University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
Spring 2025

Effect of N terminal cysteine mutations on Gα13 localization and tumorigenic signaling

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Abstract

Heterotrimeric G proteins of the Gα12/13 subfamily regulate cell proliferation, and have been implicated in cancer development. While mutations of G proteins are often implicated as drivers of tumorigenesis, simple overexpression of Gα13 causes cancerous signaling, and has been shown to aid cancer cells in developing tumor-initiating cell (TIC) properties. In order for signaling and membrane localization to occur for most G protein α subunits, enzymatic attachment of the fatty acid palmitate to one or more cysteine side chains near the N-terminus is required. For Gα13, cysteines at positions 14 and 18 in this 377-amino acid protein have been shown to undergo this palmitoylation step, however, the roles of the individual cysteine modifications in Gα13 signaling function have not been reported. In order for the specific requirements for membrane localization and signaling of Gα13 to be investigated, cysteine-to-alanine mutations were engineered in Gα13 at the individual positions (14 and 18) and in a mutant combining both mutations. Introduction of negative charges around these key cysteine residues was also investigated through mutations of surrounding uncharged amino acids to aspartic acid residues. These Gα13 variants were confirmed by DNA sequencing and transfected into human embryonic kidney (HEK) cells. Ability of Cys mutants to participate in cancerous growth signaling was assessed through serum response factor (SRF) measurements, a well-established readout of oncogenic growth signaling in human cells. Effects of Cys to Ala mutations and introduction of negative charges on the intracellular localization of Gα13 were also determined, using cell

fractionation techniques. Results suggest that mutation of either cysteine residue completely disables SRE signaling, yet does not preclude Gα13 from membrane localization. The differences in membranous vs. cytoplasmic distribution for these mutants are interesting, as they suggest individual Cys residues play roles in tumorigenic signaling beyond simply regulating Gα13 association with the cell membrane.

Introduction

Heterotrimeric G proteins are a group of molecules that act in signaling pathways that convey external cues to the cell interior. G proteins are of great interest to the cell biological and medical fields because mutations in either G proteins or G protein coupled receptors GPCRs have been identified in >20% of human tumors (Arang & Gutkind, 2020). Gα12/13 are implicated in cancer due to their regulatory role in cell proliferation and ability to endow hallmark cancerous qualities to the cell (Rasheed et al. 2022). Recent studies show that Gα13 plays a distinct role in cancer cells developing tumor-initiating cell (TIC) properties, thus making these cells more challenging to treat with chemotherapy drugs or radiation (Rasheed et al. 2018).

Heterotrimeric G proteins are comprised of three separate protein subunits: Gα, Gβ and Gγ. GPCRs on the cell surface, once stimulated by signaling molecules such as hormones or neurotransmitters, bind to the G protein and activate it by causing the G protein to release GDP and bind the more abundant GTP. This causes a conformational change resulting in a loss of affinity between Gα and G$\beta\gamma$, allowing both of these structures to regulate other signaling pathways in the cell (Wedegaertner, 2012). Gα12/13 plays an important role in the serum response element (SRE) growth signaling pathway. SRE-regulated genes are linked to oncogenic activities such as cell growth, migration, invasion, and oncogenic transformation (Jaffe, 2002). Rho guanine nucleotide exchange factors (RhoGEFs) are one of several downstream effector proteins stimulated by Gα12/13. These RhoGEFs activate RhoA causing myocardin-related transcription factor (MRTF-A) to enter the nucleus and form a complex with serum response factor (SRF). Activated SRF in turn binds the serum response element (SRE), a regulatory sequence found in several growth-factor mediated promoter regions, and initiates transcription of the associated gene (Hill et al, 1995) (Figure 1).

Wildtype Gα proteins have the intrinsic ability to hydrolyze GTP back to GDP, thus turning themselves off. However, specific mutations can cause the Gα protein to become GTPase-deficient, thus doing away with its intrinsic “off switch,” rendering the protein constitutively active. For many G proteins, these mutations have been implicated as drivers of tumorigenesis. For Gα12 and Gα13, a glutamine to leucine (QL) mutation in the C terminus causes these proteins to become GTPase-deficient, however, this mutation is rarely observed in the Gα12/13 class in human tumors and does not appear to be the driver of tumorigenesis for the Gα12/13 Subfamily. Instead, cancerous signaling by these proteins appears mainly due to overexpression of either Gα12 or Gα13 in cells, absent of GTPase-disruptive mutations (Hasan et al., 2023). The unique ability of Gα12/13 to drive tumorigenesis without mutation makes them an especially

interesting and important subfamily of proteins to study, as its mechanism of enabling tumor progression is not well-understood.

