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Prenylation-deficient G protein gamma subunits disrupt GPCR signaling in the zebrafish

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Abstract

Prenylation of G protein gamma (γ) subunits is necessary for the membrane localization of heterotrimeric G proteins and for functional heterotrimeric G protein coupled receptor (GPCR) signaling. To evaluate GPCR signaling pathways during development, we injected zebrafish embryos with mRNAs encoding $G\gamma$ subunits mutated so that they can no longer be prenylated. Low-level expression of these prenylation-deficient $G\gamma$ subunits driven either ubiquitously or specifically in the primordial germ cells (PGCs) disrupts GPCR signaling and manifests as a PGC migration defect. This disruption results in a reduction of calcium accumulation in the protrusions of migrating PGCs and a failure of PGCs to directionally migrate. When co-expressed with a prenylation-deficient $G\gamma$, 8 of the 17 wildtype $G\gamma$ isoforms individually confer the ability to restore calcium accumulation and directional migration. These results suggest that while the $G\gamma$ subunits possess the ability to interact with G Beta (β) proteins, only a subset of wildtype $G\gamma$ proteins are stable within PGCs and can interact with key signaling components necessary for PGC migration. This *in vivo* study highlights the functional redundancy of these signaling components and demonstrates that prenylation-deficient $G\gamma$ subunits are an effective tool to investigate the roles of GPCR signaling events during vertebrate development.

1. Introduction

Heterotrimeric G-Protein signaling is a versatile and broadly used mechanism in eukaryotes for the transduction of cellular signals such as those involved in the pathways controlling sight, neurotransmission and cell migration. Consisting of an alpha (α), beta (β) and gamma (γ) subunit, the heterotrimeric G-proteins are membrane-bound, cytosolically located signaling components that interact with and are activated by a diverse group of G protein coupled receptors (GPCRs). During heterotrimeric G-protein signal transduction, ligand binds a GPCR resulting in the activation of the $G\alpha$ subunit to dissociate its bound GDP and bind the more highly concentrated, free cytosolic GTP (reviewed in [1]). The activated $G\alpha$ subunit dissociates from the $G\beta\gamma$ dimer allowing both components to influence separate downstream effectors

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such as adenylyl cyclase [2-4], phospholipase C β [5,6], phosphatidylinositol 3-kinase [7,8], β adrenergic receptor kinase [9,10], and ion channels [11,12].

Heterotrimeric G proteins are capable of mediating a myriad of upstream and downstream signals, in part due to the large number of isoforms of the G protein α , β and γ subunits present in vertebrates. Humans and mice have 16 α , 5 β and 12 γ subunit isoforms [13] although not all combinations are formed. The constituents of the heterotrimeric G protein have been shown to differentially influence interactions between the heterotrimer and receptors [14,15] as well as between the G α subunit or G $\beta\gamma$ dimer and downstream effectors [16,17]. The subcellular localization of the G $\beta\gamma$ dimer has also been hypothesized to increase the specificity of signal transduction [18]. Since prior work largely focused on cultured cells, it remains unclear as to what level of cross talk and redundancy exists among GPCRs, heterotrimers and their downstream effectors *in vivo*. The present study examines the role of G γ subunit post-translational processing and GPCR mediated signaling in the complex environment of a live vertebrate.

Both G α and G γ subunits are post-translationally modified with lipids. The G γ subunits are a family of proteins that have a carboxy terminal 'CaaX' motif (where C = cysteine, a = an aliphatic amino acid, and X = a variable amino acid) whose presence makes them substrates for the prenylation enzymes farnesyl transferase (FTase) or geranylgeranyl transferase I (GGTaseI). The amino acid makeup of the CaaX motif and, in particular, the identity of the 'X' largely determines the affinity of FTase versus GGTaseI for the CaaX motif-containing protein. Prenylation is the first step in a post-translation modification that involves adding either a 15-carbon farnesyl or 20-carbon geranylgeranyl group to the cysteine of the CaaX motif followed by proteolytic cleavage of the terminal 'aaX' amino acids and methylation of the carboxy-terminal prenyl cysteine. The process of prenylation results in the increased hydrophobicity necessary for these proteins to insert into membranes and interact with other hydrophobic proteins. Prenylation of G γ subunits and palmitoylation of G α subunits confer the heterotrimeric G protein with the ability to translocate to cell membranes [19,20]. Prenylation of the G γ subunit is also necessary for the G $\beta\gamma$ dimer to interact efficiently with a G α subunit [21-23], receptors (Rhodopsin [24,25]; A1 adenosine receptor [25]), and effectors [26-30] (Phosducin (pdc) [31]; PhospholipaseC β (PLC β and PLC β 2) [32]; adenylyl cyclase [33]).

While the functions of individual members of the large G γ subunit family have been difficult to identify, some have been characterized through the creation of mouse gene knockouts (i.e. G γ 7 [34]; G γ 3 [35]). Quicker, alternative methods to determine the function of individual subunits have yielded inconsistent results. Although ribozymes targeted to individual G γ subunits have shown the ability to assay *in vitro* function [36], their efficacy *in vivo* remains to be determined. Few zebrafish heterotrimeric G proteins have been identified [37-39]. The use of morpholinos (targeted antisense oligonucleotides) in zebrafish embryos to elucidate the biological function of specific G protein subunits is complicated by poor target efficacy, maternal protein contribution, off-target effects and/or functional redundancies between G protein subunits. In this study we show that knocking down many of the individual G γ subunits is insufficient to disrupt primordial germ cell (PGC) migration, a developmental process known to rely on GPCR signaling in both insects and vertebrates [40-44]. Therefore, we evaluated an alternative method to determine the function of these important signaling proteins during zebrafish development. The tissue-specific dominant negative strategy reported here allows the study of G-protein signaling in the background of maternally provided proteins that would interfere with antisense-based methods.

