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Effect of reverting switch II amino acids in Gα13 to their ancestral forms

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Abstract

Heterotrimeric guanine nucleotide binding proteins (G proteins), upon activation by cell surface G protein coupled receptors (GPCRs) via external stimuli, transmit signals to downstream pathways regulating a variety of cellular responses, such as cell proliferation and cytoskeletal rearrangements. The G12/13 subfamily of G proteins, consisting of Gα12 and Gα13, has been demonstrated to promote uncontrolled growth of cells and play a role in metastatic cancer progression. In diverse animal taxa from sea sponge to humans, the G12/13 subfamily of G proteins showed a highly conserved Q/Q amino acid pair at a precise structural position in the switch II region of G12/13 proteins, whereas mammalian Gα13 reverted back to E/K pair common to other G protein subfamilies. The reversion was studied by examining the binding of effector proteins to Gα13 containing a substitution for the Gα12 residues Q/Q at this position. The study shows that the preferential binding of TPR and E-cadherin to Gα12 may involve these specific residues.

1. Introduction

Guanine nucleotide binding proteins are intermediate proteins in cells that pass signals from the extracellular space to effector proteins in the downstream pathways. These heterotrimeric proteins are composed of α, β, and γ subunits, and when an external stimulus, such as a hormone and neurotransmitter, is bound to the transmembrane G-protein-coupled receptor (GPCR), it causes conformational changes in the α subunit. The α subunit undergoes nucleotide exchange from GDP to GTP, making the β and γ subunits detach, and the GTP-bound α subunit affects the downstream pathways[1].

Gα proteins are classified into Gs, Gi, Gq and G12/13. G12/13 is the only known family whose wild-type form can lead to aggressive cell transformation, such as cancer cells, upon over-expression[2]. G12/13 consists of Gα12 and Gα13; Gα13 proteins have 67% amino acid identity to Gα12[1]. The two subfamilies both play roles in signaling, embryonic development, and disease physiology, but in a discrete manner[3]. Gα13 seems to play an essential role in angiogenesis of mice embryos and migratory response, while Gα12 is not essential in angiogenesis and does not affect migratory responses[4]. In addition, while Gα12 and Gα13 have several common effector proteins, many effectors are known to be Gα12 or Gα13 specific[5].

All other classes of G proteins (Gs, Gi, and Gq) have specific residues of glutamic acid and lysine (E/K) at the Switch II region, but Gα12, from sea sponge to humans, has two glutamine residues (Q/Q) at that position. These residues that are distinct to the Gα12 are known as class-distinctive residues. In contrast, mammalian Gα13 has lost these amino acids and reverted back to the non-class-distinctive residues E/K. To study this reversion, our study had previously demonstrated that Gα12 double mutant Q232E/Q234K showed less than 20% of the serum response element (SRE) activation[3]. Also, this mutant was associated with severe impairment in binding heat-shock protein 90 (Hsp90)[3]. However, the E229Q/K231Q mutant of Gα13 did not show any impairment in serum response element (SRE) stimulation, which is a transcriptional pathway activated by G12/13 signaling[3]. To understand the significance of E229Q/K231Q mutation that occurred during the evolution of Gα13, this study focused on the ability of the Gα13 mutant to bind other Gα13 interacting proteins. A myc-tagged Gα13 with QL and EQ/KQ mutations

(myc- Gα13$^{QL,\ EQKQ}$) was constructed and interacted with several Gα13-binding proteins that have been known to interact exclusively with Gα13 or with both Gα12 and Gα13[5] (Figure 1). In this study, several proteins were shown to have higher affinity to Gα12 than Gα13, but not exclusively to either one of them.

