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Ariella M. Iancu April 13, 2015

Adhesion G Protein-coupled Receptor G1 (GPR56) Regulation by Sorting Nexin 27

by

Ariella M. Iancu

Dr. Randy Hall

Adviser

Neuroscience and Behavioral Biology

Dr. Randy Hall

Adviser

Dr. Leah Roesch

Committee Member

Dr. Garry Bassell

Committee Member

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

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Adhesion G Protein-coupled Receptor G1 (G1), previously known as GPR56, plays a role in a wide variety of biological functions including cancer, immunology, and development. Mutations in G1 cause the brain developmental disorder Bilateral Fronto-Parietal Polymicrogyria (BFPP), and mutant forms of G1 associated with BFPP exhibit reduced cell surface expression and signaling. Sorting Nexin (SNX) family members typically modulate endosomal trafficking to the plasma membrane, and SNX27 has been recently identified as a potential modulator of G1 in a proteomic study. I showed that SNX27 interacts with both full-length G1 and N-terminally truncated G1 in HEK-293 cells. I also found that SNX27 increases total and surface expression of G1 and N-terminally truncated G1. Preliminary results indicate that SNX27 increases signaling of G1. Based on these results, SNX27 may be an important modulator of G1 expression and signaling *in vivo*.

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Introduction

G Protein-coupled Receptors

As the largest and most diverse group of membrane receptors, G protein-coupled receptors (GPCRs) are responsible for inter-cellular signaling mediated by many different types of molecules and also responsible for sensory signaling in the vision, olfaction, and taste systems. Characterized by their 7-transmembrane domain (7TM), GPCRs have seven alternating intracellular/extracellular α helical loops, flanked by an extracellular N-Terminus (NT) and an intracellular C-terminus (CT). GPCRs mediate cellular communication from the external environment to intracellular responses. External signals come in multitudes of forms including neurotransmitters, hormones, and photons which bind to and stabilize the active conformation of GPCRs. GPCRs transduce the external signal into a specific intracellular change by activating heterotrimeric G proteins that then modulate effector proteins [1].

Heteromeric G protein are comprised of three subunits, α, β, γ, each with different subtypes. There are over 35 subunit types, each with a specific localization pattern and function. The α subunit typically binds GDP in its inactive state. Ligands binding to GPCRs results in a shift in Gα conformation, and an accelerated hydrolysis of GDP into GTP. Ligand binding also leads to the disassociation of the G protein subunits from the GPCR. Disassociated subunits can modulate effector proteins, such as ion channels, phospholipases, and adenylate cyclase [2]. G-protein hydrolysis of GTP to GDP can be modulated by "regulator of G-protein signaling proteins" (RGS proteins) such as guanine exchange factors (GEFs), guanine nucleotide dissociation inhibitors (GDIs), and GTPase-activating proteins (GAPs) [3]. GPCRs can also be desensitized through multiple pathways including receptor phosphorylation, arrestin-mediated internalization, receptor recycling, and lysosomal and/or proteasomal degradation [1].

Split by sequence and structure, vertebrate GPCRs can be grouped into five families: Glutamate-like, Rhodopsin-like, Adhesion, Frizzled/taste2, and Secretin-like. Receptor families are distinguished by

sequence homology. While the classes vary widely in sequence, they do share the hydrophobic 7TM region, and disulfide-bridges at TMI/TMII and TMIII/TMIV [4]. With only 15 members, the Glutamate-like receptors are the smallest class of GPCRs and include metabotropic glutamate receptors, GABA receptors, single calcium-sensing receptors (CASR), and some taste receptors (TAS1) [4]. Despite the small size of this family, these receptors play important roles in the central and peripheral nervous systems, including powerful regulation of cognition and pain sensation.

The Rhodopsin-like class make up the largest class of GPCRs, with a wide array of functions but similar structures. The family is characterized by specific motifs in the 7TM region and ligand binding cavity within the 7TM (except for the glycoprotein binding receptors which bind ligands at the NT). Rhodopsin-like receptors are split further into families with many different clusters. Some examples of Rhodopsin-like receptors include muscarinic receptors, opsin receptors, cholecystokinin receptors, opioid receptors, angiotensin receptors, glycoprotein receptors, and olfactory receptors [4].

The Frizzled/taste2 class has two distinct clusters with structures and functions that vary widely between them, yet they share a few key motifs that characterize their class. Frizzled receptors signal through the Wnt pathway to control cell fate and proliferation. Including 10 family members, such as FZD1-10 and SMOH, frizzled receptors are highly divergent in sequence. Among their similarities, most are in HOX paralogy group chromosomal regions. TAS2 receptors, such as TAS2R1 and GPR59, have seven hydrophobic regions with relatively small NTs and are important for taste. TAS2 receptors have a very short NT and are localized to the tongue and palate epithelium where they are responsible for bitter taste perception [4].

Secretin-like receptors share NTs with conserved cysteine bridges that are important for the binding of their large peptide ligands. This family includes receptors such as the calcitonin receptor, the

corticotropin-releasing hormone receptor, the glucagon receptors, and the parathyroid hormone receptors. Secretin receptors typically signal in a paracrine fashion [4].

Previously grouped under secretin-like receptors, adhesion receptors are characterized by their long NTs with adhesive properties. Adhesion receptors include family members such as lectomedin receptors (LECs), Brain-specific angiogenesis-inhibitory receptors (BAIs), CD97 antigen receptor (CD97), and the EGF-like module containing receptors (EMRs) [4].

Adhesion GPCRs

Adhesion GPCRS are a subclass of GPCRs that are highly conserved across vertebrates and invertebrates. In fact, there are 33 members in humans, which can be separated into nine families, [5] and approximately 100 members in sea urchins [6]). Adhesion GPCRs have a wide array of functions including roles in development, tissue polarity, the immune system, and tumorigenesis [7]. Adhesion GPCRs are characterized by their distinct long NT which can range from 250 -2500 amino acids in length, and that allow for cell-cell and cell-matrix interactions. The long NT contains a range of adhesive domains, including EGF-like repeats, mucin-like regions, and cysteine-rich motifs [4].

Most adhesion GPCR are considered orphan receptors, in that no endogenous ligands have been identified. Of the known adhesion GPCR ligands, many are cellular or matrix-associated. In fact known ligands for G1 include the extracellular matrix component, collagen III, while CD97 and EMR2 bind the B cell surface molecule, chondroitin sulfate B, and BAI1 interacts with Gram-negative cell wall molecule, lipopolysaccharide. Langenhan *et al.* makes the case that adhesion GPCRs are “context recognizers” in that their main role is to recognize other cells and extracellular matrix structures. This function of binding to other cell surfaces is necessary for tissue orientation, migration, position, and immune response [8].

Post-translational modifications of adhesion GPCRs include glycosylation and auto-proteolysis. Due to the high level of Ser/Thr residues in the NT, adhesion GPCRs undergo O- or N-glycosylation in the ER and Golgi. The NT typically also has a GPCR proteolytic site (GPS motif), a cysteine-rich motif, which is clipped during receptor processing in an autocatalytic mechanism [5]. The auto-proteolysis occurs at the highly-conserved GPS proteolytic site. The clipped NT remains non-covalently associated to the 7TM region [9]. The GPS motif is part of a larger domain, called the GPCR auto-proteolysis-inducing (GAIN) domain, and mutations in either can lead to human disease [10]. Interestingly auto-proteolysis might occur in parallel to, or soon after, glycosylation in the ER [9]. In fact, N-glycosylation might regulate the auto-proteolysis of adhesion GPCRs. In the ER, N-glycosylation could be stabilizing the protein structure to allow for auto-cleavage [11].