In this study, we performed a comprehensive, comparative genomic analysis to identify the G β and G γ subunit families in zebrafish. We investigated the capacity of 17 prenylation-deficient G γ subunits to disrupt GPCR signaling by injecting embryos with mRNA and

assaying PGC migration. PGCs, the cells that will later give rise to the gametes, arise at a site that is distinct from the future-developing gonad and therefore must migrate long distances during early embryogenesis. The pathways involved in this long-range migration exhibit parallels to several pathologies including chronic inflammation [45-47] and cancer metastasis [48-51]. PGCs are guided by a GPCR-mediated pathway that involves migration towards increasing concentrations of the attractant chemokine Sdf1a, discovered in the zebrafish to be dynamically expressed along the developing borders of the trunk mesoderm and the pronephros [42]. The GPCR that recognizes Sdf1a, Cxcr4b, is expressed within PGCs [41,42]. Another pathway integral to the migration of PGCs involves the enzyme Geranylgeranyl transferase I [52,53]. One way that these two pathways could work in concert is if the geranylgeranylation of G γ subunits is necessary within the PGCs to localize and/or assemble the proper machinery for sdf1a signal transduction.

This study demonstrates that expressing prenylation-deficient G γ subunits in zebrafish embryos is sufficient to interfere with GPCR signaling and disrupt PGC migration, resulting in a phenotype similar to that seen when Sdf1a signaling is disrupted. The finding that the majority of prenylation-deficient G γ subunits disrupt PGC migration led us to test whether G γ subunits mediate interactions with signaling components other than their obligate signaling partner, G β . Only a subset of co-administered wildtype G γ subunits were able to restore proper migration in embryos expressing a prenylation-deficient G γ subunit, suggesting that G γ subunits can perform distinct and non-overlapping functions *in vivo*.

2. Materials and methods

2.1. Zebrafish strains and fish maintenance

Danio rerio of an AB background were outcrossed once to pet store zebrafish to restore hybrid vigor and inbred several generations for use as WT fish. Fish were raised and maintained as previously described [54,55]. All zebrafish care and experimental procedures were carried out as specified in our Institutional Animal Care and Use Committee Protocol (#647A). Fish of the AB strain were used in the calcium labeling experiments.

2.2. RTPCR and cloning of the zebrafish G-protein β and γ subunits

G β and G γ subunits were identified by performing tblastn searches of the human and mouse subunits against the current zebrafish assembly (Ensembl, Jun 2007, version 7). Reverse transcription polymerase chain reaction (RT-PCR) was performed on the G β and G γ subunits using oligo-dT amplified, 4cell-6dpf cDNA and AMV Reverse transcriptase (NEB: M0277S) according to the manufacturer's protocol. Primers for RT-PCR and for cloning of the zebrafish G γ subunits into the pT3TS & *nos1*-3'UTR vectors are reported in supplementary information (Table S2). All RTPCR primers were designed to amplify a region that spanned an intron. The pT3TS vector contains the *Xenopus* β -globin 5' & 3'UTRs that result in a highly stable mRNA which is ubiquitously translated upon injection into zebrafish embryos [56]. The *nos1*-3'UTR vector contains the 3'UTR of the *nanos1* gene which directs the transcribed mRNA to be translated in all cells at very early stages followed by mRNA degradation in somatic cells and stabilization in PGCs [57]. The G β subunits were cloned into the GFP*nos1*-3'UTR vector by disrupting the stop site of mGFP5 with the G β coding sequence.

2.3. Injection of G γ -SaaX and G γ -WT mRNA

Capped mRNA was *in vitro* transcribed from the pT3TS or *nos1*-3'UTR vectors using the T3 or SP6 mMessage mMachine kits, respectively (Ambion: AM1348, AM1340). G γ -SaaX or G γ -WT mRNAs were injected into embryos at the 1-2 cell stage. To test the ability of the wildtype mRNAs to reverse the phenotype induced by G γ -SaaX, either *gng2*-SaaX mRNA was injected at 25pg/embryo or *gng2*-SaaX(*nos*) mRNA at 50pg/embryo, after being mixed

with GFP-*nos1*-3'UTR mRNA [57] at a final concentration of 200pg/embryo. The level of fluorescence of the GFP-*nos1*-3'UTR mRNA serves as an internal control for the amount of the G γ -SaaX mRNA administered. The injected embryos were split into two groups; one group received an injection of a G γ -WT mRNA and the other group received no injection or an injection with an equal volume of control (phenol red; 0.2% final concentration). The ectopic PGC migration score for each larva was determined by counting the number of PGCs at 24-48hpf that were outside of the WT location at the anterior of the yolk extension (**0** = 0-5% ectopic; **1** = 6-19% ectopic; **2** = 20-39% ectopic; **3** = 40-59% ectopic; **4** = 60-79% ectopic; **5** = 80-100% ectopic). Only larvae with a minimum of 25 fluorescent PGCs were scored.)

2.4. In vitro translation

mRNAs were *in vitro* translated using the Retic Lysate IVT kit (Ambion: AM1200) in conjunction with ³⁵S labeled methionine and visualized using SDS-PAGE.

2.5. Microscopy

GFP-expressing larvae were imaged under brightfield and a 488nm filter on a Nikon SMZ1500 or Zeiss StereoLUMAR microscope and captured by Zeiss AxioVision software.

2.6. Fluorescence colocalization

Colocalization studies were performed using a Leica TCSSP2-DMRXE confocal at 12-18hpf. Raw images of PGCs were processed using Metamorph software to determine the fluorescence density of GFP in regions corresponding to the plasma membrane and the cytosol. The ratio of cytosolic fluorescence density to membrane fluorescence density was calculated and the means were analyzed for statistical differences (ANOVA; Gabriel post hoc test).

2.7. Time-lapse movies

Embryos were injected with 180pg mRNA encoding GFP-*nos3*'UTR or GFP-CVLL-*nos3*'UTR, a modified version of mGFP5 that has a geranylgeranyl-specific CaaX box (-CVLL) added to its C-terminus, either alone or in conjunction with *gng2*-WT(*nos*) [100pg] or *gng2*-SaaX(*nos*) mRNA [50pg]. Embryos were mounted on coverslip-bottomed dishes in 0.7% agarose and imaged at 20X on a Leica DMIRE2 spinning disk confocal scope using Metamorph software. Images were montaged from 5-6 z-stacks taken every 8 minutes and 15 seconds from 10-30hpf. Migration of individual PGCs were tracked and measured for direction and velocity using Imaris software (Bitplane).