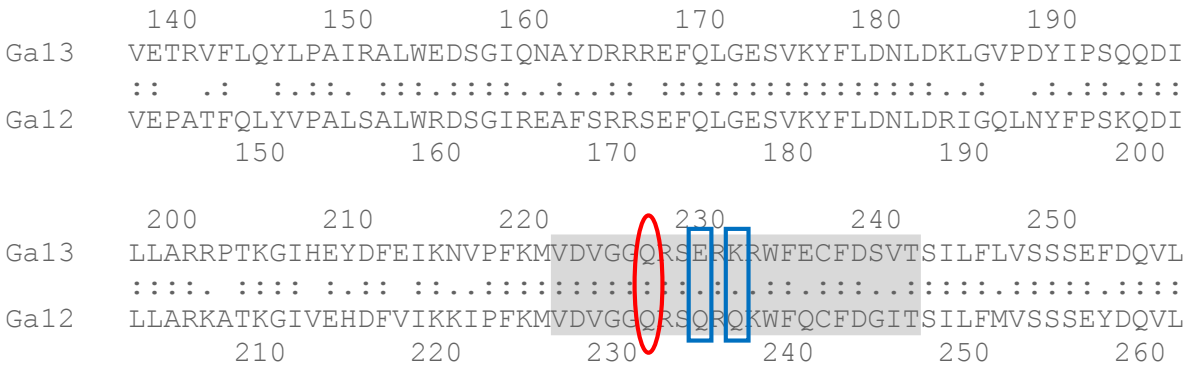

Figure 1. Comparison of non-class distinctive E229/K231 residues of Gα13 and class distinctive Q232/Q234 residues of Gα12. The blue boxes indicate the two mutations, EQ and KQ, and the red oval indicates the native glutamine (Q) that is mutated to leucine (L) to generate the activating mutation. The shaded area indicates the Switch II region.

2. Methods

A myc epitope tag (EQKLISEEDL residues) was added to all the Gα12/13 proteins under study to differentiate them from the native Gα12/13 proteins by size. These proteins also consisted of QL mutation, where the glutamine residue was substituted with the amino acid leucine, which made the proteins constitutively active by remaining in GTP-bound state. To construct a myc-tagged Gα13 with Q226L and E229Q/K231Q mutations (myc- Gα13$^{QL,\ EQKQ}$), cross-splicing was done between constructs that were previously made by other lab members. For this study, p115, radixin, leukemia-associated RhoGEF (LARG), tetratricopeptide repeat (TPR), epithelial cadherin (E-cadherin), PDZRhoGEF, HCLS1 associated protein X-1 (Hax-1), and regulator of G protein signaling 16 (RGS16) were tested for their binding to Gα13$^{QL,\ myc}$, Gα13$^{QL,\ myc,\ EQKQ}$, and Gα12$^{QL,\ myc}$. Several proteins (see section 2.2) were probed from cDNA and immobilized by sepharose bead for co-precipitation assays.

2.1 Engineering Myc-tagged Gα13$^{QL,\ EQKQ}$

Two different plasmids – Gα13 with Q226L mutation and myc-tag between amino acids 136 and 137, and Gα13 with Q226L and E229Q/K231Q double mutations – were used to construct the desired mutant. Both genes were in pcDNA 3.1 (+) plasmid (Invitrogen). The plasmids (3 µg) were digested with BglII, and then treated with CIP (calf intestinal alkaline phosphatase) to prevent self-ligation. DNA fragments of expected sizes (1760 bp and 6035 bp) were gel purified and ligated using T4 DNA ligase. The ligated plasmids were transformed into JM109 competent bacterial cells and selected on Luria-Bertani (LB) plates containing ampilicllin (100 µg/µL). Transformants were checked for directionality of the insert with NdeI and ScaI, and presence of the myc-tag and Q226L and E229Q/K231Q substitutions were verified by sequencing.

2.2 Hax-1 GST Fusion Protein Preparation

Prior studies determined that the Gα13-interacting regions were amino acid residues 176-247 on Hax-1[6], 1165-1307 on c-Jun NH2-terminal kinase-associated leucine zipper protein (JLP)[7], and 1-31 for RGS 16[8].

2.2.1 polymerase chain reaction (PCR)

Oligonucleotide primers were designed to amplify the Gα13 interacting region of Hax-1. The forward (fw) primer was designed to introduce a BamHI (GGATCC) restriction site upstream of the region. The reverse (rv) primer was introduced with a EcoRI (GAATTC) restriction site and a stop codon (TCA) downstream to terminate the translation. An arbitrary sequence of CGAA was inserted at the 5' end of each primer for stable binding of ribosome.

Fw: 5'-CGAAGGATCCGACCCCCATCCTAGAACCAGAG-3'

Rv: 5'-CGAAGAATTCTCAGCTATCTGCTTCGTGTCGGG-3'

Similarly, the primers designed to amplify Gα13-interacting region of JLP harbored BamHI and EcoRI restriction sites in fw and rv primers respectively. Both the primers were designed to also contain the arbitrary sequence.

