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Tyrosine Phosphorylation Dependent Mechanisms in the G12/13 Subfamily

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Abstract

Heterotrimeric guanine nucleotide binding proteins (G proteins) are signal transducers that have been found to have mechanistic roles either as downstream conduits of integral membrane protein receptors or regulators of various intracellular processes. In particular, the G12/13 subfamily has been implicated in a range of specialized cell growth signaling pathways. The members of this subfamily, Gα12 and Gα13, utilize novel mechanisms with regard to serine/threonine mediated phosphorylation, and recent work in our laboratory has revealed new aspects of this regulation within the G12/13 subfamily. However, regulation of these proteins by tyrosine phosphorylation has not been reported. A recent proteomic study by Chakravorty et al. (2018) identified a likely tyrosine phosphorylation event at position 44 in Gα13. To investigate this possible regulatory mechanism within Gα13, a point mutation was engineered to replace tyrosine at the 44th position with a phenylalanine, thus eliminating the hydroxyl group that allows phosphorylation with minimal disruption to the structural integrity of Gα13. At present, we are confirming this mutant through DNA sequencing. Future work with the successfully constructed Y44F mutant will focus on detecting signaling changes that occur due to this mutation. These experiments will include transcriptional activation assays to measure Gα13 signaling effects on cell growth, as well as protein interaction experiments to gauge any disruption of Gα13 binding to other proteins. Additionally, Gα12 and invertebrate G12/13 homologs will be analyzed in parallel to find if these proteins share a tyrosine-dependent regulatory mechanism with Gα13.

1. Introduction

Guanine nucleotide-binding proteins, otherwise known as G proteins, are a diverse family that are implicated as signal transducing molecules in a variety of cellular processes including oncogenesis and cell growth (1). This family of proteins take on the role of a switch within the cell causing signal transduction and creating signaling pathways that are aberrantly regulated in a range of diseases, defects, and multiple cancers. Along these pathways are G protein coupled receptors (GPCRs) that are involved in the pathophysiology of various diseases within the immune system and metabolic system; these systems are widely affected alongside infectious diseases and cancer (2). Many current areas of research focus on modulating GPCRs signaling pathway effects through clinically approved drugs; this brings the question of what inhibition does, not only to these protein families, but also to the surrounding cells.

Our laboratory has focused on these pathways through engineering mutations in the G12/13 subfamily proteins Gα12 and Gα13 to study the inactivation or overexpression in the transduction of these signals. Our laboratory has concentrated on engineering point mutations within G12/13 phosphorylation sites. Samantha Nance, a former researcher in our lab, focused on the phosphorylation of these G proteins by the protein kinases PKA and PKC. Through her studies, the importance of phosphorylation of a threonine in Gα12/13 was shown through the inhibition of PKA cell growth signaling after the phosphorylation site was mutated to alanine (Nance, 2020). Phosphorylation of target proteins has significant impact on cell growth, glycolysis, inhibition of proteins, protein degradation, regulation of GPCRs, and other essential mechanistic cell processes. When studying a protein, in this case Gα12/13, it is crucial to know everything that could happen along its signaling pathways as well as phosphorylation sites

regulating the function of that protein. Many proteins have specific phosphorylation sites that can be substituted to either mimic or inhibit phosphorylation, allowing detailed mechanistic studies of these specified functions. Through constructing these point mutations, further research can be accomplished on the regulation of a specific protein by kinases and phosphatases.