2.8. Measurement of calcium levels

Calcium levels in migrating PGCs were assayed *in vivo* by methods previously described [58].

2.9. In situ hybridization of zebrafish larvae

In situ hybridization was performed as described by Thisse et al., 2004 [59] with modifications for 2 colors according to Weidinger et al., 2002 [60].

2.10. Isolation of FLAG-tagged G γ proteins

Embryos injected at the one-cell stage with 180pg of GFP-*nos3*'UTR mRNA and either 30pg of *gng2*-WT or *gng2*-SaaX mRNA were harvested at 24hpf, dechorionated, deyolked by shaking in deyolking buffer (55mM NaCl, 1.8mM KCl, 1.25mM NaHCO₃) and washed in buffer (110mM NaCl, 3.5mM KCl, 2.7mM CaCl₂, 10mM Tris/Cl pH 8.5). Following sonication in homogenization buffer (10mM Tris pH7.4, 250 mM Sucrose, 1 mM EDTA, 1% protease inhibitor), Cytosolic and membrane proteins were isolated as described in Mumby et

al., 2002 [61] and subjected to immunoprecipitation using the FLAG-IP kit manufacturer's protocol (Sigma-Aldrich: FLAGIPT1-1KT). Proteins were separated on a gradient 10-20% SDS-PAGE gel, transferred to a PVDF membrane and subjected to Western blotting using rabbit anti-FLAG Antibody (F7425, Sigma) and ECL-HRP-linked donkey anti-rabbit secondary (Amersham: NA934).

2.11. Morpholino injections

Morpholino antisense oligonucleotides designed to target the start sites of eight gamma subunits are as follows: G γ 2 (5'-CTGTGTTGTTGGTGGCCATGAGGCT-3'), G γ 3 (5'-GTTACAGGGGTGTCTCCTTTCATC-3'), G γ 4 (5'-GTCCTTCATGATGAGTCCTGCACAC-3'), G γ 7 (5'-TGTTATTAGTCGTGGACATTCTCTG-3'), G γ 8 (5'-GACATCCTGTTTCGTTTGTAGAGACC-3'), G γ 12a (5'-TTGAGCCCGACATTTTAGAGGACAT-3'), G γ 12b (5'-GAGCTTTGCATCTTGGACAACATTT-3'), and G γ 13 (5'-GGCAAGTCCATCTCGTCCATGACTG-3'). Each morpholino was injected with GFP nos1 -3'UTR mRNA at the one-cell stage and titrated from 0.1 to 5ng per embryo.

3. Results

3.1. Zebrafish have at least 17 G Protein γ Subunits

We identified the zebrafish genes encoding G protein γ subunits. *gngt1*, *gng3* and *gng2* were previously characterized [37-39]. Identification and naming of the zebrafish G γ subunits was based on homology to their human orthologs in conjunction with phylogenetic (Figure S1) and syntenic analysis (Figure S2). In contrast to *S. cerevisiae* that has only one G γ subunit [62], or *D. melanogaster* that has two G γ subunits [63,64], the zebrafish has at least 17 G γ subunits (Table 1 and Figure S1). These 17 G γ subunits represent the vast majority of the known orthologs in the human and mouse [13,65]. While we did not find a zebrafish *gng11* ortholog, we identified two G γ subunit paralogs (*gngt2b*, *gng12b*) that likely reflect gene duplications that occurred after the separation of the teleost and mammalian lineages. Furthermore, we identified 4 G γ subunits with no clear mammalian ortholog (*gng14*, *gng15*, *gng16*, *gng17*), suggesting that these were either lost during mammalian evolution or evolved in fish after divergence from the common ancestor.

3.2. G γ -SaaX proteins are localized to the cytosol in vivo

Mutation of the cysteine of the prenylation-specific 'CaaX' motif into a serine (CaaX->SaaX) abolishes a protein's ability to be prenylated and therefore disrupts membrane localization *in vitro* [19,20,31,66-70]. To determine the effect of introducing prenylation-deficient G γ subunits during zebrafish development, the 17 zebrafish G γ subunits were separately cloned in the wildtype (WT) and mutant form (SaaX) into the pT3TS vector. mRNA *in vitro* transcribed from this construct is stable and ubiquitously translated when injected into zebrafish embryos [56]. Western blot analysis of proteins extracted from 24 hours post fertilization (hpf) larvae injected at the one cell stage with mRNA encoding N-terminally FLAG-tagged versions of G γ 2-WT or G γ 2-SaaX confirmed that the G γ -SaaX proteins lose their membrane localization (Figure 1).

3.3. G γ -SaaX mRNAs disrupt PGC Migration

To visualize the effects that introducing prenylation-deficient G γ mRNAs have on a known GPCR mediated event during development, PGC migration [71], embryos were co-injected with GFP nos1 -3'UTR mRNA (Figures 2A and 2B). Appending the 3'UTR of the *nanos1* (*nos*) gene to mRNA results in the inhibition of translation and degradation of the adjoined

mRNA in somatic cells and its stabilization in PGCs [57]. Expression of GFP $_{nos1-3'UTR}$ allows visualization of the migrating PGCs from 8hpf until well after 24hpf, the stage by which PGCs reach their final target at the anterior of the yolk extension, where the gonad will later form. Embryos injected with *in vitro* transcribed, capped *gng2-SaaX* mRNA exhibited a profound disruption of PGC migration (Figures 2C and 2D). Morphological analysis and whole mount in-situ hybridization of *sdf1a* expression showed that while PGCs mislocalize after the injection of 30pg *gng2-SaaX* mRNA, somatic structures are not disrupted, suggesting that at the doses used, the effect is specific to PGCs (Figure 2 and S3). The lack of colocalization of ectopic PGCs with regions expressing the secreted chemoattractant *sdf1a* suggests that PGCs in *gng2-SaaX* mRNA injected larvae are insensitive to this guidance cue. The rare PGCs that are ectopically localized in wildtype embryos often end up in the hindbrain, another site of high *sdf1a* expression (personal observation, Movie S1, and [42]). Injection of the remaining γ subunit mRNAs in their SaaX form indicated that 13 of the 17 γ -SaaX subunits have the ability to disrupt PGC migration (Figures 2G and 2H; Table 2 and Figures 3A and S4). Injection of the wildtype versions of the γ subunit mRNAs resulted in no obvious disruption of PGC migration (Figures 2E and 2F) with the exception of the farnesylated γ subunits, *gngt1* & *gngt2a*, which not only caused PGC migration defects at low doses but also somatic defects at higher concentrations (Figure 3B).