Adhesion GPCR auto-proteolysis might act as an endogenous ligand. In fact, N-Terminal deletions have been shown to activate receptor signaling in B2, G1, and CD97, suggesting that the NT can act as a signal suppressor [12]. Once cleaved, the NT typically remains non-covalently associated with the 7-transmembrane region to inhibit signaling. Antibodies [13] and toxins [14], which interact with the NT have similarly been shown to stimulate receptor signaling, suggesting that perhaps the binding of these molecules to the NT either changes its conformation and/or strips it from the 7-transmembrane region, activating receptor-mediated signaling [12].

Adhesion GPCRs 7 transmembrane region couple to G proteins to transduce signals through effector proteins. Adhesion GPCRs have been shown to interact with, and signal through, G-proteins such as $G\alpha_{q/11}$, $G\alpha_{12/13,}$, $G\alpha_{s}$, $G\alpha_{o}$. Another alternative signaling pathway for Adhesion GPCRs is to couple non-heterotrimeric G proteins with guanosine triphosphatase (GTPase) activity. The interaction between non-heterotrimeric G proteins and GTPase activity can be modulated through RGS proteins. Activity through small GTPases, Rho GTPase-activating proteins, and kinases can also modulate Adhesion GPCR pathways [8].

Adhesion G Protein-coupled Receptor ADGR-G1 (G1)

Adhesion G Protein-coupled Receptor G1 is an Adhesion GPCR, previously known as GPR56 and renamed ADGR-G1 (G1) under the recent nomenclature proposal [15]. G1 is widely distributed throughout the body and has a diverse set of functions. G1 is located on Chromosome 16q12.2-q21 and is 693 amino acids in length, but has at least 2 alternative splice variants [15]. G1 contains a long NT with a Serine-Threonine-Proline (STP) region, and a cysteine-rich GPS motif that allows for autocatalytic cleavage [9], [16]. N-terminus deletion results in constitutively active signaling [17]. The NT has seven N-glycosylation sites. Glycosylation at these sites are necessary for surface localization [18]. G1 is known to play a role in many biological systems specifically cancer, immunity, and development [7].

Biological Role of Adhesion G Protein-Coupled Receptor G1 (G1)

Cancer

G1 has been shown to suppress cancer growth and metastasis, while reduced expression led to increased metastasis. [19]. Xu *et al* suggests that G1 regulates the last rate limiting steps of metastasis, namely, survival in new organ, in which G1 blocks tumor growth in vivo by interacting with tissue transglutaminase (TG2). TG2 is a crosslinking enzyme found in the extracellular matrix (ECM) that binds G1 in a calcium-dependent manner. Stabilization of TG2 by binding G1 leads to the increase of ECM factors, such as fibronectins and integrins, perhaps enhancing cell adhesion as well as promoting tumor suppressor TGF-β [15]. However, contradictory reports have shown that G1 mRNA may be increased in

gliomas, colon, pancreas, and lung cancers [20] . This perhaps suggests that G1 has different effects on tumorigenesis depending on the cancer type.

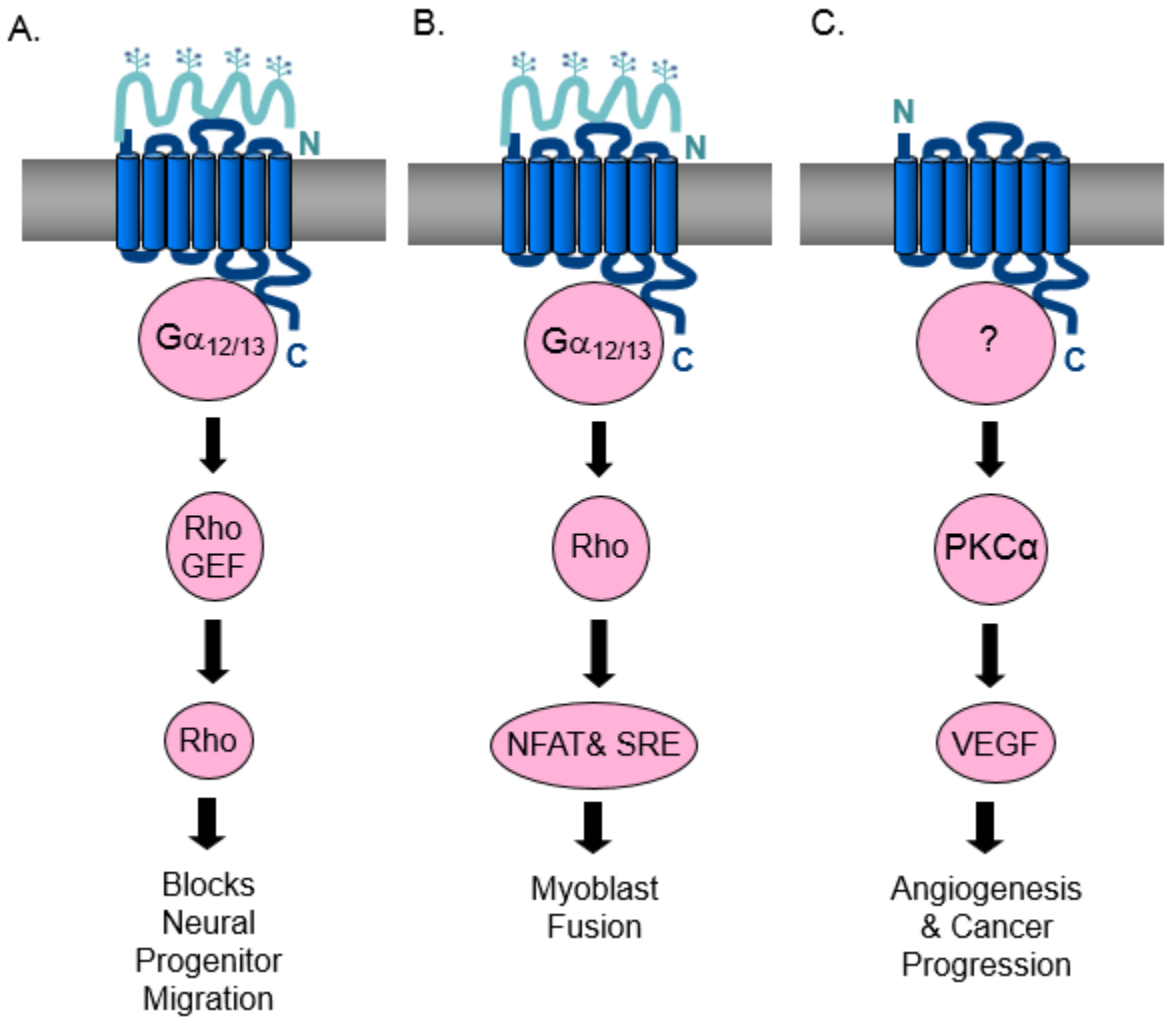


FIGURE 1 SCHEMATIC DIAGRAM OF G1 SIGNALING PATHWAYS. *G1 signals through Gα12/13 to activate RhoGEF and Rho, which block neural progenitor migration (adapted from* [18]*). B. G1 signals through Gα12/13 to activate NFAT and SRE, helping in myoblast differentiation in fusion (adapted from* [21]*). Removal of the NT from G1 leads to increased VEGF expression via PKCα, leading to angiogenesis (adapted from* [22]*.*

G1 has also been shown to inhibit angiogenesis, blood vessel formation, in melanomas by blocking VEGF [16]. Interestingly, the receptor stripped of its NT actually increases VEGF production, angiogenesis, and melanoma progression. Similar effects were seen when the Serine-Threonine-Proline (STP) region of the NT of G1, where TG2 binds, was removed(Yang & Xu, 2012), as seen in Figure 1C. A

proposed mechanism has suggested that the full length G1 binds to TG2 at the STP region, then internalizes and degrades TG2, effectively blocking TG2 tumorigenesis function [23].