There are several heterotrimeric proteins that play such roles in a wide range of signaling pathways. The alpha subunits, which provide the guanine nucleotide binding function of the heterotrimer, are divided into four subfamilies: Gs, Gi, Gq, and G12 (3). G12 is classified as Gα12/13 proteins which share various serine, threonine, and tyrosine phosphorylation sites, as well as individual phosphorylation sites that make their functions distinct although they are over 67% identical (4). Rho guanine nucleotide exchange factors (RhoGEFs), activate Rho through triggering the latter protein to release GDP and bind to GTP. G12 proteins are implicated in the regulation of RhoGEFs; Rho has essential roles in cell migration, transformation, adhesion, apoptosis, cytoskeletal rearrangement, and growth, examples seen in Figure 1 (3,5). The N-terminal region in the G12/13 subfamily has been poorly studied, which is why this experiment of engineering mutants for the purpose of exploring this region was done. By discovering which amino acids are important in RhoGEF interaction, and which amino acids are targets of phosphorylation by tyrosine kinases this investigation can be done.

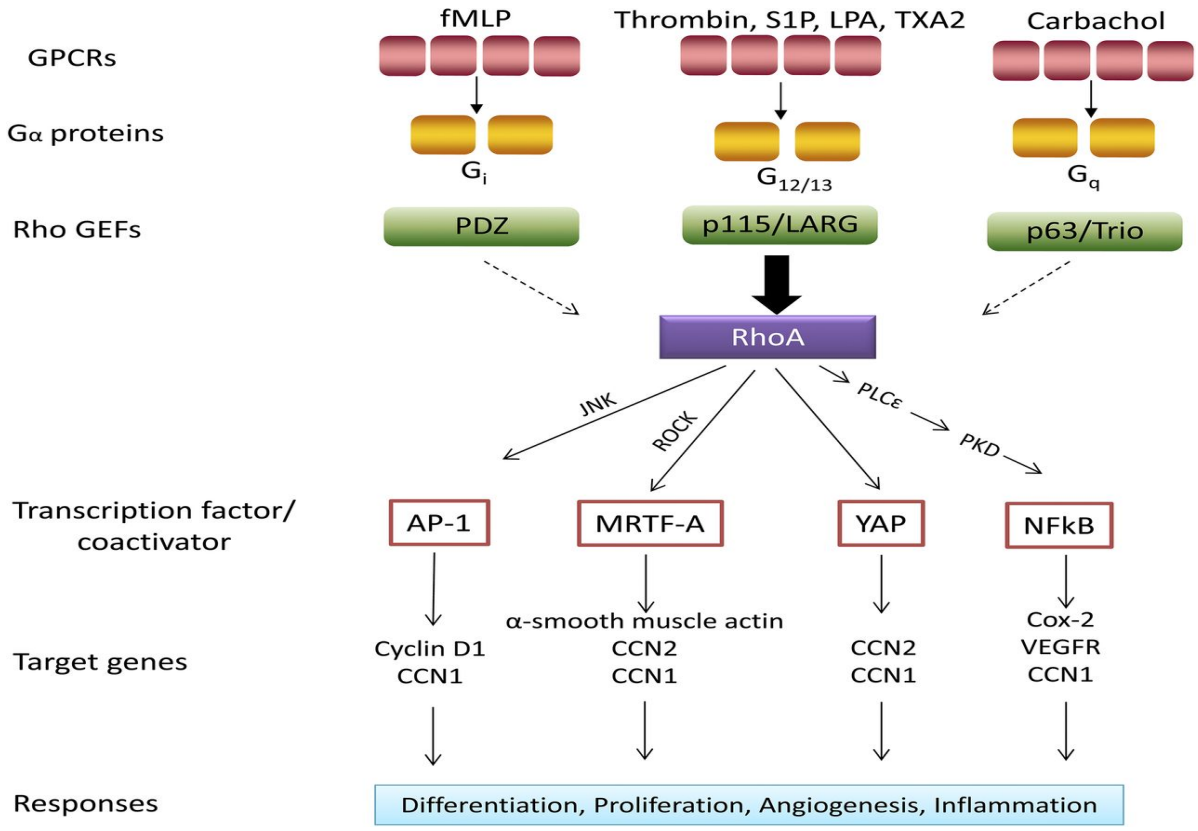

Figure 1. G alpha proteins regulate RhoA pathways that target genes resulting in various diseases (8)

Gα13 has been implicated in these misregulated processes that lead to cancer growth due to gene mutation or amplification. Gα13 harbors a predicted phosphorylation site at its 44th residue at position Y44 (6). Earlier research in relation to the G12/13 N terminus and its role in signaling to RhoA focused on the RhoGEFs engaged by Gα protein signaling pathways (Figure 1).