In vivo time-lapse movies of PGCs migrating in zebrafish injected with prenylation-deficient γ mRNA indicated that despite the PGCs' inability to reach the anterior of the yolk extension, PGCs maintain the ability to migrate actively and extend cellular protrusions. While the PGCs do not show a change in their migration speed (mean PGC speed: $\gamma 2$ -WT = 1.25 μ m/min (SD 0.37), n=266 cells tracked from 6 embryos; $\gamma 2$ -SaaX = 1.27 μ m/min (SD 0.20), n=114 cells, 3 embryos), they do show a loss of directional migration as is evident in their wildly uncoordinated migrations as well as the lack of congregation at the previously described sites of high *sdf1a* expression [60] (Movies S1-S3, Figure S3).

3.4. All G protein γ subunits are expressed in the first 24 hours of zebrafish development

To identify γ subunits important during development of the zebrafish and determine if any subunits are specifically expressed in PGCs during migration, we examined their expression by RT-PCR and whole mount *in situ* hybridization. RT-PCR analysis revealed that all of the γ subunits are expressed during the first 24hpf and, with the exception of *gngt1*, *gng3*, and *gng14*, are maternally deposited (Figure 4). *In situ* hybridization analysis revealed that none of the γ subunits have expression patterns that are restricted to the PGCs. Several of the γ subunits exhibit expression patterns that are either ubiquitous (e.g. *gng5*), in tissues where migrating PGCs are found (*gng2*[38], *gng3*[37]), or in other discrete tissues. *gngt1*, *gngt2a*, and *gngt2b* are restricted to the retina and epiphysis at later stages ([39,72]; data not shown), suggesting roles in other developmental processes.

3.5. G protein β subunits are localized to the cytosol in the presence of γ -SaaX

One hypothesis as to how the majority of γ subunits disrupt PGC migration is that they interact with a common β subunit that they sequester in the cytosol as a non-functional dimer. To test this *in vivo*, we co-injected mRNA encoding an N-terminal mGFP-fused β subunit controlled by the *nos1-3'UTR*, membrane-driven mCherry-CVLL(*nos*) mRNA, and either *gng2*-WT or *gng2-SaaX* mRNA in order to visualize any changes in the subcellular localization of the β subunit in the presence of a prenylation-deficient γ . When GFP-*gng1(nos)* was injected alone, fluorescence was observed in the cytosol of migrating PGCs (presumably due to the lack of sufficient endogenous γ ; Figure 5A). When co-injected with the wildtype form of either *gng2* or *gng5*, GFP- $\beta 1$ localized to the plasma membrane (Figure 5B). However, when GFP-*gng1(nos)* was coinjected with *gng2-SaaX* mRNA, $\beta 1$ was once again localized to the cytosol, supporting the sequestering hypothesis (Figure 5C). Quantification of the GFP fluorescence

associated with the membrane and cytosolic compartments of PGCs revealed a significant shift in localization in the $G\gamma$ -WT injected groups when compared to the $G\gamma$ -SaaX and control injected groups (ANOVA, $p < 0.0001$; Gabriel test, $p < 0.002$; Figure 5D). To investigate whether the $G\beta$ subunits have the ability to override the $G\gamma$ 2-SaaX-induced phenotype and restore normal PGC migration, we cloned nine zebrafish $G\beta$ subunits (*gnb1*, *gnb11*, *gnb2*, *gnb211*, *gnb3a*, *gnb3b*, *gnb4*, *gnb5a* and *gnb5b*; Table S1). mRNAs encoding each $G\beta$ were co-injected with *gng2*-SaaX mRNA, but none were able to reverse the SaaX-induced PGC migration defects. Injections of combinations of the $G\beta$ s were also incapable of overcoming the SaaX-induced PGC migration defects.

3.6. $G\gamma$ -SaaX induced migration defects can be reversed by a subset of $G\gamma$ -WT subunits

We postulate that $G\gamma$ -SaaX subunits perturb PGC migration through the binding and mislocalization of relevant $G\beta$ subunits. The cytosolically located $G\beta\gamma$ -SaaX dimer would be incapable of regulating $G\alpha$ and signaling downstream effectors. The addition of exogenous wildtype $G\gamma$ that binds the endogenously acting $G\beta$ to form a dimer that can interact with the upstream and downstream proteins necessary for PGC migration should be capable of reversing the $G\gamma$ -SaaX phenotype. Only the $G\beta\gamma$ dimers that form a heterotrimer with the PGC migration-mediating $G\alpha$, interact with the relevant GPCR, and become activated to signal the appropriate downstream effectors would restore proper migration. To investigate the differences among $G\gamma$ subunits in their capacity to interact with signaling partners as a $G\beta\gamma$ dimer *in vivo*, we examined whether individual $G\gamma$ -WT subunits have the ability to reverse the $G\gamma$ -SaaX induced phenotype. To accomplish this we injected embryos with *gng2*-SaaX mRNA followed by a $G\gamma$ -WT mRNA and assayed PGC migration. Embryos were injected with a mixture of GFP-*nos1*-3'UTR and *gng2*-SaaX mRNA (Figures 6A and 6B). Half of the embryos injected with this cocktail were injected a second time with mRNA encoding one of the $G\gamma$ -WT subunits. Injection of *gng2*-WT mRNA resulted in a complete reversal of the $G\gamma$ 2-SaaX induced PGC migration defects (Figures 6C and 6D). Furthermore, it was found that *gng3*, *gng4*, *gng7*, *gng8*, *gng12a*, *gng12b*, and *gng13*-WT mRNA also had the ability to reverse the $G\gamma$ 2-SaaX-induced PGC migration defects. Phylogenetic analysis of these $G\gamma$ subunits elucidated that the ability to restore proper PGC migration is limited to members of 3 out of the 5 pre-defined groups in the $G\gamma$ family [73]. All of the members of groups 2 and 5 and a subset of group 3 were able to restore proper migration (Figure 6E). The failure of the remaining $G\gamma$ -WT subunits to reverse the SaaX-induced phenotype was not due to an inability to form protein, as determined by *in vitro* translation with ^{35}S labeled Methionine and the immunoprecipitation of FLAG-tagged $G\gamma$ subunits and subsequent SDS-PAGE analysis performed on representative subunits (data not shown).