Natural Killer Cells and T lymphocytes

Cytotoxic lymphocytes, natural killer (NK) cells are a part of the innate immune system. NK cells are the early defense system against foreign particles, including cancers. NK cells migrate to inflamed areas where they then attach to the target cell. G1 is specifically expressed in $CD56^{dull}CD16^{+}$ NT cells in inflamed tissues and blood stream, though not lymph node localized NK cells. When NK cells are treated with NK activation factor IL-18, which activates NK migration, G1 expression decreased. Due to similar G1 functions in other cells, it is possible that G1 plays a role in NK migration [25]. G1 has also been shown similar increased expression in virus-specific $CD8^{+}$ T lymphocyte cells. NK cell migration, both spontaneous and induced, was inhibited by G1 ectopic expression [24].

Muscle Development-Myoblast Fusion

Myoblast fusion is a necessary step of muscle development, and G1 has been shown to play a role in this process. Muscle progenitor cells originating in the somites migrate into the limb bud where they proliferate and differentiate into skeletal muscle. Maturation and growth of progenitor cells occurs by fusion of myogenic cells [25]. G1 has been shown to be expressed during early differentiation and fusion of myoblasts, localizing to the same regions of tetraspanins CD81 and CD9, both myoblast fusion promotors. CD81 facilitates G1 signaling through the $G\alpha_{q/11}$ pathway [26] $G\alpha_{q/11}$ activates phospholipase C to hydrolyze phosphatidylinositol 4,5-biphosphate into secondary messengers diacyl glycerol (DAG) and inositol triphosphate (IP_3). DAG activates Protein Kinase C, while IP_3 goes on to open calcium channels [2]. In knockouts mice lacking G1, myoblasts do not fuse as well and has decreased signaling in the Nuclear factor of activated T cells (NFAT) and serum response element (SRE) signaling pathway [21], as seen in Figure 1b.

Progenitor Migration and Bilateral Fronto-Parietal Polymicrogyria

When neural stem cells differentiate into neural progenitor cells, they begin to migrate to their final placement as mature, fully differentiated neurons. In the cortex, progenitor cells migrate along radial glial cells from the ventral zone to the pial surface basal membrane [29]. Abnormalities in cell placement during development can lead to neurodevelopmental disorders, such as Bilateral Fronto-Parietal Polymicrogyria (BFPP)—a disease categorized by disordered cell connectivity in the cortical Fronto-Parietal area. BFPP patients may have seizures, mental retardation, ataxia, and language impairments. BFPP is associated with mutations in a single gene coding for G1 [27].

BFPP mutations are homozygous and present in the germline. There are 13 distinct BFPP causing mutations for G1, including one deletion, two splicing mutations, and 10 missense mutations. Of the missense mutations, all are found on the extracellular surface, either in the NT (6), the GPS motif (2) or on an extracellular loop (2) [27].

G1 is expressed in neuronal progenitor cells, specifically radial glia and pial basal membrane cells. The radial glia extend from the somas in the ventricular zone to the pial basal membrane of the cortex, where G1 help to guide progenitor cells and lock them in place, and maintain the connection between the pial basal membrane and radial glial endfeet [28]. It is likely that G1 is involved in the adhesion between the two cell types by binding a ligand in the extracellular matrix of the pial basal membrane [29] Typically newer neurons migrate past older neurons to form layers of the cortex. BFPP brains have a classic cobblestone-like cortical malformation: abnormally abundant, yet small and fused gyri in the cerebral cortex. Cobblestone lissencephaly is due to abnormal migration of cortical neurons. BFPP neurons break past the basal lamina to form ectopic bumps on the surface of the brain to form the cobblestone-like phenotype [28].

A possible ligand for G1 in the interaction between the radial glial cells and the extracellular matrix of the pial basal membrane is TG2. As described previously, TG2 is a binding partner of G1 that is

localized to the extracellular membrane and has been shown to express in mice at P0 in the forebrain [30]. Collagen III has been identified as a G1 ligand in the brain. Fibrillar collagen type III is a component of the extracellular matrix and is expressed in the pial basal membrane [31], [32].

G1 regulates neural progenitor cell migration by inducing the $G\alpha_{12/13}$ pathway to activate RhoA and RhoGEF. Active RhoA alters the actin cytoskeleton to inhibit neural progenitor cell migration and to change cell morphology, as Figure 1b shows. RhoA activation leads to F-actin accumulation and reorganization [13]).

In G1 and collagen III knockout mice, there is an over migration of neural progenitor cells, showing the importance of their interaction for proper neural progenitor cell migration [32]. BFPP causing mutations in the NT preventing Collagen III to bind G1, leading to incorrect migration [33]. Some BFPP-causing mutations to the N terminal region of G1 inhibit its ability to cleave itself [27]. These impaired cleavage mutants are drastically reduced at the cell surface. This may be due to their retention in the ER/Golgi network and/or a lack of recycling to the plasma membrane following endocytosis [18].

G1 Endosomal Trafficking and SNX27

Cell surface proteins, such as G1, are often internalized into the endocytic pathway to be sorted into post- Golgi structures to then be returned to the cell surface, sent to the trans-Golgi network (TGN), or sorted into the late endosome to be sent to the lysosome [34], represented in Figure 2. The trafficking of cell surface receptors to the endosome and to the plasma membrane is typically controlled by receptor-associated scaffold proteins, such as Sorting Nexin Family (SNX) members, which are characterized by their ability to bind Phosphatidylinositol phospholipids via a Phox homology (PX) domain [35].