Protein tyrosine kinases are a class of enzymes that were discovered in 1979 to catalyze tyrosine phosphorylation (7). Presently there have been studies on tyrosine kinase inhibitors that block the function of the dysregulated tyrosine kinase and build a foundation for targeted cancer therapies (7). In the current study, the goal of this investigation was to reveal an unknown tyrosine phosphorylation mechanism and its effects on the signaling pathways of Gα13.

2. Methods

2.1 SDS Page and Immunoblotting

To analyze the expression of E33/31R within Gα12 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) was performed alongside of immunoblotting. For ten minutes the cell lysates were denatured at 72° in Laemmli buffer, 4x, containing 0.1M dithiothreitol. The samples were then run at 135 volts on a polyacrylamide gel which were then transferred to a nitrocellulose membrane for immunoblotting, and probed by anti-Gα12 (Genetex) polyclonal primary antibodies, and lastly anti-rabbit secondary antibodies (Promega) conjugated to the enzyme alkaline phosphatase. The blots were then developed using nitroblue tetrazolium and 5-bromo-4- chloro-3-indolyl phosphate to detect the presence of alkaline phosphatase at specific molecular weight positions.

2.2 Phosphosite Analysis

To find specific N-terminus phosphorylation sites within Gα13 the tool 'Phosphosite' was used. Through the informative website 'Phosphosite' all phosphorylation sites of G proteins can be observed. Through this site a comprehensive list of all Gα proteins with their tyrosine phosphorylation sites were recorded.

2.3 PCR-based Mutagenesis

The Y44F point mutation of Gα13 was engineered through PCR point mutation. The starting DNA fragments were synthesized by the use of a template Gα13 cDNA containing an activating mutant (QL variant) that abolishes the ability of the G proteins to hydrolyze GTP and return to an inactive state. The resulting overhang of 21 paired bases of these fragments gave a template for secondary PCR to take place. The secondary PCR gave the engineered gene accompanied by restriction sites upstream and downstream of the start and stop codons, respectively. The PCR product was then digested with the restriction enzymes KpnI and NheI, alongside an identical digest of the plasmid vector pcDNA3.1. To clean up the digested PCR product and vector, Promega Wizard SV column purification was performed using Membrane Binding solution to equal volume to PCR sample and placed into a column tube held in a collection tube that was centrifuged at 16,000g for 1 minute. Membrane Wash was then applied to the columns at 700uL and was centrifuged at 16,000g for 1 minute, followed by another Membrane Wash of 500uL centrifuged at 16,000g for 5 minutes. The collection tube was replaced by a microfuge tube and the column was centrifuged again for 3 minutes at 16,000g. Qiagen EB buffer, 50uL, was then pipetted into the column once the collecting microfuge tube was replaced with an autoclaved microfuge tube and centrifuged at 16,000g for 1 minute. Once the samples were purified, a diagnostic agarose gel was made to check for successful recovery of the PCR product and the plasmid. As a result, the plasmids were nandropped, and sent for sequencing (Genewiz, South Plainfield, NJ).

3. Results

3.1 E33/31R SDS Page and Immunoblotting

This study began with an investigation into the N-terminus region of G12/13 subfamily because this region is poorly understood in terms of protein crystallography . By examining E31/33R charge-substitute mutants our lab can add more into the puzzle of what effects it has not only on the N-terminus, but RhoGEF regulation. This study is an important step in analyzing this mutation for changes in the regulation of multiple types of RhoGEFs within G12/13 proteins due to the charged side chains, they hold in the N-terminus region. The RhoGEFs that were used in the Western blot were LARG, PDZ, AKAP, and p115. This was achieved through performing a pull-down experiment with these RhoGEFs and the addition of separated blots to compare E31/33 mutant, and 12 QLmyc.