While G proteins localize primarily to the inner surface of the cell membrane, their location is, in fact, quite dynamic and appears intimately tied to their ability to drive other signaling pathways, such as cell proliferation (Wedegaertner, 2012). A key mechanism controlling G protein localization is via the covalent attachment of a lipid, which in turn anchors the protein at the cytoplasmic surface of the cell membrane. For most Gα subunits, this lipid is a 16-carbon saturated fatty acid termed palmitate that is enzymatically attached to a cysteine(s) amino acid near the N terminus of the protein. This palmitoylation step appears to be required for membrane localization and signaling function of Gα subunits, including Gα12 and Gα13. Interestingly, over expressed Gα12 and Gα13 have been shown to not only cause tumerogenic signaling, but to deviate from their normal localization, appearing in both the membrane and the cytoplasm (Hasan et al, 2023). Prior studies on Gα12 and Gα13 have shown that palmitoylation for Gα12 occurs on a single cysteine residue, Cys-11, while for Gα13, two cysteine residues, Cys-14 and Cys-18, appear to be palmitoylated (Bhattacharyya & Wedegaertner, 2000). This lipid attachment occurs via a thioester bond; therefore, mutation of these cysteines to amino acids lacking thiol groups eliminates palmitoylation of Gα12/13 and disrupts its oncogenic function. However, the roles of the individual cysteines in Gα13 localization and signaling have not yet been determined.

Along with better understanding the palmitoylation of Gα13, identification of the molecular agents responsible for the attachment of these lipids is of great interest. A class of 20 palmitoyl acyltransferase enzymes with a conserved catalytic aspartate-histidine-histidine-cysteine motif, thus termed DHHC proteins, have been implicated in the palmitoylation of other protein types. However, this mechanism is not well understood for many classes of G proteins, including Gα12 and Gα13 (Wedegaertner, 2012). While it appears that the N terminal cysteine residues in Gα12 and Gα13 are the site of palmitate attachment, the surrounding amino acids may affect the ability for recognition and attachment of this lipid by either DHHC proteins, or some other agents. Previous work by Meigs lab members Bailey Cook and Sam Nance, show that introduction of a negative charge next to the N terminal cysteine of Gα12 completely disabled SRE signaling, while comparable negative charge introductions to Gα13 showed no effect on SRE. This research suggests that palmitoylation mechanisms for Gα12 and Gα13 may be divergent, and or occurring via different enzymes. Understanding the precise mechanisms of palmitoylation for Gα12 and Gα13 will in turn inform strategies for inhibiting palmitoylation of these specific proteins, and thus enable disruption of their oncogenic function.

For this study we aimed to investigate the role Cys-14 and Cys-18 on tumorigenic signaling and localization of Gα13, as well as the effect of negative charge introductions on Gα13 localization. To this end, we transfected human embryonic kidney (HEK) cells with Gα13 mutants that were engineered to harbor individual cysteine-to-alanine substitutions, as well as mutants harboring aspartic acid substitutions of the amino acids around these key cysteines. Ultracentrifugation techniques were employed to track these proteins in cytoplasmic and membrane fractions. Ability of Cys mutants to participate in cancerous growth signaling was assessed through serum response factor (SRF) measurements. Results from this

research should help to illuminate the palmitoylation mechanism of Gα13, as well inform how palmitate attachment, membrane localization and tumorigenic signaling are connected, and if this differs between G proteins.