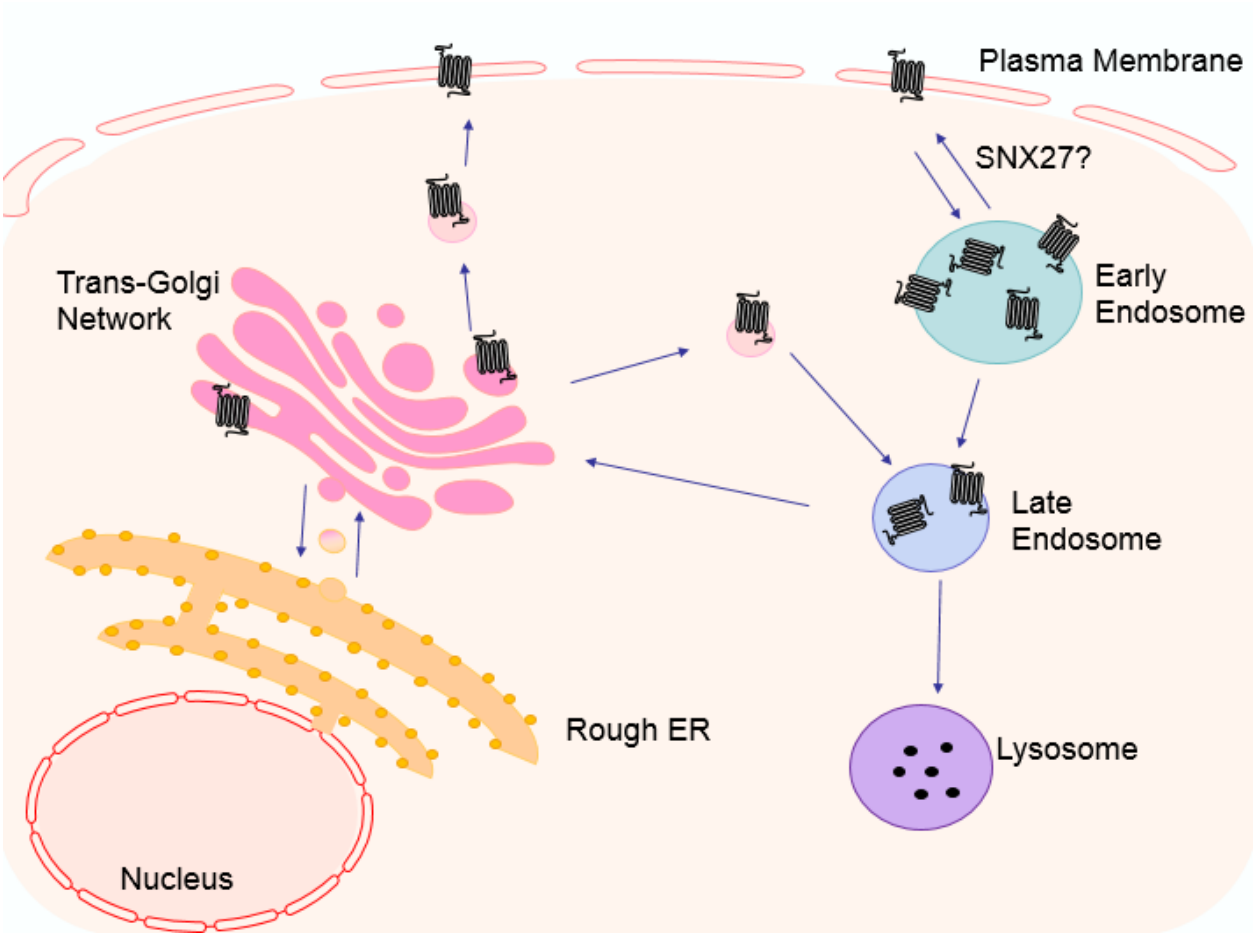


FIGURE 2 ENDOSOMAL SURFACE PROTEIN TRAFFICKING

Internalized surface proteins are sorted into the early endosome and either sent back to the plasma membrane or sent to the late endosome. From the late endosome they can be sent to the lysosome for degradation or to the Trans Golgi Network for modification.

SNX27 is localized to the early endosome due to interaction between its PX domain and phosphoinositol- 3-monophosphates (PtdINs3P) that are enriched in the early endosome [39]. In addition to a PX domain, SNX27 has a C-terminal 4.1/exrin/radixin/moesin (FERM) domain, and a Postsynaptic density 95/discs large/zonus occludens-1 (PDZ) domain. The SNX27 FERM domain allows for interactions with signaling modulator, the active Ras small GTPase [36]. The PDZ domain was made famous by PSD-95 for its role as an essential scaffold in postsynaptic density formation, stability, and function [37]. Similar to its functions in PSD-95, PDZ domains are transient scaffolding proteins that are highly expressed throughout the body and are conserved across evolution. PDZ domain containing proteins can tether receptors and RGS proteins, and modulate internalization, trafficking, recycling, and sorting to modulate signaling [38]. The structure consists of six and β-sheets and two α-helices, and characteristically binds ligands with a 3-residue PDZ motif at the C-terminus. SNZ27 mediates plasma membrane/early endosome recycling of PDZ motif containing proteins. SNX27 interacts with a heterotrimeric retromer tubule as a complex to recycle transmembrane cargos with a PDZ motif to the cell surface [40]. SNX27 is highly expressed throughout the body and has been identified in T cells [36], natural killer cells [41], the cortex and hippocampus, as well as other locations and cell lines [42]. Deficiencies in SNX27 modulation are seen in Down's syndrome, which can lead to β-Amyloid over production [43]. SNX27 function is necessary for growth; knockouts of SNX27 can be embryonically lethal or cause severe growth retardation [42].

Interestingly, SNX27 was recently identified in a wide-scale proteomic screen as a potential regulator of G1, as well as several dozen other proteins [44]. Furthermore, Steinberg et al. showed that in order to maintain surface expression levels, interaction was necessary between SNX27 and the PDZ motifs found on the C-terminal tails of membrane proteins. Thus, it is possible that SNX27 plays a role in controlling G1 localization through interactions with the receptor's C-terminus (shown in Figure 3), with potentially distinct effects on the wild-type receptor (WT) versus mutant G1.

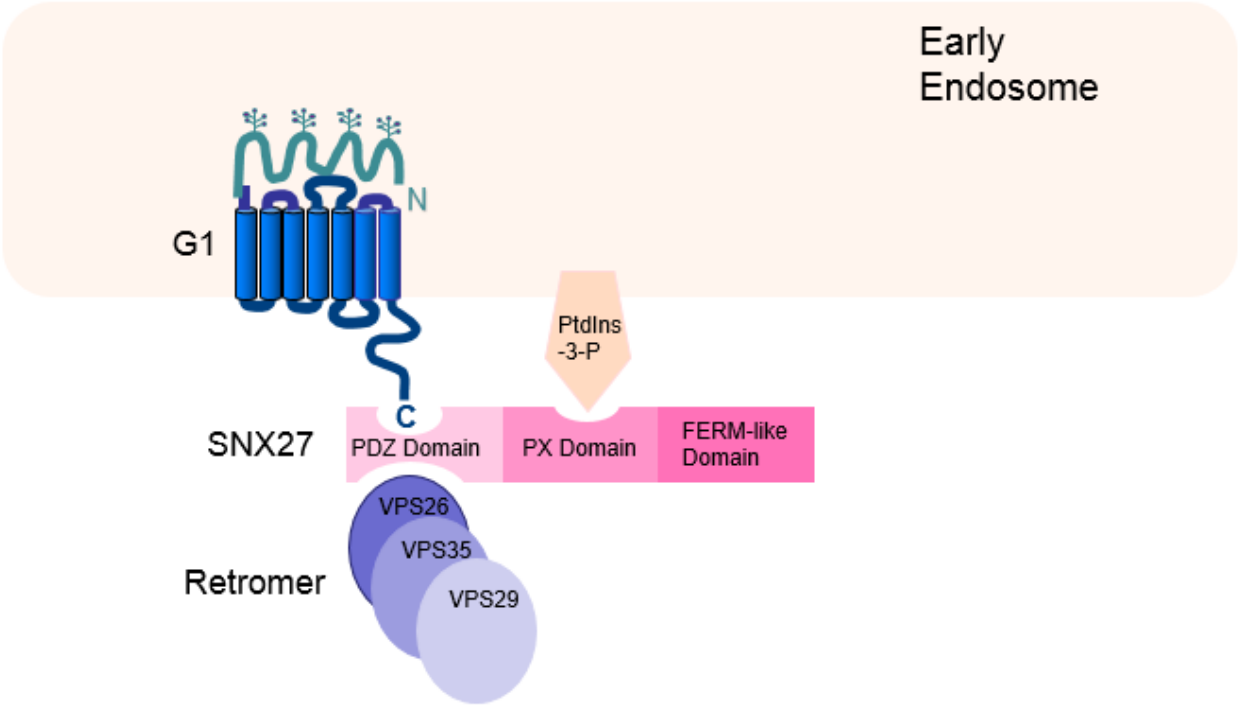


FIGURE 3 PROPOSED SCHEMATIC OF SNX27 INTERACTION WITH G1 LOCALIZED IN THE EARLY ENDOSOME
SNX27 localizes to the early endosome by binding with phosphoinositol- 3-monophosphates (PtdIns-3-P) at the PX domain. SNX27 binds the Retromer complex and the PDZ motif of cargo at the PDZ domain, a likely site for where G1 binds SNX27

Aims of This Thesis

This study aimed to examine whether SNX27 does in fact interact with G1 in cells. This study also aimed to examine how signaling and surface expression of G1 are affected by SNX27. BFPP causing G1 mutations in the NT show decreased signaling and decreased surface expression, while BFPP causing G1 mutations in the loops show comparatively normal surface expression and signaling [18]. In comparison, constitutively active G1 is expressed to the same degree on the cell surface as wild type G1, despite β-arrestin mediated internalization [17]. This suggests endosomal recycling could be related to signaling. For this reason, a protein like SNX27 that is capable of regulating endosomal recycling could potentially exert profound effects on G1-mediated signaling.

