Disruption of G-Protein \(\gamma_5\) Subtype Causes Embryonic Lethality in Mice

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Abstract

Heterotrimeric G-proteins modulate many processes essential for embryonic development including cellular proliferation, migration, differentiation, and survival. Although most research has focused on identifying the roles of the various \(\alpha\)subtypes, there is growing recognition that similarly divergent \(\beta\gamma\) dimers also regulate these processes. In this paper, we show that targeted disruption of the mouse Gng5 gene encoding the \(\gamma_5\) subtype produces embryonic lethality associated with severe head and heart defects. Collectively, these results add to a growing body of data that identify critical roles for the \(\gamma\) subunits in directing the assembly of functionally distinct G-\(\alpha\beta\gamma\) trimers that are responsible for regulating diverse biological processes. Specifically, the finding that loss of the G-\(\gamma_5\) subtype is associated with a reduced number of cardiac precursor cells not only provides a causal basis for the mouse phenotype but also raises the possibility that G-\(\beta\gamma\) dependent signaling contributes to the pathogenesis of human congenital heart problems.

Introduction

Diverse types of receptors (i.e., G-protein-coupled [1–3], frizzled [4–5], smoothened [6–8], integrin [9–10], and growth factor [11–12] receptors) converge on heterotrimeric G-proteins to coordinate embryonic development. Following activation of the upstream receptor, the G-protein undergoes conformational rearrangements to produce two signaling moieties – a GTP-bound \(\alpha\) subunit and a functional \(\beta\gamma\) dimer – that initiate bifurcating signaling cascades to yield the appropriate cellular response(s) [13–15]. Based on the known number of G-subunit genes [16], there is the potential to generate hundreds of distinct G-\(\alpha\beta\gamma\) combinations that could operate in the context of embryonic development. However, identifying which particular G-\(\alpha\beta\gamma\) heterotrimeris actually exist in vivo and how they function in various developmental processes has been challenging.

Gene targeted disruption offers a powerful approach to answer these questions. Because the functions of G-\(\alpha\beta\gamma\) heterotrimers are traditionally ascribed to the \(\alpha\) subtypes, targeted disruption of all 16 Gna genes has been performed in mice [17]. Loss of the Gnai gene produces gastrulation defects [18], while ablation of the Gna13 gene produces embryonic lethality associated with vascular problems [19]. Likewise, combinatorial disruption of the Gna11 and Gnaq genes causes cardiac hypoplasia and perinatal lethality [20], while coincident loss of all three Gna genes produces pups with skeletal defects [21]. In contrast, much less is known regarding the functions of the individual G-\(\beta\) and \(\gamma\) subtypes. Targeted disruption of two of the five Gnb genes has been carried out in mice [22–23], with loss of the Gnb1 gene producing partial embryonic lethality associated with incomplete closure of the neural tube [22]. More recently, genetic inactivation of four of the twelve Gng genes has been performed [24–29]. Although no developmental defects are reported, individual disruption of the Gng1, Gng3, Gng7, and Gng13 genes produce distinct phenotypes indicating their requisite roles in specific physiological processes that cannot be substituted by other family members [25–29]. This supports the notion that functional specificity of G-\(\beta\gamma\) dimers not only exists but further suggests that such specificity is attributable to the numerous and structurally diverse \(\gamma\) component [24,30,31].