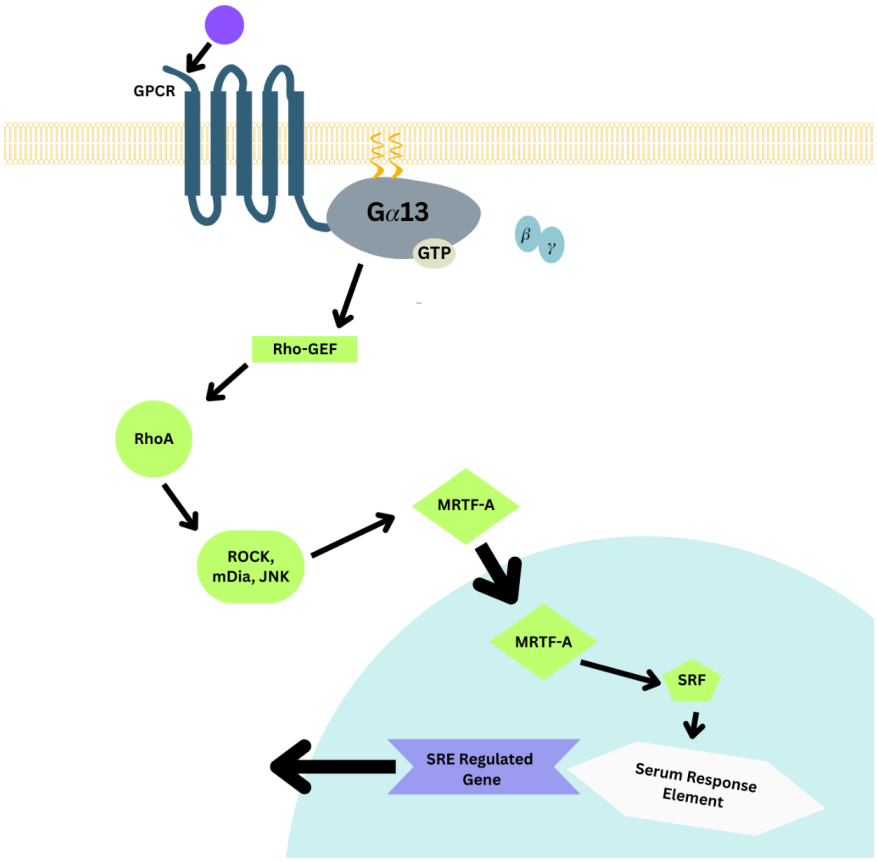

Figure 1. Serum response factor (SRF) signaling pathway. Following stimulation of the GPCR via an extracellular ligand (purple circle), Gα13 undergoes guanine nucleotide exchange and accepts GTP, resulting in the dissociation of Gα from βɣ. Remaining anchored to the cell membrane by the palmitoyl groups (orange), Gα13 stimulates RhoGEFs, which subsequently drive activation of RhoA. RhoA activates a series of downstream effectors, which results in MRTF-A entering the nucleus, binding to SRF, and forming a complex with SRE. At this time, the SRF regulated gene is promoted.

Materials and Methods

2.1. Creation of Mutants

Gα13 wt and Gα13 QL cDNA as well as C14/18A wt, C14/18A, S12D QL, S12D/G17D and S12D/P16D QL mutants were all previously engineered via polymerase chain reaction (PCR) mutagenesis. In order to create wildtype single Cys mutant plasmids, previously engineered C14A QL and C18A QL mutant plasmids were cross-spliced with a Gα13 wt plasmid provided by Forbes Fowler (Meigs lab). C14A QL and C18A QL

mutant plasmids were amplified via transformation of JM109 bacterial cells followed by Qiagen Mini-preps for plasmid isolation. C18A QL and Gα13 wt cDNA were then digested using HindIII and KpN1. Digests were visualized and isolated via agarose gel electrophoresis, and purified via Wizard SV columns with fragments then ligated together using T4 DNA ligase. A ligation lacking the PCR-generated insert was also set up and analyzed as a negative control. Transformations of JM109 cells using the ligations were then done followed by plasmid mini-preps using an affinity purification kit (Qiagen, Germantown, MD). The C14A QL cDNA was digested using HindIII and Kpn1, as well as HindIII alone. However, unexpected fragment sizes occurred causing it to be sent off for sequencing (Genewiz, NJ). Sequences showed an unintentional histidine to arginine mutation of the 107th amino acid as well as an extra HindIII and Kpn1 cut site in the untranslated region of the cDNA. To correct these mistakes the C14A mutant was made “code only” through PCR, using the C14A QL mutant cDNA and Ga13 forward and reverse oligos. PCR-amplified C14A QL was then visualized via agarose gel electrophoresis to confirm proper sequence length and PCR cleanup was performed using DNA affinity columns (Promega, Madison, WI). PCR-amplified C14A QL and pcDNA 3.1(+) were then digested using Nhe1 and KpN1. Digests were visualized and isolated via agarose gel electrophoresis, and purified via Wizard SV columns with fragments then ligated together. Transformations of JM109 cells using the ligations were then done followed by a Qiagen mini preps. The “code only” C14A QL cDNA (still containing the HP mutation) was then cross-spliced with Ga13 QL and Ga13 wt plasmids following the above procedure. Both cross-spliced single cysteine mutant wt plasmids, as well as the corrected C14A QL plasmid, were confirmed via sequencing.