Fw: 5'-GTGGGGATCCTCAGGTGTACCAGGAAATCGTC-3'

Rv: 5'-GTGGGAATTCTCACTCATTGCCATACATCAC-3'

The desired segments of the DNA were probed from the human cDNA library for Hax-1 and JLP. Due to complications in probing RGS16 from the cDNA library, pcDNA 3.1 (+) harboring the wild type human RGS16 was purchased from Missouri S&T cDNA Resource Center, transformed into JM109 bacterial cells, and the plasmid was purified. PCR was done using the engineered oligonucleotides, and the reaction tubes were divided into two each to test for better annealing temperature. Thermocycling conditions consisted of initial denaturation at 95 °C for 1 min, followed by 30 PCR cycles: denaturation at 94 °C for 45 s, annealing at 50 °C and 57 °C for 1 min, and then extension at 72 °C for 1.75 min. Finally, the reactions were run at 72 °C for 5 min for final extension and kept on hold at 5 °C until further analysis. The presence of desired fragments was verified by gel electrophoresis. To insert the DNA segments into PGEX-KG, they were ligated using T4 DNA ligase, transformed into JM109 competent bacterial cells, inoculated, and purified using miniprep. Diagnostic gels were performed, and the presence of the DNA was confirmed by sequencing.

2.2.2 glutathione-S-transferase (GST) fusion protein purification and immobilization

First, the PGEX-KG plasmids containing coding sequence for the Gα13 interacting proteins were transformed into BL21-Gold (DE3) *E. coli* and inoculated in liquid culture of LB broth containing 75 μg/ml of ampicillin. When the OD_{600} of the culture was measured to read between 0.5 and 0.8 using spectrophotometry, 0.5 mM of isopropyl-β-D-thiogalactopyranoside (Fisher Scientific) was added to trigger protein expression, and incubated with shaking. The cell pellets were collected by centrifuging at 6000 rpm and resuspended in cold GST Buffer containing 2.3 M sucrose, 50 mM Tris pH 7.7, 1 mM EDTA, and 1:500 dilution protease inhibitor (P.I.) mix. Then, a second cold GST Buffer containing 50 mM Tris pH 7.7, 10 mM KCl, 1 mM EDTA, 1mM dithiothreitol (DTT), and 1:500 dilution protease inhibitor (P.I.) mix was added to the resuspended cell. Lysozyme (4-5 mg) was used to lyse the bacterial cells, and 175 μL of 10% sodium deoxycholate, 260 μL of 1 M MgCl2, and 25 μL of 5 mg/ml DNAse I were added. When the liquid turned less viscous, the samples were centrifuged, and the supernatants were decanted into glutathione-Sepharose 4B that had been washed with T_{50}ED buffer (50 mM Tris pH 7.7, 1 mM EDTA, 1 mM DTT) 3 times. After inversion for interaction, the sepharose pellets were washed using T_{50}ED buffer containing 150 mM NaCl. The samples were snap-frozen and stored at -80 °C.

2.3 Co-precipitation Experiment

To test for interactions between Gα12/13 with different glutathione-sepharose-immobilized proteins, the GST-fusion proteins were diluted with $H_{50}E_1D_3M_{10}$ (50 mM Hepes, 1 mM EDTA, 3 mM DTT, 10 mM MgSO4) lysis buffer, and were added into human embryonic kidney cell (HEK293) lysates ($13^{QL\text{-}myc\text{-}EQKQ}$, $13^{QL\text{-}myc}$, $12^{QL\text{-}myc}$, or blank), which were also diluted with the lysis buffer. The reactions were inverted, centrifuged, and washed with HEDM buffer containing 0.05% polyoxyethylene-10-lauryl ether. The interactions were analyzed by SDS-PAGE, followed by western blotting using anti-Gα12 (Santa Cruz Biotechnology) and anti GNA 13 (EMD Millipore). The immunoblot results were quantified and the net Gaussian intensities of the bands were calculated using a Kodak Gel Logic 100 scanner and a Carestream Molecular Imaging software (New Haven, CT).