The Gng3 gene encoding the G-\(\gamma_5\) subtype shows many interesting features suggesting an important role in embryonic development. The Gng3 transcript is highly expressed in the anterior portion of the embryo giving rise to brain and heart structures (www.genat.org). Moreover, the Gng3 transcript is enriched in neural progenitor cells in both embryonic and adult brain [32–33]. Finally, the G-\(\gamma_5\) protein is present in focal adhesions important for regulating cellular adhesion, proliferation, and migration [34]. This paper shows for the first time that targeted disruption of the Gng3 gene causes complete embryonic lethality. Mutant embryos are readily identifiable by their abnormal headfolds, hypoplastic pharyngeal arches, and severe cardiac defects. These findings are novel in several respects. First, they add to a growing body of evidence that the G-\(\gamma\) subtypes are not functionally interchangeable in the context of the animal. Second, they reveal a critical requirement for the G-\(\gamma_5\) subtype in the second wave of cardiac development contributing to the formation of the right ventricle and outflow tract. Since the cardiac defects resulting from loss of the G-\(\gamma_5\) subtype are much more severe than individual or combinatorial disruption of any of the G-\(\alpha\) subtypes [17–21], these results suggest a separate
requirement for G-βγ signaling above and beyond that of any G-α pathway in this process. Although the mechanism is still being investigated, we hypothesize that G-βγ signaling may represent a point of convergence for G-protein-coupled, integrin, and fibroblast growth factor receptor signaling pathways that are critical for the expansion or survival of cardiac progenitor cells within the second heart field. This knowledge could contribute to a better understanding of human congenital heart defects arising from abnormalities within this region.

Materials and Methods

Production of Gng5 Mutant Mice

To provide the potential for conditional inactivation, the targeting vector was designed to add a loxP site upstream of the first exon and to introduce a neo' selectable marker flanked by loxP sites in the first intron of the mouse Gng5 gene (Fig. 1A). After electroporation, embryonic stem cells containing the floxed allele were injected into blastocysts to create chimeric mice (Fig. 1B,C). Following germline transmission, the mice carrying the floxed allele (Gng5<sup>+/-</sup>) were obtained on a genetic background from Caliper Life Sciences, Cranbury, NJ (Fig. 1D). Finally, after breeding to Tg(Eha-Cre) mice, the mice containing the globally disrupted allele (Gng5<sup>-/-</sup>) were produced (Fig. 1E) and loss of Gng5 expression was confirmed (Fig. 1E). Prior to characterization, the Gng5<sup>-/-</sup> mice were backcrossed to C57Bl6J mice for 10 generations to minimize genetic variability.

Genotyping

Gng5<sup>+/+</sup> mice were intercrossed to produce the three experimental groups (Gng5<sup>++</sup>, Gng5<sup>+/−</sup>, Gng5<sup>−−</sup>). For collection of embryos at specific stages, timed matings were performed, with the appearance of a vaginal plug marking embryonic day, e0.5. For genotyping of animals, genomic DNA was prepared from the yolk sac of embryos, or tails of pups. Subsequently, PCR amplification of the wild type or deleted fragment was performed with two primers that flanked the loxP site upstream of Gng5, and a third primer lying just downstream of the neo' cassette integration site (Table S1), using a PTC-100 programmable thermal cycler (MJ Research, St. Bruno, Canada).

Quantitative (q)PCR Analysis

RNA was prepared from yolk sacs, embryos, or micro-dissected, pharyngeal tissues using a Trizol-based procedure (Invitrogen, Carlsbad, CA). First-strand cDNA was prepared from 1 μg of total RNA primed with random hexamers in a reaction catalyzed by MMLV reverse transcriptase (Promega, Madison, WI). For qPCR analysis, either embryonic cDNA, or a developmental cDNA Panel (Clontech, Palo Alto, CA) was used as template to amplify G-γ transcripts and other genes of interest. For this purpose, primers were designed to span intron junctions and their sequences can be found in Table S1. All reactions were performed with iQ Sybr Green Supermix and run on the iCycler device (BioRad, Hercules, CA). Relative gene expression was calculated using the 2<sup>−ΔΔC(T)</sup> method [35]. All expression analyses were performed in triplicate and significant differences identified by Student t-test.