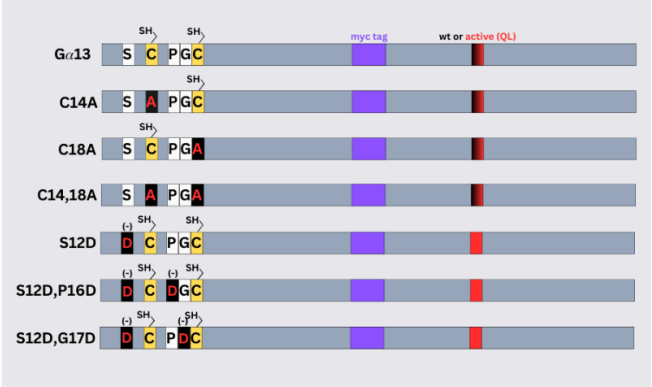

Figure 2. Schematic of Gα13 N terminal mutants. N-termini are at left, and distances are not drawn to scale. Gα13 is shown at top with native Cys residues for palmitate Attachment as well as the three surrounding amino acids. The next three constructs have Cys to Ala mutations of C14, C18, or C14,18 in order to investigate the effect of the loss of palmitoylation sites on SRE signaling and Gα13 localization. The last three constructs have Asp mutations of S12, G17 and P16 in order to investigate the effect on Gα13 localization of a negative charge next to the Cys residues for palmitate Attachment. Approximate position of the myc tag is shown in purple, and the key Gln-226 residue for mutational activation of Gα13 (QL) is black/red.

2.2. Mammalian Cell Culture and Transfection

Human Embryonic Kidney (HEK293) cells grown in Dulbecco's Modified Eagle's Media (DMEM) with 10% fetal bovine serum were split after reaching confluence then suspended in a 0.25% trypsin solution, pipetted into either 6 well (for Fractionation experiments) or 12 well (for SRE) plates, and left to incubate at 37° C until reaching ~80-90% confluence. For SRE experiments, wells were transfected with 200 ng SRE-luciferase plasmid, 20 ng of the internal standard plasmid pRL-TK (Renilla luciferase cDNA under control of a thymidine kinase promoter), and 200ng of plasmid encoding either Gα13 wt-myc, Gα13 QL-myc, C14A wt-myc, C14A QL-myc, C18A wt-myc, C18A QL-myc, C14/18A wt-myc, C14/18A QL-myc, or empty plasmid vector. Transfections were duplicated for each Gα13 encoding plasmid sample. For fractionation experiments, each well was transfected with 200ng of plasmid encoding Gα13 wt-myc, Gα13 QL-myc, C14A wt-myc, C14A QL-myc, C18A wt-myc, C18A QL-myc, C14/18A wt-myc, C14/18A, C14/18A QL, S12D QL, S12D/G17D QL, or S12D/P16D QL, or empty plasmid vector.

2.3. SRE Dual firefly luciferase assay

Approximately, forty-eight hours following transfection, cells were washed in 1 mL 1x PBS, and lysed with 250 µL passive lysis buffer while shaken for 20 minutes at 120 rpm. Cell lysates were centrifuged at 16,000 x g for 1 minute after which the top 40 µL was removed for later SDS PAGE and immunoblotting while the remaining supernatant was used to perform the SRE assay. A GloMax 20/20 luminometer (Promega) was employed for the dual luciferase assay system. Luminescence for each sample was measured first after catalyzing a luminescent reaction for the SRE-mediated firefly luciferase. And again immediately following the catalyzation of the Renilla luciferase partnered with the quenching firefly luciferase reaction to account for transfection efficiency. A ratio of the two luminescent measurements was measured to represent the strength of the SRE signal.

The 40 µL supernatant samples, removed for SDS PAGE and immunoblotting, were added to 20 µL of 4X/DTT and heated at 72°C for 10 min, after which they were frozen.

2.4. Negishi Fractionations

Approximately, forty-four hours following transfection, cells were washed in 2 mL cold PBS. Another 0.75 mL of cold PBS was added to each well and cells were scraped with a Costar 3010 scraper, moved to microcentrifuge tubes and centrifuged for 5 minutes at 500 x g 3°C. Microcentrifuge tubes were moved to icy water and the supernatant was decanted from all samples followed by addition of 100 µL of ice-cold Negishi homogenizing buffer to cell pellets. Samples were titrated then moved to ultra-microfuge tubes and submerged in liquid N 2 to. After fully thawing, 20-25 minutes in icy water, samples were spun for 15 min, 3°C, at 100,000 x g. After the ultracentrifuge run, tubes were carefully placed in a cold block on ice and 50 uL supernatant was removed and saved for SDS page and immunoblotting. Supernatant was removed down to the pellet after which 500 uL of cold Negishi Homog. Buffer was added to each pellet and titrated.