3. Results

3.1 Co-precipitation Assay

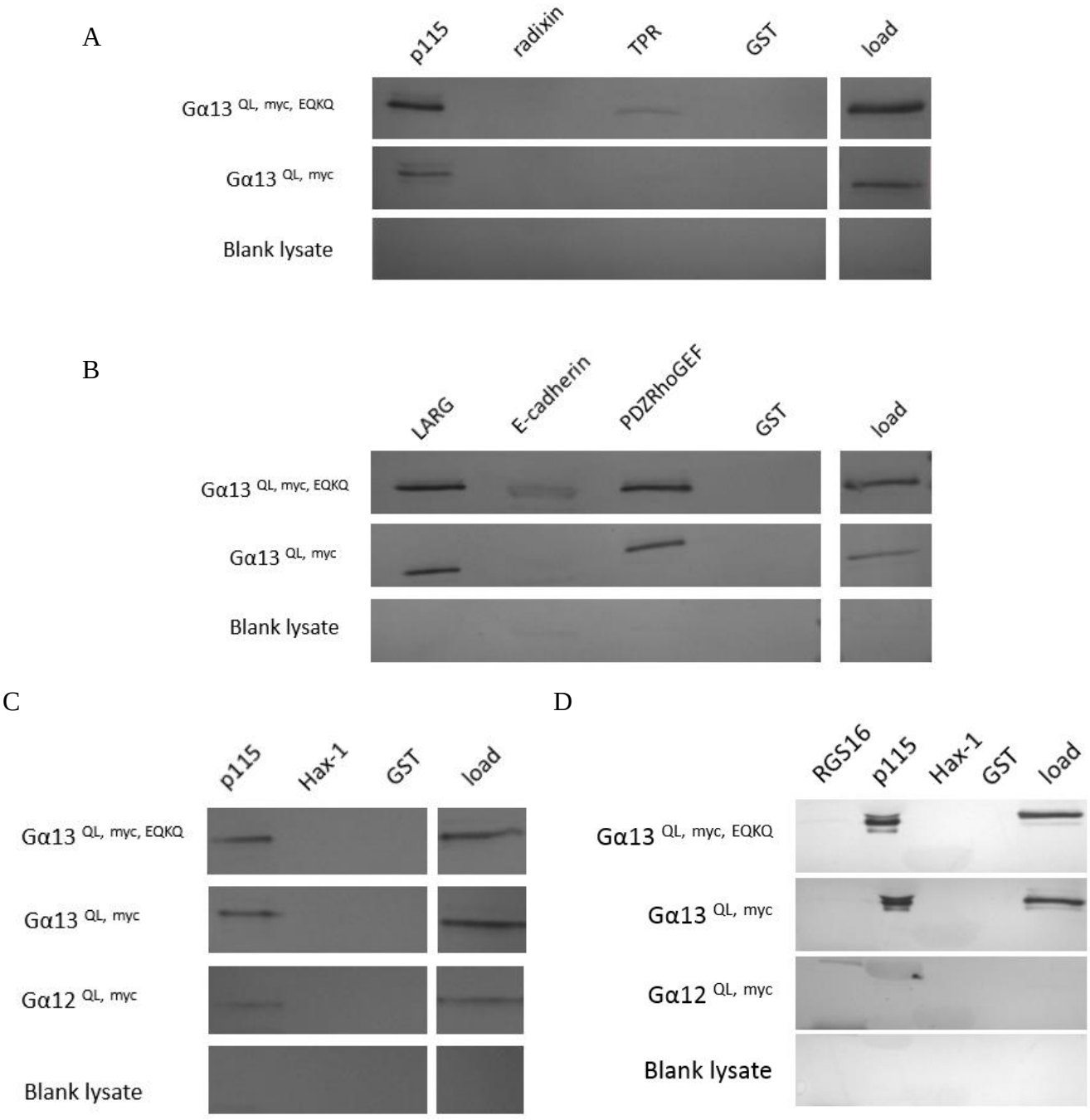

Figure 2. Immunoblots showing binding of $G\alpha13^{QL,\ myc,\ EQKQ}$ compared to $G\alpha13^{QL,\ myc}$ and $G\alpha12^{QL,\ myc}$ using several immobilized Gα12/13 effector proteins. The effector proteins were (A) p115-RhoGEF, radixin, and TPR; (B) LARG, E-cad, and PDZRhoGEF; (C, D) Hax-1 and RGS16. For each HEK293 lysate, a set percentage was analyzed separately as load. The amount of interaction was analyzed by the intensity of the bands. Each lysate was co-precipitated with GST alone to ensure that there was no binding. Blank lysates were used as negative controls to detect any irrelevant signals. For (C) and (D), p115 was used as a positive control to compare the affinity of Hax-1 and RGS16.