RNA in situ Hybridization and RNAscope Analyses

Embryos were fixed in 4% paraformaldehyde, dehydrated with ethanol, and paraffin-embedded. Subsequently, embryos were sectioned (6-μm), processed for RNA in situ hybridization, or stained with Hematoxylin and Eosin. To detect expression of the Gag5 and fibroblast growth factor 8 (Fgf8) genes, RNA in situ hybridization was performed on sectioned embryos using the RNAscope Brown 2.0, In Situ hybridization kit (Advanced Cell Diagnostics, Hayward, CA). For this purpose, the Gag5 probe was designed to transcript NM_010318.2 (nt 2-474), while the Fgf8 probe was designed to transcript NM_010205 (nt 317-1008). Prior to in situ hybridization, the slides were baked at 58°C (1-hour) to soften the paraffin; cleared in xylene (2×5-minutes), rinsed in 100% ethanol (2×3-minutes), and then air dried. After circling tissues with a hydrophobic barrier pen, the RNAscope protocol was performed according to manufacturer’s recommendation (Advanced Cell Diagnostics), with the following exceptions: Pretreatment 2 was performed at 95°C for 10-minutes; Pretreatment 3 was diluted 1:3 with 1X phosphate-buffered saline and carried out at 40°C for 30-minutes; and Ammonia wash was extended for increased contrast between DAB staining and tissue. Subsequently, the slides were hybridized with test probes (Fgf8 or Gag5) at 40°C for 2-hours, using the following incubation conditions: Amp 1 at 40°C for 30-min; Amp 2 at 40°C for 15-min; Amp 3 at 40°C for 30-min; Amp 4 at 40°C for 15-min; Amp 5 at room temperature for 30-min; and Amp 6 at room temperature for 15-min. All incubations were carried out using the HybEZ hybridization oven with humidifying chamber (Advanced Cell Diagnostics). Between each step, the slides were washed in the provided wash buffer (2×2-minutes). Finally, the colorimetric reaction was performed with the 1:1 DAB solution (equal volumes of DAB-A and DAB-B were mixed directly before addition to the tissue) at room temperature for 10-minutes. The slides were rinsed with water, stained with 50:50 Myers Hematoxylin/H2O2, rinsed with water, and then rinsed again with 0.01% ammonia water. After drying tissue by dehydration with 70% ethanol (2-minutes), rinsing with 100% ethanol (2×2-minutes), and clearing with xylene (5-minutes), the coverslips were mounted with cytoseal for microscopic assessments.

Proliferation and TUNEL Analyses

Somite-stage-matched embryos were processed as described previously [36]. To prepare cryosections, embryos were fixed in 4% paraformaldehyde and then protected in a sequential series of 10, 20 and 30% sucrose/PBS solutions, oriented in OCT (Tissue Tek) filled molds, frozen, and then cut into 10-μm sections. Subsequently, sections were washed with PBS, blocked in 2% bovine serum albumin with 0.5% Triton-X100 and incubated overnight with anti-phosphohistone antibody (anti-HH3; 1:500; Millipore #06-570). After washing, sections were washed, blocked, and incubated with AlexaFluor 488 conjugated secondary antibody (1:500; Jackson ImmunoResearch #711-545-152). Simultaneous TUNEL was performed by adding TMR Red in situ cell death detection reagents (Roche) to the secondary antibody incubation. Sections were preserved in Vectashield anti-fading reagent (Vector Laboratories) and captured by confocal microscopic analysis at different magnifications.
Figure 1. Production of Gng5 mutant mice. A. Wild type Gng5 allele (top bar) illustrating the three Gng5 exons (black boxes), homology arms of the targeting vector (grey boxes) and 5' and 3' probes for Southern blotting (striped boxes). Floxed Gng5 allele (middle bar) illustrating insertion of loxP sites (triangles) and neomycin resistance cassette (NeoR). Deleted Gng5 allele (bottom bar) illustrating deletion of sequence between 1st and 3rd loxP site including the first exon of Gng5 and NeoR cassette. Also illustrated are expected sizes of fragments following digestion with BamHI (B) or XhoI (X), along with the primers used for PCR (a,b,c). Primer sequences can be found in Table S1.

B. Southern blot of DNA prepared from five properly-targeted ES cell clones (C3-H8) and one wild type clone (WT). DNA was digested with BamHI and probed with the 5' probe. Properly targeted clones displayed both the 7.8 kb floxed allele and the 11.4 kb wild type allele.