Samples were spun again for 15 min, 3°C, at 100,000 x g. After the ultracentrifuge run, tubes were carefully placed in a cold block and supernatant was removed down to the pellet. 75 uL of 1.33X w/DTT added to each pellet and titrated while 25 uL of 4X w/DTT was added to each of the 50 uL supernatant samples then flicked to mix. Supernatants and pellets were heated at 72°C for 10 min and then frozen.

2.4 SDS-Page and Immunoblotting

In order to analyze protein expression, and localization for fractionation samples, samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Frozen denatured protein samples from the SRE and Negishi Fractionation experiments were thawed and run on a 12% polyacrylamide gel at 135 volts. For Negishi Fractionation samples supernatant and pelleted membrane samples of the same transfected plasmid were loaded in adjacent wells, with an empty well between different plasmid samples. Furthermore, all Negishi Fractionation samples were loaded twice in order to create a duplicate gel for immunoblotting of the membrane resident protein E-cadherin (E-cad) and the cytoplasmic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Once separated, the samples were transferred from the polyacrylamide gel to nitrocellulose membrane for immunoblotting. Blots were probed anti-Gα13 (Millipore) polyclonal primary antibodies, followed by secondary anti-mouse, for Gα13 and GAPDH, or anti- rabbi, for E-cad, antibodies (Promega). Western blots were developed using 5-bromo-4- chloro-3-indolyl phosphate, nitroblue tetrazolium, and alkaline phosphatase before being imaged using a Kodak Gel Logic 100 system.

Results

3.1. SRE-mediated Growth Signaling in Gα13 wt requires both key N terminal Cys residues

Consistent with previous results, overexpressed Gα13 wt and Gα13 QL caused robust SRE signaling in human embryonic kidney (HEK293) cells, with Gα13 QL showing higher signaling than Gα13 wt (Figure 3). Also consistent with previous results, double cysteine mutants (C14/18A) of wt and QL disabled SRE signaling to comparable or lesser values than the control (plasmid only, no cDNA) sample. Mutation of either C14 or C18 to Ala in Gα13 wt completely disabled SRE signaling with renilla ratio less than in the control sample, and comparable signaling to that done by the C14/18A mutants for both wt and QL variants. C14A and to a greater degree C18A mutants for the QL variant showed higher SRE signals than for control and wt counterparts, despite C14/18A mutants of both wt and QL variants decreasing signaling to the same degree. Expression of Gα13 for all transfected SRE samples was confirmed through immunoblot analysis.