Through a Coomassie Blue gel analysis it can be seen that, in this experiment, each RhoGEF (AKAP, PDZRG, P115, LARG) shows the expected protein levels at the conclusion of the pull-down procedure. As seen in Figure 3 the blank load, the control, did not show bands at the molecular weight of Ga12, indicating the bands seen in the transfected samples were likely showing the actual Ga12 variants. The mutated QL12myc showed pulldown from

each RhoGEF indicating regular binding while the GST only remained clear indicating accuracy. The E31/33R mutation shows binding to AKAP and p115, but lacks binding to PDZRG, and LARG, figure 3.

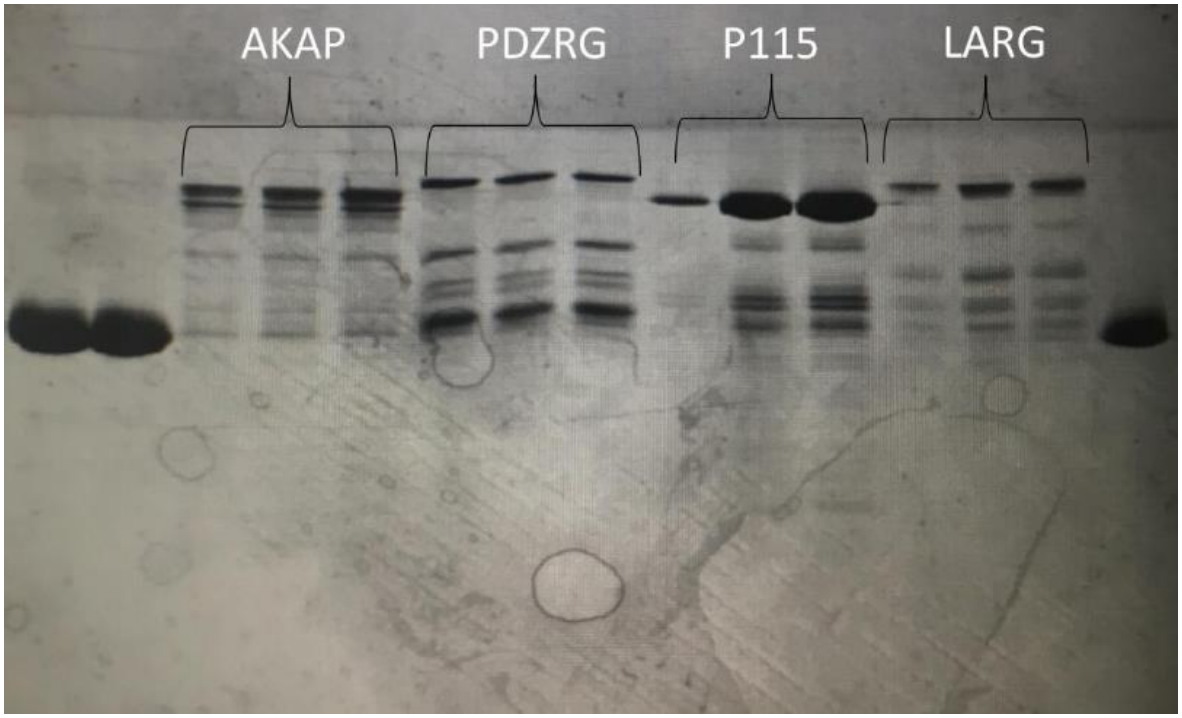

Figure 2. Coomassie Blue gel analysis with 12QLmyc pulldown of AKAP, PDZRG, P115, LARG

Blot	RhoGEF Distinction
Blank	LARG p115 PDZ AKAP GST only Ladder Blank Load
12 QLmyc	LARG p115 PDZ AKAP GST only Ladder 12QL myc Load
E31/33R	LARG p115 PDZ AKAP GST only Ladder E31/33R Load

Figure 3. Blank load indicating a control for the experiment, 12 QLmyc shows regular binding to all RhoGEFs tested along with an GST only well for control, E31/33R Load showing binding to p115 and AKAP but lacks binding to LARG and PDZRG. Each G12 band pull down to 44 kDa.