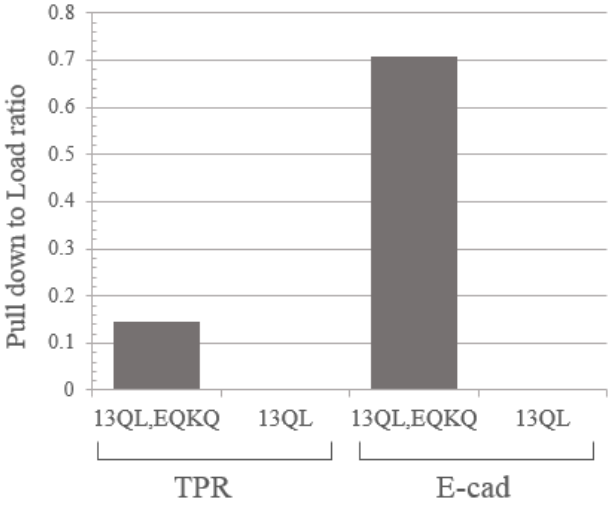

Figure 3. Pulldown-to-Load ratios of TPR and E-cadherin comparing their interaction with $G\alpha13^{QL,\ myc,\ EQKQ}$ and $G\alpha13^{QL,\ myc}$.

Interactions between the effector proteins and constitutively active Gα proteins, including the $G\alpha13^{QL,\ myc,\ EQKQ}$ mutant, were determined by co-precipitation assays. The results of immunoblotting are shown in figure 2. JLP was successfully probed and inserted into PGEX-KG plasmid, but it had not yet been immobilized by sepharose beads, so it was not used for co-precipitation assays.

The blots for the GST-fused p115, LARG, and PDZRhoGEF indicated strong bindings in both $G\alpha13^{QL,\ myc,\ EQKQ}$ and $G\alpha13^{QL,\ myc}$ (figure 2 A and B). At the same time, radixin showed no bands. The bands for TPR and E-cadherin showed interaction with $G\alpha13^{QL,\ myc,\ EQKQ}$ but the $G\alpha13^{QL,\ myc}$ did not show any detectable interaction with either TPR or E-cadherin (figure 2 A and figure 3).

In figure 2 C, Hax-1 was co-precipitated with different cell lysates, but there were no signals. Thus, the experiment was repeated again, along with RGS16 (figure 2 D). Hax-1 did not show any detectable bands again. A faint band was visible for interaction between RGS16 and $G\alpha13^{QL,\ myc,\ EQKQ}$ (figure 2 D), although it was not detected by the software. The $G\alpha12^{QL,\ myc}$ cell lysate that was used for the experiment was found to be malfunctioning, and thus, no relevant reactions were visible in the $G\alpha12^{QL,\ myc}$ blot.

4. Conclusion

Rho-specific GEFs (p115, LARG, and PDZRhoGEF) were previously reported to interact with both Gα12 and Gα13 when activated[5]. Thus, it is expected that the reversion of non-class distinctive residues E/K in Gα13 to ancestral residues Q/Q of Gα12 does not have significant effect on the binding to these RhoGEFs. Surprisingly, radixin, a protein known to interact with both Gα12 and Gα13, did not show any sign of interaction. For confirmation, radixin will be studied again, alongside with Gα12 as the positive control.

Results from this study demonstrate that TPR and E-cadherin bind slightly better with $G\alpha13^{QL,\ myc,\ EQKQ}$ than $G\alpha13^{QL,\ myc}$. Preliminary results from our lab showed that interactions between E-cadherin and Gα12 had stronger signals than with Gα13. If this holds true, since E-cadherin, and possibly TPR, has better interaction with Gα13 when the double mutation is present (figure 2 B), the ancestral Q/Q residues in Gα12 could play an important role in binding to these effector proteins. Further studies will be done to compare the binding of these effector proteins between Gα13 and Gα12. GFP tag will be added into $G\alpha13^{QL,\ EQKQ}$ for it to have an identical tag as GFP-tagged $G\alpha12^{QL}$ so that it allows the use of a common antibody to test for TRP and E-cadherin interaction.