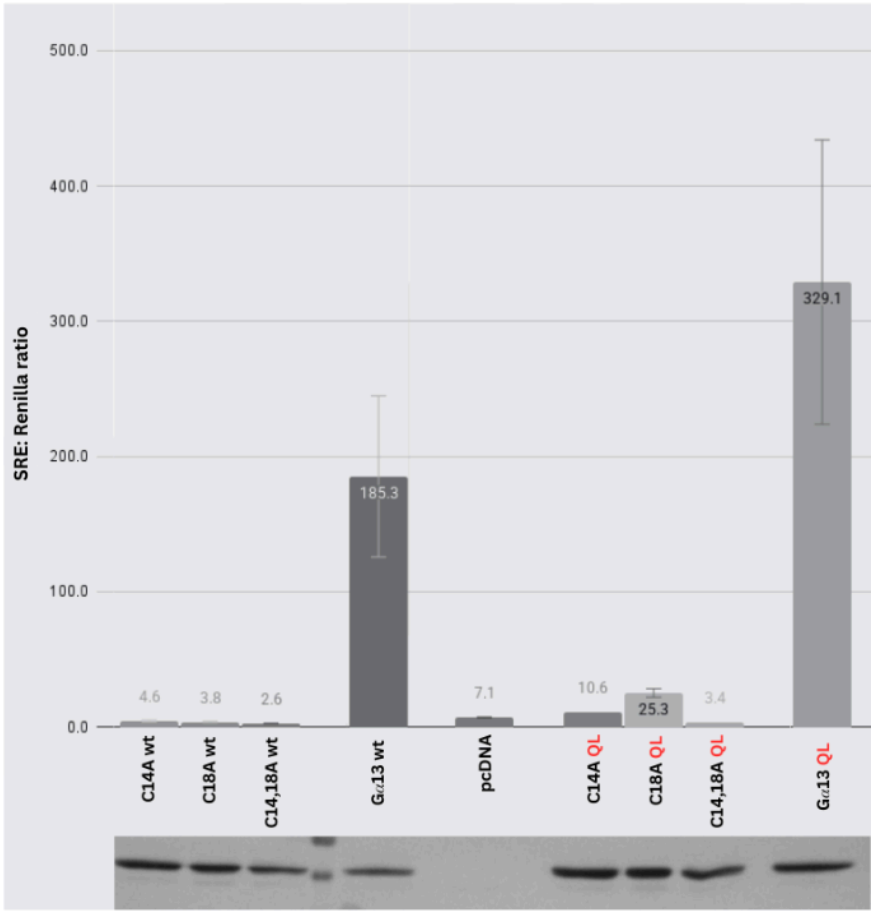

Figure 3. Ability of Gα13 N terminal Cys mutants to stimulate SRF. Gα13 wt and QL mutant plasmids (see Figure 1.). HEK293 cells were transfected with SRE-L and pRL-TK as described above, plus 200 ng of plasmid encoding Gα13 wt and QL N terminal Cys mutant plasmids (see figure 1.). Luminometry values (firefly luciferase activity divided by Renilla luciferase activity) are graphed for each Cys mutant. Constructs were examined in ≥4 trials, and mean ± s.e.m. is shown. Immunoblot analysis of whole cell lysates was used to verify expression of all wildtype and QL plasmids, and is shown under the graph with blots corresponding to labeled Gα13 constructs (immunoblot is from a representative experiment).

3.1 Localization of Gα13 is impacted by disturbances to Key N terminal cysteines and their surrounding amino acids

Consistent with previous results, though faint, immunoblotting of fractionation samples showed all endogenous Gα13 appearing in the membrane fractionation, confirmed by its smaller size, with the vector control (pcDNA) only showing endogenous Gα13 (Figure 4). Furthermore, the C14/18A mutant for both wt and QL variants appeared exclusively in the supernatant sample. Unfortunately, wt gels developed poorly, however, it can still be observed that for both wt single Cys to Ala mutants (C14A wt and C18A wt) some Gα13 appeared in the membrane fractionation along with some in the supernatant

(Figure 4A). No blots appeared for Gα13-myc wt in either the membrane or supernatant samples, we believe this is due to the poor development of the blot. For the QL single Cys mutants (C14A QL and C18A QL) Gα13 appeared in both membrane and supernatant samples (Figure 4B). Gα13 concentration was greater in the supernatant fractionations than in the membrane fractionations for both C14A QL and C18A QL. Gα13 C18A QL membrane concentration appeared greater than Gα13 C14A QL Gα13 membrane concentration. Consistent with previous results Gα13 QL appeared in both the supernatant and membrane fractionations, without a marked difference in concentration. For Asp QL mutants Gα13 appeared in both membrane and supernatant fractionations (Figure 4C). Concentration of Gα13 appeared comparable between S12D QL and S12D/G17D QL, with no marked difference marked in concentration between membrane and supernatant fractionations. S12D/P16D QL, however, showed a marked difference between membrane and supernatant fractionations, with a greater concentration of Gα13 in the supernatant. Immunoblots of GAPDH and E-cadherin confirmed fractionations contained only membrane or cytoplasmic proteins.