This study, which led to the phosphorylation mutation experiment presented, found that with the mutation of E31/33R RhoGEFs LARG and PDZ were not retained but binding to AKAP was not affected. With this result it was concluded that AKAP along with p114 form different RhoGEF which are not as well studied as RH-RhoGEFs. This conclusion, along with Samatha Nace's finding on phorylation, brought the study to investigate the N-terminus function with phorylation sites in the N-terminus of Gα12/13. To do this the site Phosphosite was used which produced an unexpected result of a tyrosine in the 44th position of Gα13.

3.2 Phosphosite Analysis

After the E31/33R mutation results, the next step was to research phosphorylation sites found in Gα proteins. Figure 6 exemplifies all known Gα proteins with tyrosine phosphorylation sites and where exactly those sites are found. One of these sites within Gα13 is Y44 confirming an N terminus phosphorylation site. It can also be noted that Gα12 does not share this phosphorylation site at the N terminus. Once this phorylation site within Gα13 was confirmed the PCR products were made with the mutation Y44F.

Gα Protein	Number of Tyrosine Phosphorylation Sites	Phosphorylation Sites
Gα11	3	Y103, Y291, Y365,
Gα12	1	Y121
Gα13	3	Y44, Y145, Y189
Gα14	2	Y147, Y156
Gα15	1	Y316
GαO	2	Y69, Y74
GαOLF	0	-
GαQ	3	Y128, Y291, Y356
GαS	3	Y311, Y339, Y360
GαZ	4	Y75, Y147, Y155, Y156
Giα1	1	Y155
Giα2	2	Y61, Y69
Giα3	3	Y61, Y69, Y167

Figure 4. A comprehensive compilation of all tyrosine phosphorylation sites found within Gα proteins

3.3 Creation of Y44F mutation in Gα13

Once Y44 was found in Gα13, the amino acid selected to replace it was phenylalanine, due to its similar aromatic structure that lacks the hydroxyl group required for phosphorylation. A plasmid map was constructed in order to depict the mutation in the Gα13 sequence, figure 4. The first PCR performed was with the forward oligo and the reverse end oligo that annealed to Gα13 plasmid past its stop codon. The second PCR included thet reverse oligo strand and the forward end oligo hat annealed to the Gα13 plasmid just before the start codon. With the two reactions performed there were two DNA fragments formed. One fragment encoded Gα13 up to the engineered Y44F mutation, while the other fragment encoded from the mutation to the C terminal end of Gα13. Resulting fragments were gel purified through the Promega Wizard column purification procedure. Once the samples were purified, the end oligos were used to create restriction sites within the DNA, with the NheI site positioned upstream of the Ga13 start codon and the KpnI site downstream of the Ga13 stop codon. The PCR Gα13-Y44-F product was digested alongside the plasmid

vector, pcDNA3.1, which resulted in the mutation being cloned into pcDNA3.1. After this digestion, gel-purification was performed on each DNA sample and liagations were set up. Then the ligations were transformed into JM109 bacteria and plated on LB-ampicillin plates. Here there was a result of four colonies growing on the edge of the Y44F plate and no colonies growing on the control plate made of a ligation without the Gα13-Y44-F molecule.