To address whether the failure of these wildtype $G\gamma$ subunits to restore proper migration in the presence of $G\gamma$ 2-SaaX is due to their inability to compete with $G\gamma$ 2 for PGC migration mediators (such as a common interacting $G\beta$ subunit, upstream receptor or downstream effector) rather than their inability to interact with those mediators, wildtype mRNA of the $G\gamma$ subunits incapable of reversing the $G\gamma$ 2-SaaX phenotype were injected in the presence of their own $G\gamma$ -SaaX mRNA. While mutation of the CaaX motif of $G\gamma$ subunits or truncation of the protein so that it is no longer prenylated does not affect the tight interaction of $G\gamma$ with its obligate partner, $G\beta$ [74], we expect wildtype $G\gamma$ subunits would have a higher affinity for upstream or downstream effectors than their SaaX mutant counterparts due to the increased hydrophobicity imparted by the prenyl moiety. Wildtype $G\gamma$ subunits incapable of reversing the $G\gamma$ 2-SaaX induced phenotype (*Gyt1*, t2a, 5, 10, 15, 16, 17) were also incapable of reversing the phenotype induced by their own $G\gamma$ -SaaX counterpart, suggesting that while they can interact with the endogenous $G\beta$ subunit necessary for PGC migration (as assayed by their ability to disrupt PGC migration), they have an inability to interact with other essential PGC

migration mediators. Alternatively these G γ subunits could have reduced stability in the PGCs that would make them incapable of reversing the SaaX phenotype.

3.7. PGC-specific disruption of GPCR signaling results in ectopic PGCs

To address whether G γ -SaaX proteins are acting within PGCs or within somatic cells to perturb PGC migration, we cloned wildtype and SaaX mutant G γ subunits into the *nanos1*-3'UTR vector to drive their expression selectively in PGCs. While overexpression of *gng3*-WT mRNA in the PGCs has no effect on somatic development or PGC migration (Figures 7A and 7B), injection of *gng3*-SaaX(*nos*) resulted in PGC migration defects (Figures 7C and 7D). Similar results were found when these experiments were repeated with *gng2*-WT(*nos*) and *gng2*-SaaX(*nos*) (Figure 8). All of the *nos1*-3'UTR-directed wildtype and SaaX mRNAs tested (*gng1*, *gng2*, *gng3*, *gng4*, *gng5*) behaved identically to their T3TS counterparts (except for the added ability of the *nos1*-3'UTR fused mRNAs to be injected at much higher concentrations before causing somatic disruption). This data is consistent with a cell-autonomous need for G γ -mediated signaling within PGCs for proper migration. To assay for the disruption of somatic components of PGC migration, we tested the ability of a PGC-driven WT subunit to reverse the phenotype induced by ubiquitously driven *gng2*-SaaX. If G γ 2-SaaX is also disrupting GPCR signaling in somatic cells, a PGC-driven *gng2*-WT would be unable to reverse the SaaX-induced phenotype. At the low concentrations of ubiquitously translated *gng2*-SaaX necessary to specifically disrupt PGC migration, injection of the *nos1*-3'UTR controlled *gng2*-WT mRNA was able to restore proper PGC migration (Figures 7E, 7F, 8). However, injection of *gng5*-WT(*nos*) mRNA failed to reverse the SaaX-induced phenotype, providing additional evidence that G γ subunits can have distinct signaling roles (Figures 7G and 7H).

3.8. G γ -SaaX disrupts calcium accumulation in the protrusions of migrating PGCs

Disrupting Sdf1a signaling by targeting the chemokine *sdf1a* or its receptor *cxc4b* with antisense morpholinos results in a decrease in calcium accumulation in the protrusions of migrating PGCs [58]. To investigate whether G γ -SaaX expression disrupts this essential signaling pathway, we assayed calcium levels in the protrusions of PGCs migrating in larva injected with *gng2*-SaaX mRNA. Injection of *gng2*-SaaX mRNA resulted in a significant decrease of calcium accumulation in protrusions when compared to wildtype levels (Figure 9; Movie S4). This decrease is similar to that seen when embryos are injected with morpholinos targeted to *cxc4b* and can be reversed by injection of *gng2*-WT mRNA (Movie S5). While injecting *gng2*-WT mRNA alone increases the amount of calcium present in these protrusions, it has no detectable effect on PGC migration (Movie S6).

4. Discussion

We have described an *in vivo* method to disrupt G-protein coupled signaling in a tissue-specific manner and investigate the signaling components involved in a conserved cell migratory pathway. Here we show that introducing prenylation-deficient G γ subunits results in the reduction of calcium within the protrusions of migrating PGCs and an inability of PGCs to migrate directionally.

4.1. Mechanism of G γ -SaaX action

G γ subunits have specific and overlapping affinities for the less-numerous G β subunits [68, 75-83]. The ability of the majority of G γ -SaaX subunits to disrupt PGC migration is consistent with the hypothesis that G γ -SaaX binds and sequesters the PGC migration-mediating G β subunits into nonfunctional, cytosolically located G $\beta\gamma$ -SaaX dimers. G $\beta\gamma$ dimer formation occurs prior to the prenylation and proteolytic cleavage of the carboxy terminal 'aaX' amino acids [23] and is thus unaffected by the loss of G γ lipid modification. Sequestration of G β into G $\beta\gamma$ -SaaX dimers may result in the inactivation of not only G $\beta\gamma$ signaling but also downstream

effectors of $G\alpha$ subunit signaling as has been suggested in *Saccharomyces* [84] and *Arabidopsis* [85]. PGC defects may result from an inability of the $G\beta\gamma$ -SaaX dimer to form a heterotrimer with the $G\alpha$ subunit or to interact with specific GPCRs and/or downstream effectors. Future studies will investigate whether the sequestration of $G\beta$ alone or in concert with other signaling partners determines the ability of a $G\gamma$ -SaaX subunit to disrupt PGC migration. Swapping the central region of the $G\gamma$ subunits, the main site of interaction with the $G\beta$ subunit [78,86,87], may confer the ability to restore or disrupt PGC migration. The ability of the transducin orthologs (*gngt1* and *gngt2*) to disrupt PGC migration when expressed in their wildtype form suggests that they may direct the $G\beta\gamma$ dimer to an alternative subcellular localization in their wt form, similar to the 'SaaX' form, or they may interact with signaling molecules not involved in PGC migration.