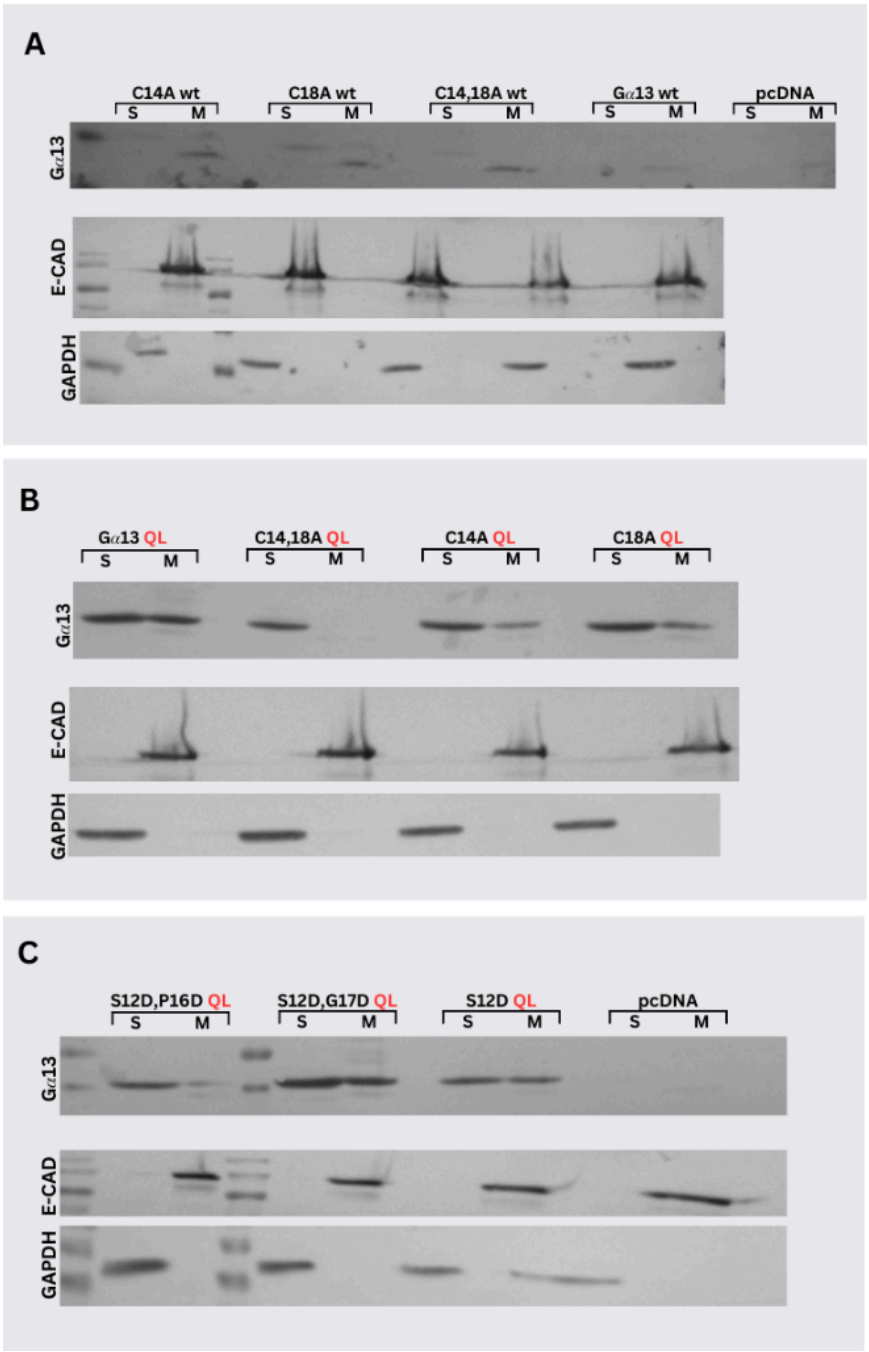

Figure 4. Intracellular localization of Gα13 wt Cys mutants (A), Gα13 QL Cys mutants(B) and Gα13 QL Asp mutants. Gα13 mutant plasmids (see Figure 1.), as well as a vector control (pcDNA3.1), were transfected and subsequently expressed in HEK293 cells and subjected to fractionation, as described in Materials and Methods. Immunoblots detected both endogenous Gα13 (though faint) and myc-tagged, Gα13 variants. Marker antibodies for cytoplasm (GAPDH) and membranes (E-cadherin) were used to confirm pure membrane and cytoplasm samples were obtained through fractionation.

Discussion

Both localization and SRE signaling were affected by mutation of either Cys-14 or Cys-18 to an Ala residue. Yet, SRE results for these single mutants differed between wt and QL variants, and between the Cys mutants. SRE signaling was completely disabled in both single Cys wt mutants, suggesting that both Cys residues are required for SRE signaling by Gα13wt. However, C14A and C18A QL produced SRE signaling above normal levels, though comparatively weak to signaling by non Cys mutants. These results are especially interesting as QL double cysteine mutants (C14/18A QL) consistently exhibit total loss of SRE signaling comparative to their wt counterparts. These results suggest that while normally SRE signaling appears impossible for Gα13 with loss of both or either Cys residue, if constitutively activated signaling can occur if one Cys is present. Furthermore, greater signaling was produced by the C18A QL mutant, suggesting that Cys-14 may play a more crucial role in signaling than C18. The nature, and greatly the degree, of this importance cannot be determined by these experiments, however, there are many possible reasons.

All Gα subunits are classified based on similarities in amino acid sequence, reflecting an evolutionary connection and a common protein ancestor. Early in vertebrate evolution the Gα12/13 subunits arose from gene duplication (Krishnan et al, 2015), as Gα12 has a single palmitoylated Cys residue, Cys-11, and Cys-14 in Gα13 is able to produce a stronger SRE signal when left as the sole key Cys residue in QL variants, the ancestral form of Gα12/13 may have had a single Cys residue that underwent palmitoylation, with the importance of the more N terminal Cys retained in Cys-14. Conversely, the ancestral form may have had two key Cys residues, with the more N terminal one being more important and thus solely retained in Gα12. Cys-14 may endow a stronger attachment to the membrane, help with recognition by DHHC proteins, or create a conformational change leading to better attachment of Cys-18, either to the membrane or allowing it to be better palmitoylated. A further possibility for the differences in SRE signaling between C14A and C18A is that Cys-14 and Cys-18 may be palmitoylated by different enzymes, with the enzyme palmitoylating Cys-18 either slightly less effective than the one palmitoylating Cys-14 or somehow influenced by either presence or previous palmitoylation of Cys-14.