C. DNA from these same clones was digested with XhoI and probed with the 3' probe. Properly targeted clones displayed both the 13 kb floxed allele and the 18.9 kb wild type allele.

D. PCR analysis of DNA from tail biopsy of a wild mouse Gng5+/+ , a floxed mouse Gng5+/fl, and offspring of a cross between a floxed mouse and Tg(EIIa-cre) mouse showing varying degrees of recombination Gng5/fl→del or Gng5/fl→del. The first lane is the φX HaeIII molecular weight marker. Primers a and b produce a 241-bp band from the wild type (+) allele or a 333-bp band from the floxed (fl) allele. Primers a and c produce a 454-bp band from the deleted (−) allele resulting from recombination between the 1st and 3rd loxP site. E. RT-PCR of Gng5 (primers d and e) and eukaryotic elongation factor (EF) from two wild type (Gng5+/+), two heterozygous (Gng5+/fl), and two homozygous knockout (Gng5−/−) embryos, confirming the absence of Gng5 mRNA transcript in knockout embryos.
Results

Successful Targeting of the Gng5 Locus

Careful design of the targeting strategy was necessary to remove only exon 1 of Gng5 and to minimize any impact on contiguous genes (Fig. 2A). The Gng5 gene (orange blocks) lies within an intron of the chitosiase (Cibs) gene (blue blocks) that produces two transcripts containing the last two exons of the Gng5 locus (Fig. 2B) [37,38]. Also, the Gng5 gene resides in a head-head arrangement with the spermatogenesis-associated (Spata1) gene (yellow blocks) that generates multiple transcripts arising from different non-coding exons [39]. To confirm successful targeting of the Gng5 locus, heterozygous mice were intercrossed to produce the three genotypes (Gng5+/+, Gng5−/−, and Gng5+/−/− embryos). Using Gng5 gene-specific primers (d,e; Fig. 2B), RT-PCR analysis confirmed the absence of Gng5 transcript in knockout embryos (Fig. 1E). In contrast, all three genotypes showed similar expression of Cibs-Gng5 (Fig. 2C) and Spata1 (Fig. 2D) transcripts, whose identities were confirmed by DNA sequence analysis (Fig. S1). Taken together, these results validate the targeting strategy by showing both loss of Gng5 expression and preservation of expression from the two contiguous loci.

Embryonic Phenotype

Heterozygous Gng5−/− intercrosses produced no homozygous Gng5−/− pups (Table 1). To determine when knockout embryos died, timed matings were set up and embryos collected at different gestational stages. Between embryonic days, e8.5 and e10.5, all three genotypes were present at the expected Mendelian frequency. However, all Gng5−/−/− embryos were severely compromised or dead by e10.5 (Table 1) and were readily identifiable by their morphologic defects that included abnormal headfolds, hypoplastic pharyngeal arches, and severe cardiac defects.

To better understand the embryonic requirement for the G-protein γ5 subunit, we performed RNA in situ hybridization on whole or sectioned embryos (Fig. 3). In e8.0 whole embryos, Gng5 transcript was broadly distributed in the anterior portion of the embryo (Fig. 3A). Of particular interest, the Gng5 transcript was detected in cardiac precursors residing in the cardiac crescent (white arrowheads). Attesting to the specificity of the hybridization signal, no Gng5 transcript was detectable in knockout embryos (Fig. S2). Next, we employed the RNAscope method to visualize the Gng5 transcript in sectioned embryos. Compared to standard in situ RNA hybridization, this method is more sensitive and more quantitative since the amount of chromagen is directly correlated with the number of transcripts in each cell [40]. Sectioned embryos confirmed expression in cardiac precursors (Fig. 3B, black arrowheads), and also in the adjacent pharyngeal epithelia (boxed region magnified in Fig. 3C). In e9.5 whole embryos, Gng5 transcript continued to be expressed throughout the embryo although levels in the heart proper were relatively lower than other regions (Fig. 3D). Sectioned embryos again confirmed Gng5 expression in the cardiac precursors residing in the splanchnic mesoderm dorsal to the heart (Fig. 3E, black arrowhead and boxed region magnified in Fig. 3F). Thus, Gng5 transcripts are found in regions relevant to mouse cardiogenesis.