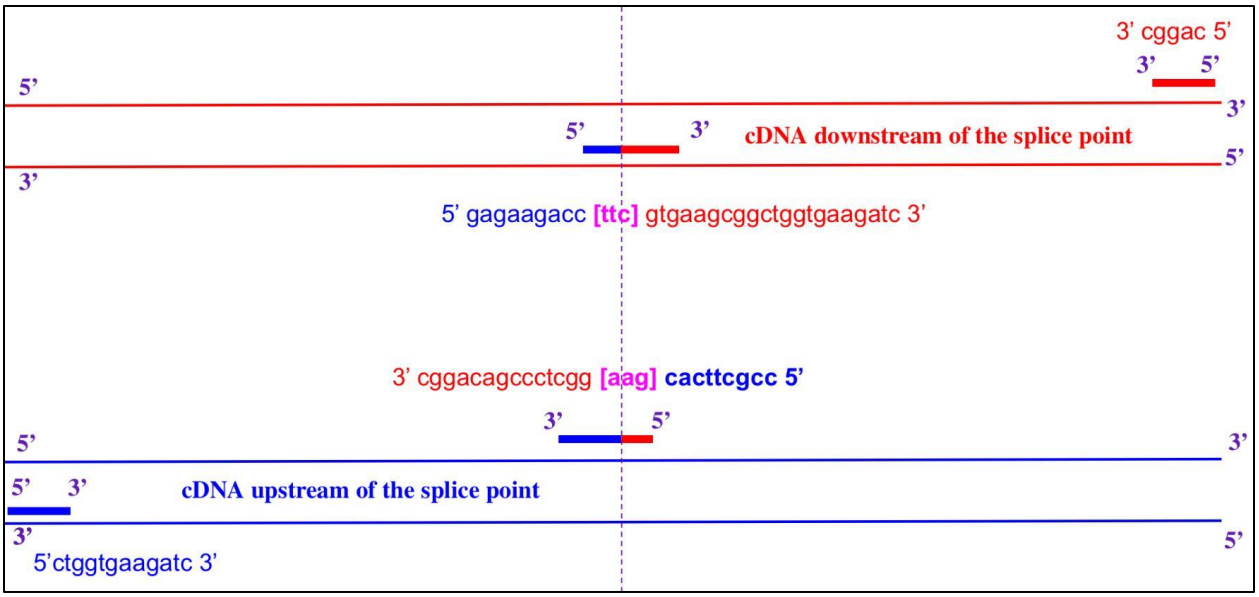

Figure 5. Each strand, thin red or blue lines, represent Gα13 cDNA. The top strand in red represents the PCR product encoding for the tyrosine to phenylalanine mutation denoted by pink codon [ttc].

The bottom strand has the reverse complement to the first strand. This figure shows the overlap between the forward oligosaccharide strand and the reverse complement to that mutated strand. This overlap gave the forward end oligo, denoted by the 5'-3' blue strand in the bottom left corner, and the reverse end oligo, denoted by the 3'-5' red strand top right corner. These oligos were used in the PCR that created restriction sites for the restriction enzymes to later cut through digestion of the Y44F mutation and vector plasmid.

Once the mutation was made, the samples were tested by Nanodrop UV spectroscopy to confirm the concentrations each mutation was expected to have. With the concentration of all samples measured and evaluated the mutants were sent off for sequencing. The reaction successfully produced the full coding sequence of Gα13 with the 44th tyrosine mutated into a phenylalanine.

4. Discussion and Conclusions

The heterotrimeric guanine nucleotide binding proteins Gα12 and Gα13 are involved in numerous pathways that, if engineered correctly, can be mutated for further advancing research. This mutation in regards to phosphorylation is what initiated the investigation of Y44F mutation in the N-terminal region of Gα13. Since phosphorylation is an essential process in the regulation of numerous disease-implicated proteins, it is likely that through studies of phosphorylation at specific sites within the protein family in question, modern day medicine can come closer to specific solutions. These solutions, just like inhibition of protein tyrosine kinases and implications in drug research, can be used to advance pharmaceuticals in their means of inhibition. The N-terminus of a G protein can be manipulated through genetic engineering to model structure and function of these proteins. Future experiments on this region can help to increase the possibilities of pharmacological intervention on disease.