The ability of wildtype $G\gamma$ subunits to reverse the $G\gamma$ -SaaX-induced phenotype suggests that $G\gamma$ -SaaX proteins are acting as competitive inhibitors of GPCR signaling that can be overcome by simultaneous overexpression of wildtype $G\gamma$ subunits. The finding that only a subset of the wildtype $G\gamma$ subunits reverse the $G\gamma$ -SaaX phenotype is consistent with the hypothesis that while many $G\gamma$ subunits can interact with the less-variable $G\beta$ subunits, $G\beta\gamma$ dimers consisting of different $G\beta$ or $G\gamma$ members do not interact with the same pool of GPCRs and effectors [15,30,32,88-96]. This is evidenced by the ability of both $G\gamma 2$, which does reverse the $G\gamma$ -SaaX phenotype, and $G\gamma 5$, which does not reverse the $G\gamma$ -SaaX phenotype, to drive GFP- $\beta 1$ to the plasma membrane.

Previous studies, primarily in cell culture, support the hypothesis that $G\gamma$ -SaaX could exert its potent effect on PGC migration by sequestering $G\beta$ subunits, thereby, creating a pool of non-functional $G\beta\gamma$ -SaaX dimers incapable of mediating the interactions essential for directed migration. However, when we overexpressed the identified $G\beta$ subunits either individually or together in embryos injected with $G\gamma$ -SaaX mRNA, the PGC migration defects remained. Although unlikely, it is possible that we were unable to express enough $G\beta$ to reverse the effect of $G\gamma$ -SaaX mRNA ($G\beta$ mRNA was injected in quantities 1-10 times more than the molar equivalent of $G\gamma 2$ -SaaX). Alternatively, there may be an unidentified $G\beta$ that is required for PGC migration. The failure of $G\beta$ overexpression to reverse the $G\gamma$ -SaaX phenotype suggests $G\beta\gamma$ -SaaX dimers exert a dominant negative influence on GPCR signaling as opposed to being inactive in the cytosol. $G\beta\gamma$ -SaaX could exert a dominant negative effect by either sequestering the available $G\alpha$ into a nonfunctional heterotrimer or sequestering other proteins such as the corresponding GPCR, and/or downstream effectors into larger nonfunctional units. Further studies will be necessary to elucidate the *in vivo* interactions between $G\gamma$ subunit family members and their signaling partners essential for PGC migration.

4.2. Morpholinos are insufficient to study $G\gamma$ function

Historically, morpholinos have been a powerful antisense tool to study genetics in zebrafish. However, individual or combinatorial injection of morpholinos targeting the ATG sequence of the 8 $G\gamma$ -WT subunits that reverse the $G\gamma$ -SaaX phenotype had no effect on PGC migration. It is unclear whether this is due to an abundance of maternally deposited protein and mRNA or whether the morpholinos were unable to knock down their intended targets sufficiently. Until zebrafish lines containing mutations in each of the $G\beta$ and $G\gamma$ subunits are established, viable alternative methods of disruption such as injecting $G\gamma$ -SaaX mRNA can be used to investigate the roles of heterotrimeric G proteins during development.

4.3. $G\gamma$ -SaaX mRNA injection as a general tool for GPCR disruption

Given the studies reporting differences in the specificities and affinities of $G\gamma$ subunits for $G\beta$ subunits, we would expect that the $G\gamma$ subunits have variable efficiency in disrupting GPCR signaling pathways. Overexpression of a number of the $G\gamma$ -SaaX subunits results in failure of

the dorsal tissues to converge at the midline, multiple tail tips, cyclopia, and gross edema. The amount of mRNA necessary to induce such phenotypes was variable between $G\gamma$ -SaaX subunits, which could either suggest a difference in translation efficiency *in vivo* or differences in their individual affinities for the $G\beta$ and/or interacting partners.

4.4. Why are the PGCs so sensitive to $G\gamma$ -SaaX?

We expected that PGC-specific expression of $G\gamma$ -SaaX would be necessary to prevent disruptions in the somatic cells but found that the amount of ubiquitously translated mRNA injected could be titrated down to levels that exhibited a PGC migration-specific phenotype. Of all the signaling events that could be disrupted by $G\gamma$ -SaaX, such as those involving the mitogen-activated protein kinases ERK1 and ERK2 [97], $G\alpha$ or sdf-mediated signaling necessary for convergence and extension [98] or endoderm migration [99], the signaling pathways operating in the PGCs seem to be especially sensitive to disruption by injection of $G\gamma$ -SaaX mRNA. One hypothesis why PGCs may be more susceptible to perturbation involves a limited level or delay of transcription in this cell type in comparison to the somatic cells such as has been described in *Drosophila* during early stages of development [100]. A smaller pool of endogenous wildtype $G\gamma$ could be more easily outcompeted by $G\gamma$ -SaaX in PGCs that are unable to synthesize new $G\gamma$.

4.5. Signaling pathways that influence PGC migration

The chemokine SDF1 was recently shown to promote the accumulation of calcium in the protrusions of migrating PGCs [58]. The finding that $G\gamma$ -SaaX mRNAs also disrupt the accumulation of calcium in the protrusions of PGCs provides strong evidence that Sdf1a, $G\gamma$ and thus GGTaseI participate in the same pathway by functioning downstream of 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR). In this model, HMGCR acts within the PGCs to synthesize precursors of the isoprenoid geranylgeranyl diphosphate, which is post-translationally added by GGTaseI to the C-terminus of $G\gamma$ subunits necessary for the transduction of the Sdf1a signal (Figure 9E). Future experiments will explore whether abrogating prenylation through treatment with HMGCR inhibitors, such as atorvastatin, also disrupt calcium signaling.