Defective Cardiogenesis

At e9.5, morphologic analysis of Gng5−/− embryos revealed a specific set of anatomic defects, including severely hypoplastic pharyngeal arches and an unlooped cardiac tube (n = 25/25), which were never observed in their littermate controls (n = 17/17) (Fig. 4 A–D). Since an impaired cardiovascular system is the most common cause of death at this stage [41], we examined the overall structure of the heart and vasculature in more detail. At e9.5, histologic examination of control littermates revealed fully looped hearts characterized by four, primitive chambers along with distinct inflow and outflow tracts (n = 60/60) (Fig. 4 E,F). However, knockout littermates had unlooped hearts characterized by a primitive atrium caudal to a single ventricle with no demonstrable outflow tract (n = 16/16) (Fig. 4 G, H). These results demonstrate for the first time that Gng5 disruption produces embryonic lethality reflecting an essential role for the G-protein γ5 subtype in right ventricle (RV) and outflow tract (OFT) formation.

To probe the basis for this phenotype, we assessed the integrity of the second heart field normally giving rise to RV and OFT formation [42]. Integral to this process, fibroblast growth factor signaling (ie, Fgf8/Fgf10) drives continued proliferation of cardiac precursor cells within the pharyngeal mesoderm that are required for RV and OFT formation [36,43–47]. Using the RNAscope procedure [40], we compared Fgf8 expression within the pharyngeal mesoderm of control and knockout embryos sectioned transversely (Fig. 5A–C). At e8.5, the control embryo exhibited intense Fgf8 expression in the numerous cardiac precursor cells within the pharyngeal mesoderm (Fig. 5A, brown staining, black arrowheads), as well as other cells within the pharyngeal endoderm and ectoderm. In contrast, Gng5−/−/− littermates showed only faint Fgf8 expression reflecting both fewer numbers of cardiac precursor cells and markedly less chromagen present in the remaining cells (Fig. 5B–C, brown staining, black arrowheads). In the top panel of the mutant (Fig. 5B), the red arrowhead labels the rostral portion of the heart tube and the absence of OFT. In the bottom panel (Fig. 5C), the red arrowhead labels more caudal mesoderm adjacent to the left side of the heart tube (Fig. 5C). Taken together, these data demonstrate that loss of the G-γ5 subtype is associated with a defect in the second heart field, which is consistent with both the nature and severity of the cardiac defects observed in Gng5−/−/− embryos.

To characterize the molecular events underlying this defect, we examined gene expression in pharyngeal tissues encompassing the second heart field that had been microdissected from control and mutant embryos (Fig. 5D). Since sustained proliferation of cardiac precursor cells within this region is critical for RV and OFT formation [42], we first assessed cell proliferation by determining the relative levels of several proliferative markers [48,49] in pharyngeal tissues from control and mutant embryos. Notably, all three proliferative markers were significantly reduced in mutant pharyngeal tissues (Fig. 5D). Next, we assayed cell proliferation and apoptosis by immunohistochemical staining of control and mutant embryos (Fig. 6). Cryosections were stained for DNA (DAPI, blue), cells in mitosis (anti-PHH3, green), and cells undergoing apoptosis (TUNEL, red) [36,43]. This analysis revealed globally decreased proliferation in Gng5−/−/− embryos and multiple regions of abnormal apoptosis. Although no apoptosis was detected in the heart tubes of the mutants, nearly 10% of cardiomyocytes in control heart showed anti-pHH3 staining, whereas only occasional proliferating cells were present in the hearts of mutants. Even more striking, the second heart field mesoderm lying dorsal to the heart exhibited both decreased cell proliferation and increased cell death in the mutants (Fig. 6C versus D). Taken together, these results identify a novel role for the G-γ5 subtype in regulating the expansion and/or survival of cardiac precursor cells. Consistent with previous reports showing enriched Gng5 expression in neural stem cells [32,33], our data also support a similar role in other cell populations, including neural progenitor cells.
Discussion