This study identified the 44th position of Gα13 and its ability to be mutated through point mutation that led to the substitution of phenylalanine for the 44th tyrosine. In engineering this substitution, phosphorylation at this specific site was inhibited. Future steps of this finding will include examining this mutation against QL Gα13 for its ability to

interact with surrounding Gα13 binding proteins, as well as for its ability to affect growth signalling. For the future research to be done on this Y44F mutation, the mutant Gα13 protein will need to be expressed in cultured kidney cells, which will be lysed and used to prepare extracts containing the Y44F construct and its non-mutant control. Once this step has been completed, a pulldown will be done to examine whether QL Gα13 and the Y44F mutation have similar binding to several target proteins, including RhoGEFs. Here the goal is to look for the ability of this mutation to co-precipitate with, previously expressed in bacteria, proteins. Lastly, SREL will be used to study the Y-to-F Gα13 for pathways affected by this mutation in human kidney cells, including but not limited to the Serum Response Element pathway. Once these studies have been concluded there will be more data to report on the inactivation of the point mutation.

Other than Gα13, there are a range of G proteins that have potential tyrosine phosphorylation sites. Due to my previous research done on Phosphosite, we know that the following proteins have multiple Y phosphorylation specific sites: Gα11, Gα12, Gα14, Gα15, GαO, GαQ, GαS, GαZ, Gαi1, Gαi2, Gαi3. Knowing what we do about tyrosine phosphorylation and the inhibition of these specific sites involved in drug intervention we can continue the research to heterotrimeric guanine nucleotide binding proteins and the pathways they signal.

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6. References

1. Meigs TE, Fields TA, McKee DD, Casey PJ. Interaction of Galpha 12 and Galpha 13 with the cytoplasmic domain of cadherin provides a mechanism for beta -catenin release. Proc Natl Acad Sci U S A. 2001 Jan 16;98(2):519-24. doi: 10.1073/pnas.021350998. Epub 2001 Jan 2. PMID: 11136230; PMCID: PMC14619.
2. Heng, B.C., Aubel, D. and Fussenegger, M. (2013) An overview of the diverse roles of G-protein coupled receptors (GPCRs) in the pathophysiology of various human diseases. Biotechnol. Adv. 31, 1676–1694 https://doi.org/10.1016/j.biotechadv.2013.08.017
3. Neurosignals. 2009 Feb; 17(1): 55–70.Published online 2009 Feb 12. doi: 10.1159/000186690
4. Strathmann MP, Simon MI. G alpha 12 and G alpha 13 subunits define a fourth class of G protein alpha subunits. *Proceedings of the National Academy of Sciences*. 1991;88(13):5582-5586. doi:10.1073/pnas.88.13.5582.
5. Juneja J, Casey PJ. (2009) Role of G12 proteins in oncogenesis and metastasis. Br J Pharmacol 158:32–40
6. Yu OM, Brown JH. G Protein–Coupled Receptor and RhoA-Stimulated Transcriptional Responses: Links to Inflammation, Differentiation, and Cell Proliferation. *Molecular Pharmacology*. 2015;88(1):171-180. doi:10.1124/mol.115.097857.
7. David Chakravorty, Sarah M. Assmann; G protein subunit phosphorylation as a regulatory mechanism in heterotrimeric G protein signaling in mammals, yeast, and plants. Biochem J 15 November 2018; 475 (21): 3331–3357. doi: https://doi.org/10.1042/BCJ20160819
8. Yu J-S. From discovery of tyrosine phosphorylation to targeted cancer therapies: The 2018 Tang Prize in Biopharmaceutical Science. Biomedical Journal. 2019;42(2):80-83. doi:10.1016/j.bj.2019.03.004