Methods

Cell Culture

For all experiments, Human Embryonic Kidney 293 (HEK-293T) cells were used. Cells were maintained at 37°C with 5% CO_2, in a 'complete media' made up of Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal Bovine Serum, and 1% penicillin/streptomycin. For transfections, the cDNA of interest was placed in serum-free DMEM with Mirus TransIT-LT1 according to the manufacturer's protocol. The transfected cultures incubated for 24-48 hours before experiments were performed.

Plasmids

The plasmids used include pcDNA 3.1 (Empty Vector), Human Wild Type G1 (WT), human G1 a.a. 383-693 (ΔNT) and HA-tagged SNX27.

The SNX27 plasmids in pcDNA3.1 are from Mark von Zastrow's group at University of California, San Francisco. The G1 plasmids have been previously described [17].

The ΔNT plasmid was amplified with a forward primer: 5'-GCA AAG AAG CTT ATG ACC TAC TTT GCA GTG CTG ATG-3'; and reverse primer: 5'-GCA AAG TCT AGA CTA GAT GCG GCT GGA CGA GGT-3'. The primers included restriction enzyme sites for HindIII and Xbai to match restriction enzyme sites found in the MCS of pcDNA3.1. The plasmids were sequentially digested by restriction enzyme and then ligated with T4 DNA ligase (New England Biolabs). XL2-Blue ultracompetent cells transformed with ligation preparation under ampicillin selection and colonies were grown O/N for plasmid preparation. Plasmids were sequenced (Genewiz) to verify.

Antibodies

Antibodies purchased from manufacturers include: Anti-HA (Roche) and Biotinylated anti-G1 NT (R&D Systems). A custom antibody against G1 CT was developed by Orbigen, Inc by injecting the antigen CSNSDSARLPISSGSTSSSRI into rabbits (Orbigen Inc.). The antibody was then purified by an affinity column with the peptide as the immunogen.

Western Blotting

Samples were run on 4-20% Tris-glycine gels in SDS-PAGE electrophoresis. Proteins were then transferred to nitrocellulose membranes. To block background signal, the membranes were washed for 30 min at room temperature in blocking buffer, made up of 2% nonfat dry milk, 50 mM NaCl, 20 mM HEPEs, and 0.1% Tween 20. The membranes were then incubated in primary antibody for either 1 hour at room temperature, or 16 hours at 4°C. Before the membranes were incubated in secondary antibody, they were washed three 5-minute washes with blocking buffer. The membranes were then incubated in HRP-conjugated secondary antibody for 30 min. The wash step was repeated with blocking buffer. The membranes were visualized with ECL reagent and exposure to film.

Coimmunoprecipitation

HEK-293T cells were transfected with 2 µg cDNA, as described above, and incubated for 24 hours. The cells were then washed with 1x PBS, scraped, and resuspended in lysis solution (1% Triton X-100, 150 mM HEPES, 10 mM $MgCl_2$, 1mM EDTA, and protease inhibitor tablet (Roche)). Cells were incubated in the lysis solution for 10 min at 4°C and then cleared by high-speed centrifugation for 15 minutes. 6x Laemmli buffer was added to a small sample of soluble lysate. The rest of the soluble lysate was incubated with 30 µL anti-Ha agarose beads (ThermoScientific) for 60-90 minutes at 4°C, then washed three times with lysis solution. The antigens were then eluted off the beads 2x Laemmli buffer. Co-immunoprecipitated samples were then analyzed via Western blot, as described above.

Cell-Surface Biotinylation

To measure presence of protein on the cell-surface, extracellular proteins were biotinylated and later pulled down by streptavidin beads. HEK-293T cells were transfected with 1.5 µg cDNA constructs, as described above, and incubated for 24 hours. The cells were then washed with 1x PBS three times. To biotinylate surface proteins, the cells were incubated with 2 mM Sulfo-NHS-LC-Biotin (Thermo Scientific) in PBS for 25 minutes. Negative control cells were incubated in PBS. The reaction was then quenched with 100mM Glycine in 1x PBS. Cells were then washed, scraped, and resuspended in lysis solution (as

described above). Cells incubated in the lysis solution for 10 min at 4°C and were then cleared by high-speed centrifugation for 15 minutes. 6x Laemmli buffer was added to a small sample of soluble lysate. The rest of the soluble lysate was incubated with 30uL streptavidin beads (Pierce) for 1 hour at 4°C, then washed three times with lysis solution. Proteins were then eluted off the beads using 2x Laemmli buffer.

Cell-Surface biotinylation was detected by Western blot, as described above. Expression was quantified by densitometric analysis using Image J. Band intensities were normalized to background before comparison.

Gene Reporter Assay: Dual-Glo Luciferase Assay

The Dual-Glo Luciferase Assay was used to measure reporter genes linked to transcription factors that are affected downstream of receptor activity. Nuclear Factor of Activated T-cells (NFAT) is a transcription factors that is upregulated downstream of G1 activity(Wu, Doyle et al. 2013). The reporter gene included Renilla Luciferase and transcription factor reporters for NFAT. Each reporter encoded the firefly luciferase reporter gene which was modulated by a minimal CMV promoter and tandem repeats. The Dual-Glo Luciferase Reagent (Promega) was a substrate for firefly luciferase. The luminescence created by the interaction between firefly and the reagent was measured by spectroscopy.

The studies were done in 96 well plates that had been each seeded with 35,000 HEK-293 cells, one day before transfection. HEK-293 cells were transfected, as described above, with 10 ng cDNA constructs, 10 ng of Renilla, also known as pRL-SV40, 50 ng of NFAT, and 2 µL Mirus. Cells were incubated for 48 hours after transfection. After incubation, Luciferase Reagent was applied to each well according to the manufacturer's protocol, and then the luminescence was measured. The Luminescence reaction was quenched by Dual-Glo Stop & Glo Reagent (Promega), according to the manufacturer's protocol, and the luminescence of the Renilla-Luciferase activity in the cell was measured. Each trial had 3 replicates. GPCR activity is measured by calculating Firefly luciferase activity normalized to background noise of Renilla-luciferase activity.

Statistical Analysis

Results from the Cell-Surface Expression and gene reporter signaling assays were analyzed with a Leven's test, followed by a Student's *t* test (Microsoft Excel). Data are expressed as means±SEM. $p<0.05$ was considered as a statistically significant difference.

Results

SNX27 Interacts with G1

A recent proteomic study identified SNX27 as a potential modulator of many surface-expressed proteins, including G1(Steinberg, Gallon et al. 2013). Co-immunoprecipitation was used to determine if SNX27 and G1 interact within the cell. HEK293 cells were transfected with either G1 (Figure 4A) or empty vector (pcDNA 3.1) and either HA tagged SNX27 or empty vector. Cells were harvested 24 hours later and SNX27 was pulled down from the solubilized cell lysates using anti-HA agarose. To determine if SNX27 interacts with G1, the pull-down samples were probed for expression of G1 via Western blot using a G1 C-terminal antibody. To determine if the interaction holds true for an actively signaling receptor, HEK293 cells were transfected with the empty vector or SNX27 and either empty vector or an N-terminally truncated G1 with increased activity (ΔNT) (Figure 4b). Cell lysates were immunoblotted with anti-G1 CT antibody to show levels of expression, and for anti-HA antibody to show levels of expressed SNX27.