Homozygous disruption of the Gng5 gene encoding the G-γ5 subtype produces a complex phenotype characterized by severe defects in the head, heart, and other developing structures (Fig. 4). In this paper, we focus on its requirement in cardiac development since the impaired cardiac performance limits our ability to examine any direct role in brain development. Notably, we found that all mutant embryos have severe cardiac defects that are incompatible with survival (Table 1). The observation that e8.5 mutant embryos have an overtly normal linear heart tube suggests normal specification and deployment of precursor cells from the first heart field [50], although further analyses of this structure will be needed to rule out any molecular or functional changes. In contrast, both looping of the heart tube and formation of the RV and OFT are notably absent in e9.5 knockout embryos. Our

![Figure 2. Successful targeting of the Gng5 locus. A. Region of mouse chromosome 3:146,110,000–147,170,000 containing the Gng5 locus from the UCSC genome browser. B. The top bar of this schematic illustrates the arrangement of exons of Gng5 (orange boxes), Ctbs (blue boxes), and Spata1 (yellow boxes). The bars underneath illustrate the Gng5 mRNA transcript, the two Ctbs splice variants, the two Ctbs-Gng5 splice variants, and the three Spata1 splice variants (not drawn to scale). Letters indicate RT-PCR primers which can be found in Table S1. C. RT-PCR of the Ctbs-Gng5 fusion transcript (primers f and g) from the same embryos shown in Fig. 1E, confirming that expression of this fusion transcript is preserved in knockout embryos. Identification of the amplified products marked Ctbs-Gng5 was confirmed by DNA sequence analysis (Figure S1). The first lane on both gels is a molecular weight marker, φX digested with HaeIII. D. RT-PCR of Spata1 from the same embryos (primers f and h), demonstrating that expression of Spata1 is preserved in knockout embryos. doi:10.1371/journal.pone.0090970.g002](http://www.plosone.org/doi/10.1371/journal.pone.0090970.g002)
Table 1. Genotype distribution of embryos from Gng5+/− intercrosses.

| Age     | Number of pups or embryos observed (expected) | X² value | P value |
|---------|---------------------------------------------|----------|---------|
|         | Total                                       | +/+      | +/−     | −/−     |
| Postnatal Pups | 89                                      | 32(22)   | 57(44)  | 0(22)   | 29.8  | 3.5 × 10⁻⁷ |
| E8.5    | 44                                          | 7(11)    | 27(22)  | 10(11)  | 2.68  | 0.26    |
| E9.5    | 48                                          | 18(12)   | 19(24)  | 11(12)  | 4.13  | 0.13    |
| E 10.5  | 36                                          | 5(9)     | 21(18)  | 10(9)   | 2.39  | 0.30    |

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Figure 3. Developmental expression of Gng5. A, Ventral view of e8.0 mouse embryo after whole mount RNA in situ hybridization (blue signal) to detect Gng5 mRNA. White arrowheads denote location of cardiac progenitors. HF, head fold. B, Section of e8.5 embryo through the region of the pharynx. Brown signal indicates Gng5 transcripts in HF, endoderm (En), ectoderm (Ec) and cardiac progenitors (black arrowheads). There is little signal in the heart proper (RV, right ventricle; OFT, outflow tract). C, Magnification of the region boxed in B containing cardiac progenitors in splanchnic mesoderm (SM). D, Left lateral view of e9.5 mouse embryo after whole mount RNA in situ hybridization (blue signal) to detect Gng5 mRNA. Widespread expression is present with less in the heart (white line denotes dorsal inflow region). E, Section of e9.5 embryo through the region of the pharynx. Brown signal indicates Gng5, endoderm (En), ectoderm (Ec) and cardiac progenitors (black arrowhead). There is also some signal in the heart (HT). F, Magnified view of pharyngeal and splanchnic mesoderm corresponding to boxed region in E.