Consistent with previous results, double Cys mutants of both wt and QL variants appeared exclusively in supernatant fractionations, supporting that palmitoylation of these key Cys residues is required for membrane localization and subsequent signaling function of Gα subunits. However, single Cys mutants of both wt and QL variants appeared in both membrane and supernatant fractionations, though concentration appeared greatest in supernatant fractionations.These results suggest that while single mutations of either Cys-14 or Cys-18 have an effect on membrane localization, they do not prohibit Gα13 from embedding in the membrane. This marked difference in localization between single and double Cys mutants, coupled with observed abolishment, or near abolishment, of SRE signaling for single and double Cys mutants, suggests the role of palmitoylation in SRE signaling may be more complex than simply allowing Gα13 to adhere to the membrane.

Localization results for the Asp mutants add further complexity to the connection between membrane localization, palmitoylation and SRE signaling of Gα13. Research

by previous lab members suggests that while SRE signaling for Gα12 is abolished with introduction of a negative charge next to its key N terminal Cys residue, SRE signaling by Gα13 is minimally affected for comparable negative charge introductions. In fact, Gα13 SRE signaling appeared slightly elevated for Ql mutants harboring either S7D, S9D or S12D mutations. While all Gα13-QL Asp mutants studied appeared in both membrane and supernatant fractionations, suggesting ability of mutants to adhere to the membrane, concentrations of Gα13 in membrane vs supernatant fractionations differed between the three mutants. S12D QL and S12D/G17D QL showed appearingly equal localization of Gα13 in the membrane and cytoplasm, comparable to non-mutant Gα13 wt and QL variant localization, suggesting presence in the cytoplasm to be due to overexpression and not decreased ability to adhere to the membrane. However, for the S12D/P16D QL mutant, a marked difference between membrane and supernatant fractionations was observed, with a greater concentration of Gα13 in the cytoplasm than the membrane. This suggests an impeded ability of the S12D/P16D QL mutant to adhere to the membrane, comparable to impediment caused by Cys to Ala mutations of Cys-14 or Cys18. Just like the observed difference in SRE signaling by C14A and C18A QL mutants, the nature and degree of this impediment will require further investigation. While the relationship between palmitoylation, membrane localization, and SRE signaling is still unclear, the results of this study suggest that SRE signaling is independent from membrane localization, and that Cys-14 and Cys-18 may play larger roles than just palmitate attachment points. Furthermore, these results suggest that SRE signaling and palmitoylation requirements are different for Gα13 and Gα12, thus perhaps palmitoylation occurs via different enzymes.

It is important to note that localization results are preliminary and unreplicated due to time constraints, equipment malfunctions, and unresolved blot development issues. Replication is required for any true conclusions to be formed. While results with a new Gα13 antibody for recent blots (Aspt mutants and QL variants) are much better, suggesting that wt blots with this new antibody should be promising, employment of a myc-antibody may be helpful if future issues are encountered. Future work should focus on further classification of the requirements for both membrane localization and SRE signaling in Gα13. While introduction of a negative charge appears to have no effect on signaling, yet seemingly at certain locations it affects localization, introductions of positive charge should be investigated. Furthermore, it would be interesting to investigate how introduction of a negative charge, paired with a key Cys mutation, may affect Gα13 signaling and localization, especially for Ql variants.

Acknowledgments

I would like to thank UNCA's undergraduate research program for funding and supporting this project. I would also like to thank my reviewers Dr. Greene and Dr.Clark-Hachtel. A great thanks is due to all the Meigs lab members, both past and present, for their research, especially the lab mates I have had the pleasure to work with. A huge shoutout goes out to Forbes Fowler for his gracious lending of Gα13-myc tag wt and QL plasmids, as well Maya Nakano and Gavin Perez for their help, support and wonderful company throughout my research. Finally, I want to thank Dr.Meigs for his amazing mentorship, hard work, knowledge, care, and genuine passion for science. Having the opportunity to do research, especially with such a great professor and lovely lab mates, has been the best, most rewarding experience of my time at UNCA.

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