If signaling through Sdf1a and its receptor CXCR4b is the same pathway disrupted in the presence of Geranylgeranyl transferase inhibitors (GGTIs), we might expect the migration defects caused by $G\gamma$ -SaaX and GGTI-2166 to be identical, which is not the case. $G\gamma$ -SaaX mRNA injection results in directional migration defects and not in a decrease in PGC migration speed as was previously observed with GGTI-2166 treatment of zebrafish embryos [52]. These differences may result from maternally deposited, prenylated $G\gamma$ being present in the PGCs and unaffected by GGTI-2166. The lower concentrations of inhibitor required to maintain normal somatic development might be insufficient to completely block the geranylgeranylation of $G\gamma$ within PGCs. Alternatively, the geranylgeranylation of several protein targets may be necessary to orchestrate a cellular event as complex as the migration of PGCs *in vivo*.

5. Conclusion

Our findings provide evidence that prenylation of $G\gamma$ subunits is necessary within the PGCs for directed migration. These findings also suggest that multiple $G\gamma$ subunits present in the PGCs could act redundantly or as reserves to a primary $G\gamma$ subunit(s) during migration. It would be evolutionarily advantageous for an organism to have redundancies in the mechanisms involved in PGC migration given the limited ability of PGCs to synthesize new proteins coupled with the necessity of PGC migration for propagation. While there is currently no simple and efficient method for assaying the role of endogenous $G\gamma$ subunits in the zebrafish, the

injection of Gy-SaaX mRNAs provides robust data implicating the function of GPCR-mediated signaling during development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

The membrane localization of Gβ and Gγ is disrupted when the CaaX motif of Gγ is mutated to SaaX. Embryos injected with mRNA encoding FLAG-tagged versions of the Gγ proteins were harvested at 24hpf, separated into cytosolic (C) and membrane (M) fractions and subjected to IP.

Fig. 2.

PGC migration in embryos overexpressing WT and SaaX mutated $G\gamma$ subunits. Bright-field image of an embryo injected with GFP-nos1-3'UTR mRNA [150pg] (**A**) results in GFP fluorescence restricted to the PGCs located at the anterior of the yolk extension (**arrow in B**). Bright-field image of an embryo injected with a mix of *gng2*-SaaX mRNA [20pg] driven ubiquitously (ubiq.) by the *Xenopus* β -globin 5'UTR & 3'UTR and the PGC driven GFP-nos1-3' UTR mRNA (**C**). Fluorescent image of (**C**) reveals PGC migration defects (**D**) that are not observed when embryos are injected with *gng2*-WT mRNA [400pg] (**E & F**). Bright-field image of an embryo expressing *gng8*-SaaX [40pg] reveals no obvious alteration in somatic development (**G**) but fluorescent microscopy of the same embryo indicates PGC migration is perturbed (**H**). All embryos shown were injected with mRNAs at the 1 – 4 cell stage and subjected to microscopy at 24hpf.

Fig. 3. Ability of the $G\gamma$ subunits to disrupt PGC migration when injected as mRNA in their SaaX (A) or wildtype forms (B). Wildtype PGC migration is reflected by GFP-*nos3*'UTR injection alone (A). Injections of the remainder of the $G\gamma$ SaaX mRNAs are summarized in Figure S4. Each concentration is a summary of 2 – 5 experiments with a minimum of 20 embryos scored. (Ectopic PGC migration score is represented as Mean $\pm$ S.D.)

Fig. 4.

All Gγ subunits are expressed during the first 24 hours of development. With the exception of *gngt1*, *gngt2a*, *gng3*, and *gng14*, all the Gγ subunits are maternally deposited. The zebrafish gamma subunits are 204-228bp in length. All have a single intron interrupting their coding sequence after approximately 90 coding base pairs, as has been reported in humans and mice [101].

Fig. 5.

The membrane localization of G β 1 is lost in the presence of G γ -SaaX. Confocal images of PGCs migrating *in vivo* after embryos were injected with the plasma membrane-localized mCherry-CVLL(*nos*) mRNA (column 1) and GFP-*gng1* mRNA (column 2). Embryos were co-injected with either phenol red as a control (A), *gng2*-WT mRNA (B), or *gng2*-SaaX mRNA (C). Ratios of the GFP fluorescence density in the cytosol to the GFP fluorescence density in the membranes of embryos injected with or without G γ subunits (D) (CFD = cytosol fluorescence density; mfd = membrane fluorescence density; * $p < 0.002$ when compared to phenol red and *gng2*-SaaX injected groups). (For interpretation of the references to color in this figure the reader is referred to the web version of this article.)

Fig. 6.

Several wildtype G-protein γ subunits have the ability to reverse the $G\gamma 2$ -SaaX induced disruption of PGC migration. Bright-field microscopy of a larvae ubiquitously expressing *gng2*-SaaX [20pg] reveals no somatic defects (**A**) while PGC migration in this same embryo is perturbed (**B**). Bright-field image of a larva ubiquitously expressing both *gng2*-WT mRNA [200pg] and *gng2*-SaaX mRNA [20pg] (**C**). The fluorescent image of (**C**) indicates a restoration of proper PGC migration (**D**). All embryos shown were injected at the 1 – 4 cell stage with mRNAs as described above, together with GFP-nos1-3'UTR mRNA and subjected to microscopy at 48hpf. Phylogenetic analysis of the zebrafish $G\gamma$ subunits and their ability to reverse the $G\gamma 2$ -SaaX induced disruption of PGC migration (**E**). Method: Neighbor Joining;

Best Tree; tie breaking = systematic. Distance: Poisson-correction; Gaps distributed proportionally. The $\text{G}\gamma$ subunits are divided into five groups according to their secondary structure as previously described [73]. Data was summarized from 2 – 5 experiments with a minimum of 20 embryos scored for the presence of ectopic PGCs at 24hpf. (For interpretation of the colors in this figure the reader is referred to the web version of this article.)

Fig. 7.