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Figure 4. \textit{Gng5} \textsuperscript{2/2} mutants fail to form the cardiac outflow tract and right ventricle and have severely hypoplastic pharyngeal arches. \textbf{Panels A–D}, images of e8.5 whole mount wild type and mutant embryos. By comparing the left lateral (A, C) and ventral (B, D) views of intact wild type and \textit{Gng5} \textsuperscript{2/2} embryos, respectively, the unlooped heart tube is clearly evident (B, D). \textbf{Panels E–H}, images of e9.5 wild type and mutant embryos. The left parasagittal section from a control embryo shows the inflow tract and left side of common atrium, endocardial cushion in the atrioventricular canal, and left ventricle (E). The red arrowheads mark the first pharyngeal arch, also labeled 1. The midline section shows the outflow tract connecting to aortic sac in the second pharyngeal arch (bracket), right ventricle, the right portion of the common atrium, and the sinus venosus in the control embryo (F). The left parasagittal section of a \textit{Gng5} \textsuperscript{2/2} mutant shows severely hypoplastic but vascularized first pharyngeal arch (red arrowhead), dilated heart tube with atrial chamber caudal to ventricle, narrow inflow, and paucity of cells in the pharyngeal mesoderm (G). The midline section shows the unlooped, dilated heart tube and no outflow tract; cardiac chamber opens directly into dilated aortic sac (bracket) in a \textit{Gng5} \textsuperscript{2/2} embryo (H). TB, tail bud; HF, head fold; OFT, outflow tract; RV, right ventricle; LV, left ventricle; A, atrium V, ventricle; EC, endocardial cushion; SV, sinus venosus. doi:10.1371/journal.pone.0090970.g004

Figure 5. \textit{Gng5} \textsuperscript{2/2} mutants show loss of second heart field and reduced expression of proliferative markers. \textbf{Panels A–C}, control and mutant embryos stained for \textit{Fgf8} expression in the second heart field. As shown by \textit{in situ} hybridization for \textit{Fgf8} transcripts, the e8.5 control embryo (A) displays numerous pharyngeal mesodermal cells exhibiting \textit{Fgf8} expression (brown staining; black arrowheads). These cells represent progenitors of the right ventricle (RV) and outflow tract (OFT) that reside dorsal to the heart. \textit{Fgf8} expression is also detected in the lateral pharyngeal endoderm (En) and pharyngeal ectoderm (Ec). In stark contrast, \textit{Gng5} \textsuperscript{2/2} mutants (B, C) have fewer pharyngeal mesoderm cells and reduced \textit{Fgf8} expression in this region. The red arrow (B) indicates the most rostral portion of heart tube and the absence of OFT; the black arrowhead denotes absent \textit{Fgf8} expression in the thin layer of pharyngeal mesoderm dorsal to the heart. In a more caudal section (C), the heart tube (HT) is visible and faint \textit{Fgf8} expression is detected in the mesoderm adjacent to the heart tube (black arrowhead) and in the most proximal portion of the left side of the heart tube and adjacent mesoderm (red arrowhead). HF, head fold, P, pharynx; En, endoderm; Ec, ectoderm. \textbf{Panel D}, relative expression of proliferative markers in pharyngeal region of e9.0 control and \textit{Gng5} \textsuperscript{2/2} mutant embryos. As shown by qPCR analysis, \textit{Gng5} \textsuperscript{2/2} mutant embryos display reduced expression of three proliferative markers in the second heart field (* \textit{p}<0.002; ** \textit{p}<0.0001 by Student’s \textit{t}-test), using elongation factor 1 (\textit{Eef1a1}) as the housekeeping gene. All primer sequences can be found in Table S1. doi:10.1371/journal.pone.0090970.g005
subsequent studies confirmed loss of G-$\gamma_5$ signaling disrupts the expansion and/or survival of cardiac precursor cells that contribute to chamber specification and OFT formation [42].

