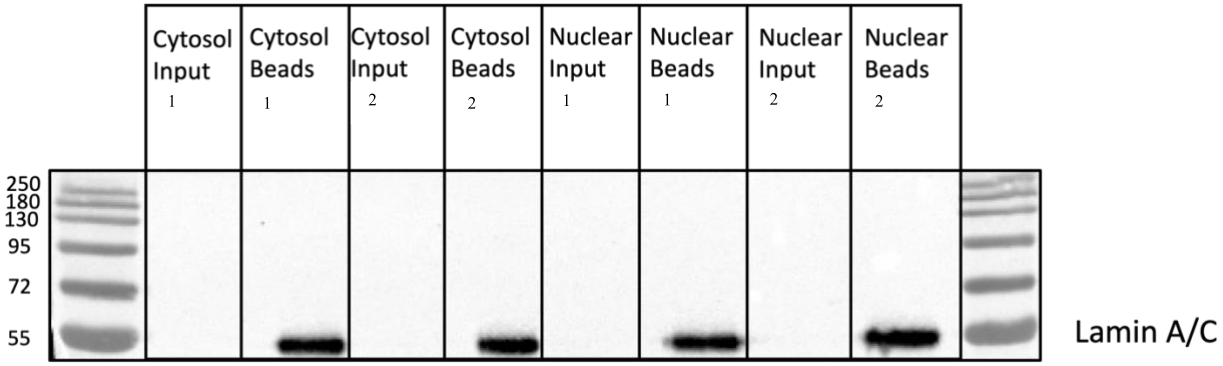


Figure 3.7: Cell fractionation of whole mouse brain hemispheres is not effective when fractionated using the same procedure as previously used with hippocampi. For both (A) and (B), gray bands on either side of the figure indicate the standard protein ladder used to determine the size (kDa) of the proteins in the experimental samples. (A) shows staining of GAPDH and (B) staining of Lamin A/C in whole brain fractions. Column titles "Cytosolic," or "Nucleic" indicate the expected cellular contents of the sample in that lane of the blot. Column titles "Beads" or "Input" indicates that the sample did or did not undergo immunoprecipitation, respectively. Column titles "1" and "2" indicate that the tissue was homogenized in lysis buffer with or without NP-40 detergent, respectively. "GAPDH" and "Lamin A/C" indicate the name of the protein detected in the gray or black bands between the protein ladders.

Confirmation of RGS14 Immunoprecipitation

The immunoprecipitation protocol was also tested to confirm successful immunoprecipitation of RGS14. All samples underwent the same immunoprecipitation protocol except for homogenization in different solutions. Western blotting with anti-RGS14 antibody was performed on protein samples to evaluate levels RGS14 in cytosolic and nuclear fractions of mouse hemisphere tissue homogenized in lysis buffer with NP-40 detergent or without NP-40 detergent. As can be seen in **Figure 3.8**, strong bands just under the 55 kDa protein standard band were detected in all samples that were subjected to immunoprecipitation. No bands were detected in any input samples. The bands were expected to represent RGS14 protein, which is 61 kDa, but the band was detected at a much smaller size. More analysis is needed to confirm successful immunoprecipitation.