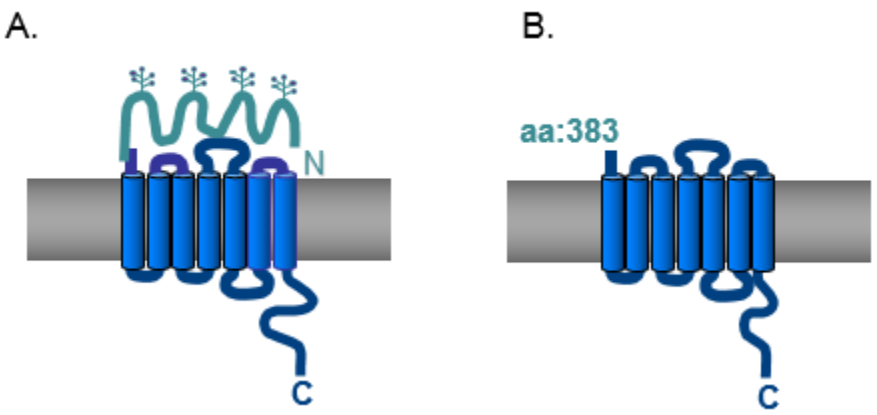
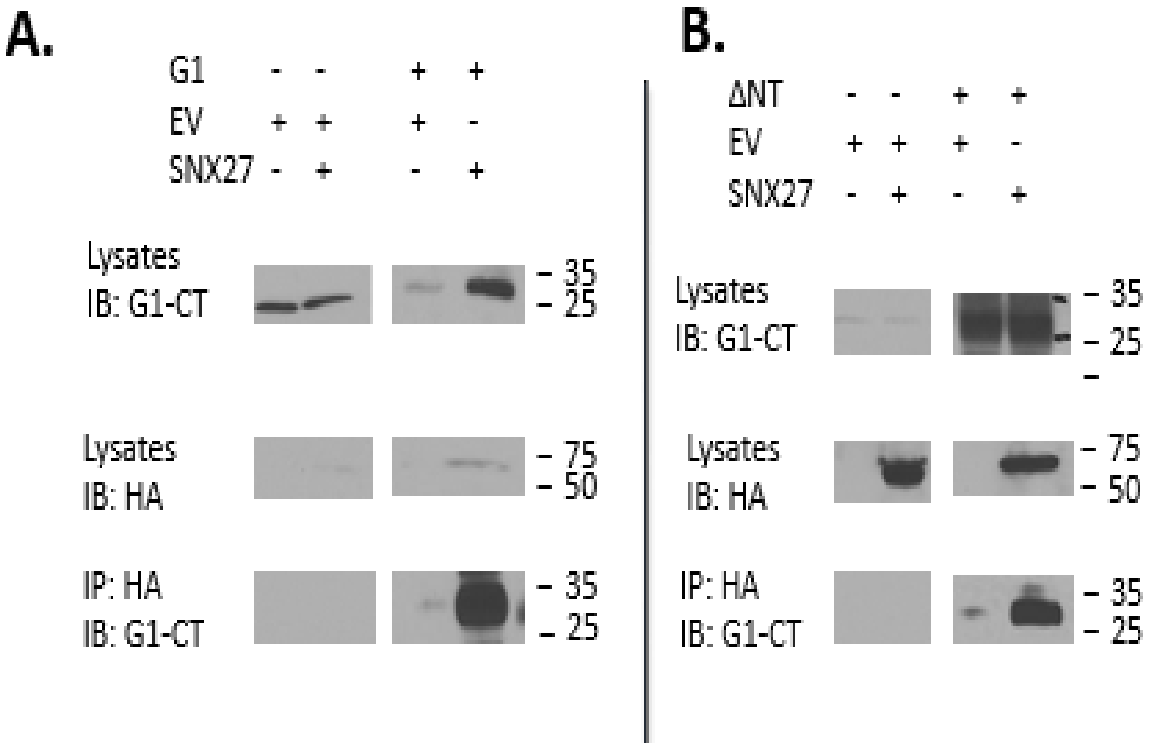


FIGURE 4 SCHEMATIC OF G1 AND ΔNT VECTORS
Schematic of Wild Type human G1 vector (A) and constitutively active, N-Terminally truncated G1 vector (B).

FIGURE 5 G1 AND ΔNT INTERACT WITH SNX27



(A) HEK-293 CELLS WERE TRANSFECTED WITH EMPTY VECTOR OR G1, AND EMPTY VECTOR OR SNX27 AND CELL LYSATES WERE PROBED WITH ANTI-G1 CT ANTIBODY OR ANTI-HA ANTIBODY. IMMUNOPRECIPITATION WAS PERFORMED WITH ANTI-HA BEADS AND SAMPLES WERE PROBED WITH ANTI-G1 CT ANTIBODY. THE POSITIONS OF MOLECULAR WEIGHT MARKERS ARE INDICATED BY THE NUMBERS ON THE RIGHT. (B) SIMILAR EXPERIMENT TO THAT SHOWN IN PANEL A, EXCEPT THAT THE CELLS WERE TRANSFECTED WITH EMPTY VECTOR OR ΔNT RATHER THAN EMPTY VECTOR OR FULL-LENGTH G1.

Immunoblot analysis of cell lysates for G1 CT and SNX27 verified expression of constructs in each condition (Figure 5). Immunoprecipitation with anti-HA agarose and immunoblotting for G1 CT showed that SNX27 interacts with both G1 (n=3; Figure 5A) and ΔNT (n=6; Figure 5B).

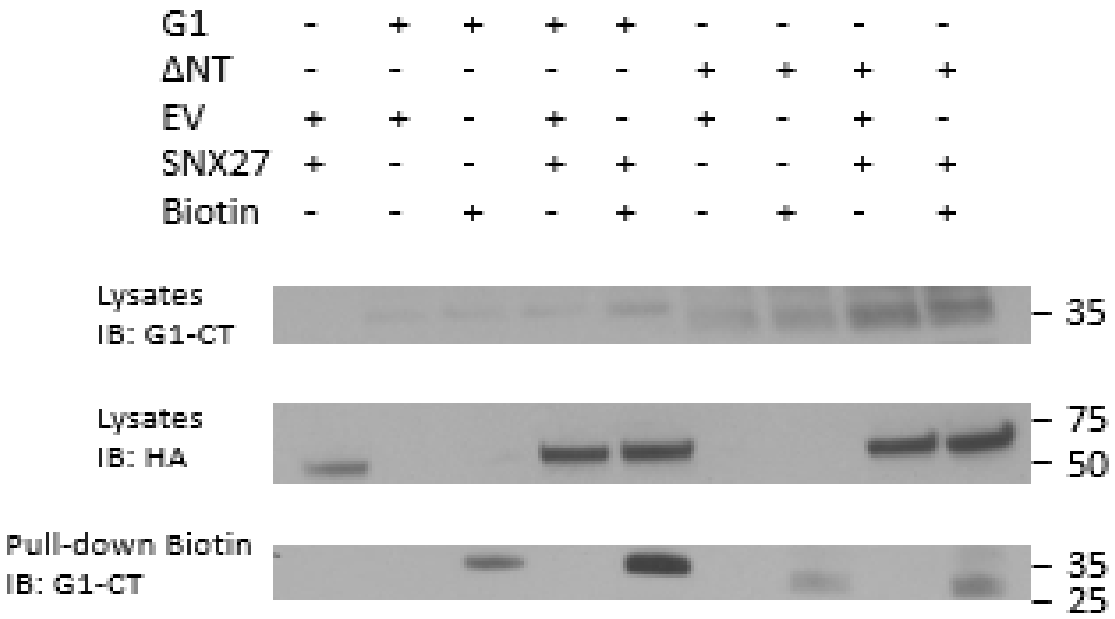
Total and Surface Expression of G1 Increase with SNX27

SNX27 is essential for recycling of signaling receptors from the early endosome to the plasma membrane [45] . As G1 is a surface expressing signaling receptor, it is possible that SNX27 modulates its recycling between the plasma membrane and the early endosome, affecting its surface expression. A surface biotinylation assay was preformed to determine if SNX27 affects surface expression of G1. HEK-293 cells were transfected with EV, G1, or ΔNT, and EV or SNX27. Surface proteins were biotinylated

with membrane-impermeable Sulfo-NHS-LC-Biotin and pulled down with Streptavidin conjugated agarose. Expression was quantified by densitometric analysis using Image J. Band intensities were normalized to background. Then expression with SNX27 was compared to expression without SNX27 for both total and surface expression of G1 and ΔNT.