G γ -SaaX expression specifically within the PGCs results in migration defects that can be reversed by injection of one of several G γ -WT mRNAs. Overexpression of *gng3*-WT [300pg mRNA injection] specifically in the PGCs has no effect on either somatic development as assayed by bright-field microscopy (A) or PGC migration as revealed in the corresponding fluorescent image (B). *gng3*-SaaX expression [150pg] specifically in the PGCs also has no effect on somatic development (C) but profoundly disrupts PGC migration (D). The effect of *gng2*-SaaX on PGC migration is reversed by injection with PGC-driven *gng2*-WT mRNA [200pg] (E & F) but not PGC-driven *gng5*-WT mRNA [550pg] (G & H). All embryos shown were injected at the 1 – 4 cell stage with mRNAs as described above, together with GFP-nos1-3' UTR mRNA and subjected to microscopy at 48hpf.

Fig. 8. Representative experiments exhibiting the reversal of the *Gy*-SaaX phenotype by subsequent injection with *Gy*-WT mRNA. When *gng2*-WT is driven either ubiquitously or in the PGCs (*nos*), it has the ability to reverse both the ubiquitously translated *gng2*-SaaX phenotype (**A**) and the phenotype resulting from *gng2*-SaaX being translated specifically in the PGCs (**B**). Each concentration is summarized from 2 – 5 experiments with a minimum of 20 embryos scored. (Ectopic PGC migration score is expressed as mean $\pm$ S.D.)

Fig. 9.

Overexpression of *gng2*-SaaX attenuates calcium accumulation within the protrusions of PGCs migrating in vivo. Injection of embryos at the 1-cell stage with the PGC-driven *gng2*-WT mRNA [75pg] results in an increase in the accumulation of calcium in the protrusions of PGCs migrating in vivo (A). PGC-driven *gng2*-SaaX mRNA [50pg] results in a suppression of the calcium accumulation in these protrusions (B), which is reversed by injection with *gng2*-WT mRNA [75pg] (C). Calcium levels were quantified with the calcium indicator Oregon green 488 BAPTA-1 [500 μM]. To identify the PGCs, embryos were co-injected with vasa-DsRedex1-nos1 RNA [180 pg] (A-C) and data is summarized in (D). (All PGCs described above were assayed 9-11hpf.) Model of a cell autonomous mechanism for HMGCR's role in PGC migration (E). Internalization of the extracellular signal Sdf1a is transduced through a GPCR that utilizes heterotrimeric G-Proteins of which the γ subunit is a substrate for geranylgeranylation. (HMGCoA = 3-hydroxy-3-methylglutaryl-Coenzyme A; HMGCR = HMGCoA Reductase; GGDP = geranylgeranyl diphosphate synthase; GGTaseI = geranylgeranyl transferase I) (For interpretation of the colors in this figure the reader is referred to the web version of this article.)

Table 1

Zebrafish G protein γ subunit genes

Subunit	Accession #		Chromosome	CaaX Motif	Homology to Human Gamma Subunits	
	Nucleotide	Protein			% Identity	% Similarity
<i>gng1</i> [†]	NM_199967	NP_956261	19	*CVIC		66.7 / 81.3
<i>gng2a</i>	BC059612	Q6PBR4	3	*CIIT		70.0 / 88.6
<i>gng2b</i>	none	none	12	*CIIT		1 54.3 / 78.6
<i>gng2</i> [‡]	DQ109815	Q15KE4	13	CAIL		94.4 / 98.6
<i>gng3</i> [§]	NM_131841	NP_571916	21	CALL		93.4 / 98.7
<i>gng4</i>	XM_001335940	XP_001335976	13	CTIL		88.2 / 100
<i>gng5</i>	-	NP_999886	2	CSFL		84.1 / 92.8
<i>gng7</i>	-	NP_001002397	2	CIIL		94.2 / 98.6
<i>gng8</i>	zgc:110316	Q503Q6	21	CTVL		80.3 / 97.2
<i>gng10</i>	XM_001923181	XP_001923216	5	CTLL		76.8 / 92.8
<i>gng12a</i>	NM_213312	NP_998477	2	CTIL		76.7 / 89.0
<i>gng12b</i>	none	none	6	CTIL		78.1 / 87.7
<i>gng13</i>	XM_001333427	XP_001333463	3	CVLL		75.0 / 91.2
<i>gng14</i>	XM_001338349	XP_001338385	1	CVIL		2 55.9 / 73.5
<i>gng15</i>	XM_681703	XP_686795	6	CVLL		3 54.9 / 78.1
<i>gng16</i>	none	none	1	CGLL		4 56.2 / 69.9
<i>gng17</i>	none	none	3	CVLL		4 49.3 / 70.4

The identified zebrafish $G\gamma$ subunit genes, their associated sequences, chromosomal location and CaaX motif. The zebrafish $G\gamma$ subunits that lack an obvious human ortholog are compared to the human subunit that has the highest homology

- ¹ *gng2b* was compared to *GNGT2*
- ² *gng14* was compared to *GNGI3*
- ³ *gng15* was compared to *GNG7*
- ⁴ *gng16* was compared to *GNG5*
- ⁵ *gng17* was compared to *GNG10*.

* = CaaX motifs that are preferred by farnesyl transferase over geranylgeranyl transferase.

[†] Previously identified zebrafish subunits: Chen et al., 2007

[‡] Leung et al., 2006

[§] Kelly et al., 2001.

Table 2

Summary of the G γ subunits' ability to disrupt PGC migration in their WT and/or SaaX forms.

Gamma Subunit	PGC Disruption by γ -WT (T3TS)*	PGC Disruption by γ -SaaX (T3TS)?
<i>gng1</i>	+	+
<i>gng12a</i>	+	+
<i>gng12b</i>	—	—
<i>gng2</i>	—	+
<i>gng3</i>	—	+
<i>gng4</i>	—	+
<i>gng5</i>	—	+
<i>gng7</i>	—	+
<i>gng8</i>	—	+
<i>gng10</i>	—	#
<i>gng12a</i>	— *	+
<i>gng12b</i>	—	+
<i>gng13</i>	—	+
<i>gng14</i>	—	—
<i>gng15</i>	—	##
<i>gng16</i>	—	##
<i>gng17</i>	— *	##

* somatic disruption at high concentrations [x > 400pg/embryo]

PGC disruption only at relatively higher concentrations [x > 300pg/embryo]

PGC disruption at high concentrations potentially secondary to somatic disruption; See Figure S4 for detailed results