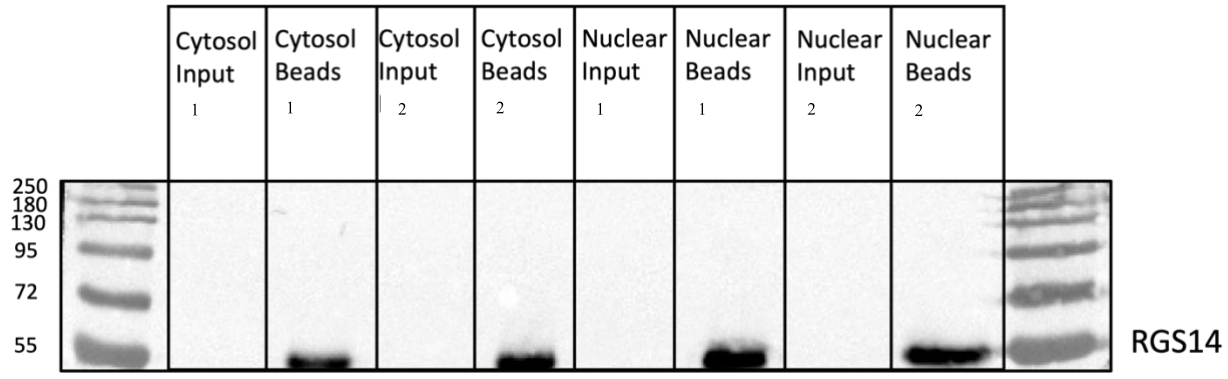


Figure 3.8: Confirmation of immunoprecipitation of RGS14 from mouse brain hemispheres is inconclusive. Gray bands on either side of the figure indicate the standard protein ladder used to determine the size (kDa) of the proteins in the experimental samples. Staining is shown for anti-RGS14. Column titles "Cytosolic," or "Nucleic" indicate the expected cellular contents of the sample in that lane of the blot. Column titles "Beads" or "Input" indicates that the sample did or did not undergo immunoprecipitation, respectively. Column titles "1" and "2" indicate that the tissue was homogenized in lysis buffer with or without NP-40 detergent, respectively. "RGS14" indicates the name of the protein detected in the gray or black bands between the protein ladders.

4. DISCUSSION

The overall goal of this study is to identify novel protein binding partners of RGS14 in the nucleus thereby allowing us to better understand RGS14's roles in the nucleus. Thus far, this study has successfully optimized protocols for cellular fractionation and biotin labeling of HEK293 cells and cellular fractionation of mouse hippocampi. In order to accomplish the goal of the study, successful collection of fractionated HEK293 cells confirmed to undergo successful transfection and subsequent expression of the DNA vectors HA-Turbo, HA-Turbo-NLS, HA-Turbo-RGS14, and HA-Turbo-RGS14-L505R must occur, with follow-up LC-MS/MS identification of biotinylated proteins. A parallel complimentary approach will be to immunopreciptate native RGS14 from mouse brain, specifically from CRISPR knock-in mice expressing RGS14-L504R in the nuclei. I have fractionated whole mouse brain, but this must be optimized at which point I will need to proceed to the immunoprecipitation steps. These troubleshooting studies are ongoing.

The HA-Turbo and HA-Turbo-NLS, and HA-Turbo-RGS14 cDNA vectors have functioned successfully in HEK2933 cells as expected. However, the HA-Turbo-RGS14-L505R construct has not so far. The HA-Turbo-RGS14-L505R cDNA vectors is being regenerated to account for any degeneration or errors that may have occurred in their initial construction. The streaks present in blots used to test for these constructs may also be due to a faulty HA antibody, and so a new antibody will be used going forward.

A new procedure for the homogenization and cellular fractionation of whole mouse brain tissue is being developed to account for the poor results of the original procedure when performed on mouse brain hemispheres. The new procedure will include steps to remove brain tissue debris to allow cellular fractionation to be performed on isolated cells which should allow for distinct

cytosolic and nucleic fractions.

Once these steps are taken to resolve the issues in the current protocol, samples can be collected and sent to proteomics (Dr. Nick Seyfried, Emory Proteomics Core) where the binding partners of RGS14 in the cytosol and the nucleus of the cell can be detected. Detected RGS14 cytosolic and nuclear Turbo-alone binding partners will be removed from data sets, and remaining detected nucleic binding partners will become the focus of further study. I expect multiple proteins to consider, and these proteins will be analyzed by pathway analysis to determine their known behaviors and cell processes that they are primarily involved in. Leading candidates will be tested for direct binding to RGS14 by co-immunoprecipitation and/or as purified proteins. This will allow us to begin to narrow down the major processes that RGS14 is involved in within the cellular nucleus.

RGS14 is part of a family of G protein regulators that primarily interact with proteins at or near the cellular membrane. RGS14 is unusual within this family in that it contains domains for nuclear import and export of the protein. Several other RGS proteins have also been reported to localize to the nucleus (20). A regulator protein that almost exclusively acts at the cellular membrane would not typically need domains to allow for travel between the cytosol and the nucleus, so this data will allow us to determine why RGS14 has these domains and what cellular processes it may be involved in that are thus far unknown and occur within the cell nucleus.

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