**Developmental Function**

How G-$\gamma_5$ influences the number of cardiac precursor cells is not entirely clear. Multiple signaling cascades ensure the proper balance between cellular proliferation, differentiation, and survival [42,46]. Of these, Fgf8 is one of the most important factors involved in this process [46]. Therefore, the finding that Gng5$^{-/-}$ mutants have fewer Fgf8 expressing precursor cells in the pharynx and diminished Fgf8 expression in the few remaining cells (Fig. 5) offers a causal basis for this phenotype. Cross talk between G-protein and growth factor signaling cascades could provide a

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**Figure 6. Gng5$^{-/-}$ mutants have decreased cell proliferation and survival.** Sagittal cryosections of e9.5 control and mutant embryos after immunohistochemical staining for DNA (DAPI, blue), cells in mitosis (anti-pHH3, green), and cells undergoing apoptosis (TUNEL, red). In all panels, rostral is at right, ventral at top. **Panels A, D** represent 10X views of control (A) and mutant (D) embryos, while **Panels B,C,E,F** show 20x views of control (B,C) and mutant (E,F) sections. The red arrowheads in C and F denote second heart field pharyngeal mesoderm and adjacent endoderm. The pharyngeal arches are numbered. H, heart; T, telencephalon; HB, hindbrain; O, otocyst; OFT, outflow tract; IFT, inflow tract; HT, heart tube (mutant only). doi:10.1371/journal.pone.0090970.g006
At this stage, the identity of the G-αβγ heterotrimer that functions in the context of cardiac progenitor cells is not known. None of the sixteen Gna subunit genes that have ablated in mice [17] recapitulate the cardiac defects seen upon disruption of the G-γ subtype. This suggests that the G-βγ dimer performs a separate role above and beyond that of its G-γ partner in this process. Providing additional support for this possibility, genetic ablation of the Drosophila ancestral G-γ subunit also blocks heart development [36,57]. Likewise, neither the Gnb1 or Gnb5 genes that have targeted in mice [22,23] phenocopies the cardiac defects observed upon loss of the G-γ subtype. This implies that one of the three remaining G-β2, β5 or β4 subtypes partners with the G-γ5 protein, or that the closely related G-β subtypes can substitute for one and another in this particular context.

Clinical Relevance
Congenital heart defects, including OFT malformations, occur in >1% of live births [58,59]. Thus, the identification of G-βγ signaling as a major player in this process is translationally significant since successful intervention will only come from a better understanding of the signaling cascades driving OFT formation. This finding could also be clinically relevant since statins block the function of the G-γ subtypes [33,56] and inadvertent use of statins during pregnancy is reportedly associated with increased incidence of infants with head and heart defects [60]. In fact, statins produce cardiac defects that are phenocopied by genetic ablation of the ancestral gng gene in flies [56,57]. Assuming the Gng5 gene performs a similar role in mammals, our results could be important in guiding the use of statins that are increasingly being administered to women of child-bearing age [61].

Supporting Information

Figure S1 Preservation of the Ctbs locus. As confirmed by DNA sequence analysis of amplified PCR product, Ctbs-Gng5 transcripts are expressed in e9.5 knockout embryos even though Gng5 transcripts are lost (Fig. 1E).

Figure S2 Validation of in situ RNA hybridization procedure. Gng3 transcripts are widely expressed in anterior portion of e8.5 wild type embryo (left panel). Attesting to the specificity of signal, no staining is observed in stage-matched, knockout embryo (right panel).

Table S1 List of PCR primers. Gene-specific expression was detected using indicated primer pairs.

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Author Contributions
Conceived and designed the experiments: AM WS JR. Performed the experiments: AS KS AF. Analyzed the data: AM AS WS JR. Contributed reagents/materials/analysis tools: AM JR. Wrote the paper: AM JR.
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