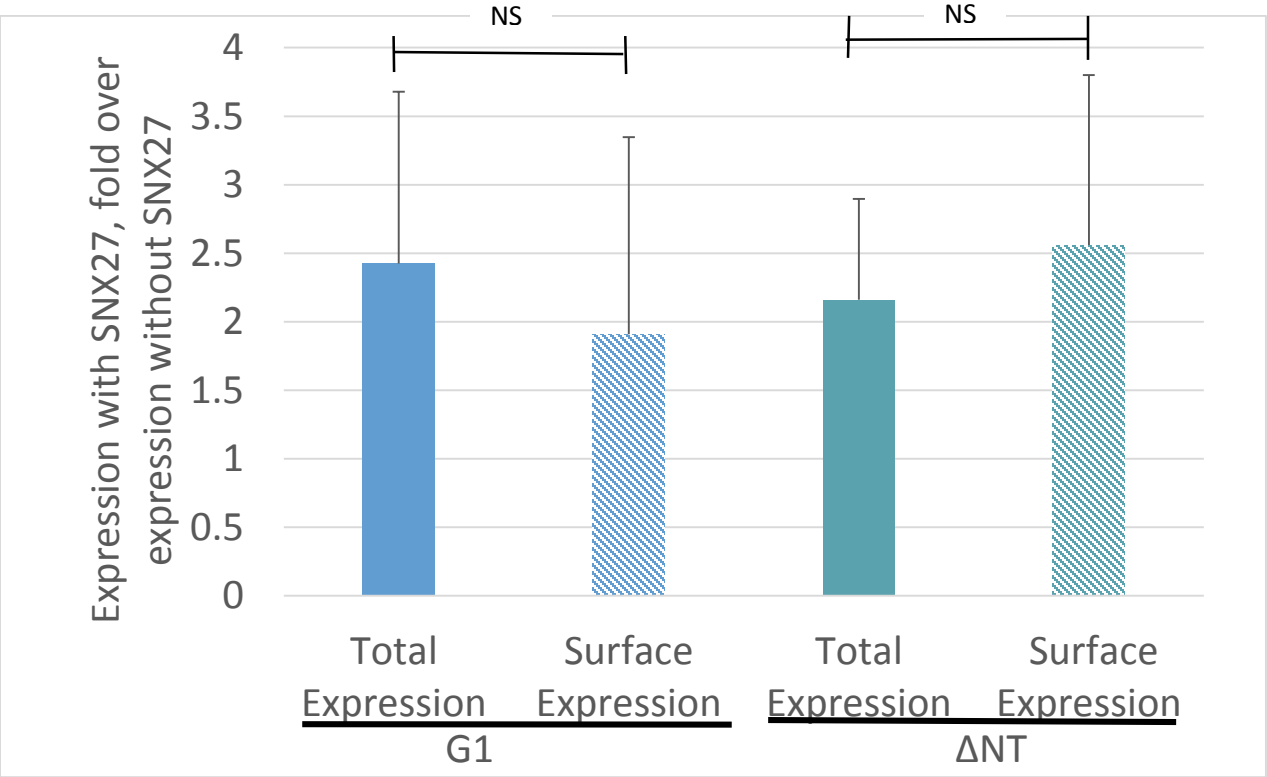
Immunoblots of the cell lysates with anti-G1 CT antibody revealed that total expression of G1 increased with SNX27 (Figure 6) by 2.4±0.5-fold increase (n=3), and for ΔNT: 2.2±0.2-fold increase (n=4) (Figure 7). Similarly, G1 surface expression increased with SNX27 (Figure 6) by 1.9±0.9-fold increase (n=4), and for ΔNT by 2.6±0.8-fold increase (n=4) (Figure 7). A Levene's test was used to measure for homogeneity of variance between total and surface expression, followed by a student's *t* test to determine if the increase in total and surface expression were statistically different. The increase in total and surface expression were found to be from the same population for G1 ($F=3.2$, $p>0.05$) and ΔNT ($F=2.9$, $p>0.05$) (Figure 7). The increase between total and surface expression are not statistically different, and are therefore related.

FIGURE 6 SNX27 INCREASES BOTH TOTAL AND SURFACE EXPRESSION OF G1 AND ΔNT



CELLS WERE TRANSFECTED WITH EMPTY VECTOR, G1, OR ΔNT, AND EMPTY VECTOR OR SNX27. LYSATES SHOW TOTAL EXPRESSION OF CONSTRUCT. CELLS WERE EITHER TREATED WITH PBS OR BIOTINYLATED TO LABEL SURFACE PROTEINS. PULL-DOWN ASSAYS WERE THEN PERFORMED WITH STREPTAVIDIN BEADS AND THE BEAD ELUATES WERE BLOTTED FOR G1-CT TO ASSESS EXPRESSION OF G1 AND ΔNT ON THE SURFACE.

FIGURE 7 QUANTIFICATION OF INCREASE IN G1 TOTAL AND SURFACE EXPRESSION WITH SNX27



DENSITOMETRY QUANTIFICATION OF INCREASE IN TOTAL AND SURFACE EXPRESSION OF G1 AND ΔNT WITH SNX27 COMPARED TO WITHOUT SNX27. N=4, NS= NON-SIGNIFICANT