Dhanasekaran et al., in 2004, have confirmed that Hax-1 interacted only with Gα13 of Gα subunit families[6]. Their study demonstrated that both wild-type and constitutively active (with QL mutation) Gα13 physically interacted with Hax-1. Moreover, the constitutive Gα13 enhanced the interaction with Hax-1[6]. Their research also demonstrated that the amino acid residues 176–247 of Hax-1 were sufficient to interact with Gα13. Nevertheless, our study showed a very mild interaction between the constitutively active Gα13 and Hax-1. In comparison with our

study, their experiment involved S-epitope-tagged Hax-1[6]. The S-epitope tag (KETAAAKFERQHMDS) has 15 residues compared to a myc-tag (EQKLISEEDL), a 10 residue peptide used for this study. The presence of a longer peptide could have led to stronger binding. Like Hax-1, RGS16 also showed a light interaction with their cell lysates, and did not show a notable difference between $G\alpha13^{QL,\ myc}$ and $G\alpha13^{QL,\ myc,\ EQKQ}$. However, when compared to the blank lysate, the bands for RGS16 seem to be irrelevant data. This could suggest that even though it was previously reported that amino acids 1-31 at N-terminus of RGS16 was necessary and sufficient to interact with Gα13[8], it may not be sufficient. Their studies used a His-tagged N-terminus of RGS16[8], which could have stabilized the short peptide to bind better with Gα13. Thus, addition of His-tag for this study might show different results.

To follow up with the results of this study, further research will be performed, including co-precipitation assays to assess the interaction for E-cadherin and TPR with Gα13 and Gα12. The experiment for Hax-1 and RGS16 interaction with Gα12 will be repeated using a more recently made functional myc-tagged $G\alpha12^{QL}$. Also, GST-fused JLP will be made and tested for its binding ability with Gα12, Gα13, and the mutant Gα13. Future research on interaction of $G\alpha13^{EQKQ}$ will help in understanding the purpose of this non-class distinctive reversion in Gα13.

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6. Literature Cited

1. Strathmann, M. P. and Simon, M. I. 1991 July 1. Gα12 and Gα13 subunits define a fourth class of G protein α subunits. *Proc Natl Acad Sci USA* 88 (13): 5582-5586.

2. Xu, N., Bradley, L., Ambdukar, I., and Gutkind, J. S. 1993 July 15. A mutant alpha subunit of G12 potentiates the eicosanoid pathway and is highly oncogenic in NIH 3T3 cells. *Proc Natl Acad Sci USA* 90 (14): 6741-6745.

3. Montgomery, E. R., Temple, B. R., Peters, K. A., Tolbert, C. E., Booker, B. K., Martin, J. W., Hamilton, T. P., Tagliatela, A. C., Smolski, W. C., Rogers, S. L., Jones, A. M., and Meigs, T. E. 2014 April. Gα12 Structural Determinants of Hsp90 Interaction Are Necessary for Serum Response Element-Mediated Transcriptional Activation. *Mol Pharmacol* 85 (4): 586–597.

4. Gu, J. L.**,** Muller, S., Mancino, V., Offermanns, S., and Simon, M. I. 2002. Interaction of Gα12 with Gα13 and Gαq signaling pathways. *Proc Natl Acad Sci USA* 99: 9352–9357.

5. Kelly, P., Casey, P. J., and Meigs, T. E. 2007 Jun 12. Biologic functions of the G12 subfamily of heterotrimeric g proteins: growth, migration, and metastasis. *Biochem* 46 (23): 6677-6687.

6. Radhika, V., Onesime, D., Ha, J. H., and Dhanasekaran, N. 2004. Gα13 stimulates cell migration through cortactin-interacting protein Hax-1. *J Biol Chem* 279 (47): 49406-49413.

7. Kashef, K., Radhakrishnan, R., Lee, C. M., Reddy, E. P., and Dhanasekaran, D. N. 2011 April. Neoplastic transformation induced by the gep oncogenes involves the scaffold protein JNK-interacting leucine zipper protein. *Neoplasia* 13 (4): 358-364.

8. Johnson, E. N., Seasholtz, T. M., Waheed, A. A., Kreutz, B., Suzuki, N., Kozasa, T., Jones T. L. Z., Brown J. H., and Druey, K. M. 2003. RGS16 inhibits signaling through the Gα13–Rho axis. *Nature cell biology* 5 (12): 1095-1103.