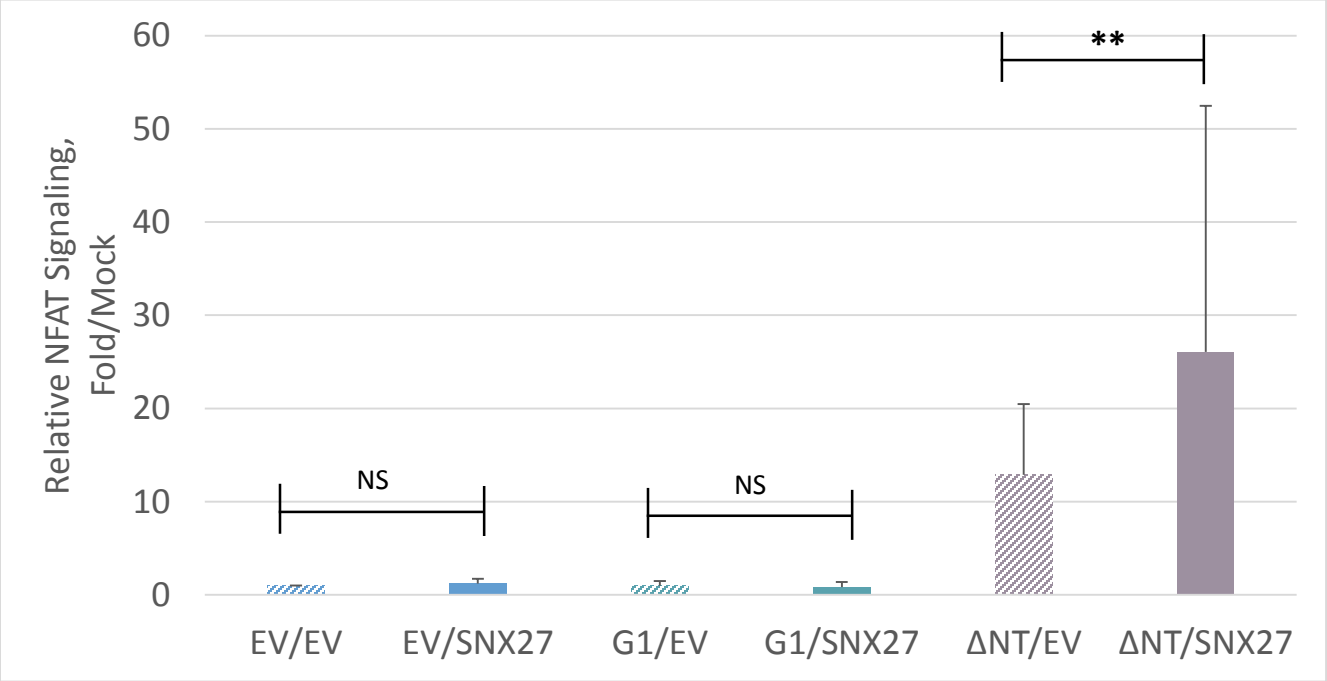
SNX27 Increases G1 Signaling

If SNX27 affects expression of G1, it is possible that SNX27 modulates G1 signaling by decreasing its internalization and degradation. To determine if G1 signaling is affected by SNX27, a gene reporter assay was used. NFAT expression is upregulated downstream of G1 signaling activity, and therefore NFAT expression can be used as a measure of G1 signaling [21]. In this assay, the NFAT promoter is paired with luminescent reporter gene firefly luciferase, which is normalized to total cell expression by measuring the luminescence of Renilla luciferase expression. Relative activity is measured by comparing NFAT luminescence to Renilla luminescence. HEK-293 cells were transfected with EV, G1, and ΔNT with and without SNX27, as well as NFAT-responsive firefly luciferase and Renilla luciferase.

Currently, only 3 experiments have been performed, and thus the results are preliminary, as seen in figure 8. Transfection of full-length G1 and empty vector resulted in no increase in luciferase

activity in either the absence or presence of co-expression of SNX27. Transfection with ΔNT induced an increase in NFAT-luciferase activity of 15±2 fold over signaling observed upon transfection with empty vector. When SNX27 was co-transfected, ΔNT signaling was 32±8 fold over empty vector activity. A student's T test was used to determine if the difference in ΔNT signaling with and without SNX27 was statistically significant. A Levene's test was used to measure for homogeneity of variance between the two conditions. Thus far, the preliminary evidence indicates that SNX27 may enhance ΔNT signaling (F=0.08, p=0.059, N=3).

FIGURE 8 G1 SIGNALING WITH AND WITHOUT SNX27



*RELATIVE NFAT-LUCIFERASE ACTIVITY FOR HEK-293 CELLS TRANSFECTED WITH EV, G1, AND ΔNT WITH AND WITHOUT SNX27. NS=NON-SIGNIFICANT **P=0.059*

Discussion

BFPP-causing mutations in the NT of G1 exhibit reduced surface expression, perhaps due to retention in the ER/Golgi or endosomal compartments [18]. SNX27, a regulator of early endosome-plasma membrane recycling, has recently been identified as a potential regulator of G1 in a wide-scale

proteomic screen [44]. This study aimed to assess whether SNX27 can interact with and regulate G1. The conclusions derived from the data shown above are that SNX27 can bind to G1 (Figure 5), increase both total and surface expression of G1 (Figure 6,7), and likely increase G1 signaling (Figure 8).

The data revealed that SNX27 binds to both full-length G1 and the constitutively-active truncated form of G1, ΔNT (Figure 5). These findings suggest that SNX27 does not bind to the N-terminus of G1, which is expected since SNX27 is a cytoplasmic protein and the G1 N-terminus is extracellular. SNX27 is the only Sorting Nexin family member with a PDZ domain, which binds cargo with a C-terminal PDZ motif [40]. Thus, it is likely that SNX27 binds to G1 at the receptor's C-terminus, most likely at the G1 PDZ motif, although that idea has not yet been confirmed. Future co-immunoprecipitation experiments will be performed with a mutated version of G1 lacking the PDZ motif and/or a mutated version of SNX27 lacking the PDZ domain in order to confirm the necessity of the PDZ interaction for G1-SNX27 binding.

SNX27 is involved in early endosome-plasma membrane recycling, suggesting that perhaps it can increase presence of G1 on the plasma membrane following receptor internalization. Indeed, the data shown above reveal that SNX27 increases G1 surface expression as well as total expression (Figure 6,7) by approximately 2-fold. It is possible that SNX27 interaction with G1 increases stability, and therefore leads to lower levels of receptor degradation, thereby increasing total expression. More expression of G1 could therefore lead to higher levels of receptor of concentration, pushing the exocytosis pathway forward. On the other hand, higher levels of G1 present on the surface, due to plasma membrane trafficking by SNX27, could allow for G1 to interact with scaffold proteins and G-proteins increasing its stability on the surface. The increased stability could lead to less internalization and degradation of G1 increasing total G1 expression. Steinberg et al have shown that SNX27 is critical in surface expression and stability of a number of different proteins. When SNX27 was knocked down, its various cargo proteins exhibited increased degradation. In addition, SNX27 cargo with PDZ motifs, such as GLUT1,

ATP7A, STEAP3, MCT1, SLC30A1, SLC4A7, KIDINS220, and CD97 were shown to require the PDZ motif-domain interaction to avoid internalization and be rescued from lysosomal degradation [44]. It is likely that the SNX27 interaction with G1, perhaps at the PDZ motif, increases G1 stability on the surface by blocking its sorting to the lysosome for degradation.

As G1 makes it to the surface without overexpression of SNX27 (Figure 6), it is likely that there is endogenous SNX27 or other endosome-plasma membrane trafficking modulators of G1, such as the retromer complex. To determine if indeed endogenous SNX27 is necessary for G1 surface expression, the effects of knockdown of SNX27 by siRNA have started to be studied in context of G1 expression and signaling. If SNX27 is necessary for G1 surface expression, siRNA knockdown will compromise G1 signaling and surface expression. If SNX27 is not necessary, the retromer complex and other endosome-plasma membrane trafficking modulators will be studied.

Co-expression of SNX27 with G1 has so far has resulted in increased signaling of the constitutively active receptor G1, ΔNT, approaching significance (Figure 8). G1 is not shown to signal in this assay, likely because of the non-covalent association of the NT to the receptor that inhibits its signaling. With the ΔNT mutant, the NT is not present and so G1 is constitutively active. Given a minimal sample size of 3, it is difficult to achieve sufficient power to conclusively detect functional effects of the co-expressed SNX27. However, the difference in signaling constitutes a sufficiently strong signal to warrant the collection more data. These are still preliminary studies and more NFAT signaling assays are planned. To look at other signaling pathways of G1, other gene reporter assays are also planned, such as a Serum Response Factor and Serum Response Element- Luciferase assays, both of which have been shown to be activated downstream of G1 Signaling([13], [22]). Increased surface expression by SNX27 trafficking could explain increased signaling of G1. The more receptor that is on the surface, the more it can interact with its G-proteins, such as $G\alpha_{12/13}$, to mediate signaling. To further validate the role of SNX27

in endosomal trafficking of G1, we plan to use confocal imaging to localize SNX27 with G1, ΔNT, and BFPP causing G1 mutants within the cell.

Due to the large role G1 plays in many biological functions, including cancer, immunology, and development, it is important to understand the trafficking of G1 to the plasma membrane for increased signaling. Disease-associated G1 mutants have lower surface expression as well as reduced signaling, leading to BFPP [18]. In addition, G1 plays roles in myoblast fusion [21], melanoma metastasis [22], and natural killer cell function [46]. By understanding G1 surface expression and signaling, it may perhaps be possible to manipulate G1 surface expression to modulate G1 function in controlling the migration of cancer cells, natural killer cells, and neural progenitor cells. Here I have shown that the interaction of G1 and the endosome-surface trafficking modulator SNX27 increases G1 expression and signaling. These findings suggest that SNX27 may be a key regulator of G1 function *in vivo*.

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