Heterotrimeric G Proteins Regulate a Noncanonical Function of Septate Junction Proteins to Maintain Cardiac Integrity in *Drosophila*

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SUMMARY

The gene networks regulating heart morphology and cardiac integrity are largely unknown. We previously reported a role for the heterotrimeric G protein γ subunit 1 (Gγ1) in mediating cardiac-pericardial cell adhesion in *Drosophila*. Here we show G-o147A and Gβ13F cooperate with Gγ1 to maintain cardiac integrity. Cardial-pericardial cell adhesion also relies on the septate junction (SJ) proteins Neurexin-IV (Nrx-IV), Sinuous, Coracle, and Nervana2, which together function in a common pathway with Gγ1. Furthermore, Gγ1 signaling is required for proper SJ protein localization, and loss of at least one SJ protein, Nrx-IV, induces cardiac lumen collapse. These results are surprising because the embryonic heart lacks SJs and suggest that SJ proteins perform noncanonical functions to maintain cardiac integrity in *Drosophila*. Our findings unveil the components of a previously unrecognized network of genes that couple G protein signaling with structural constituents of the heart.

INTRODUCTION

Cardiogenesis is a remarkably conserved process at both the morphological and the molecular level (Bodmer and Venkatesh, 1998; Olson, 2006). The *Drosophila* heart, or dorsal vessel, is a linear contractile tube formed by a layer of myoepithelial cells (cardial cells). Two rows of pericardial cells (PCs) flank the cardial cells (CCs), and carry out both structural and excretory functions (Cripps and Olson, 2002). The linear dorsal vessel in *Drosophila* is morphologically equivalent to the vertebrate heart prior to looping and chamber formation (Cripps and Olson, 2002; Zaffran and Frasch, 2002).

The gene regulatory networks directing heart cell fate specification have been extensively studied in *Drosophila* (Olson, 2006), however the molecular mechanisms regulating heart tube morphogenesis remain largely unknown. We previously performed a heart-specific genetic screen and identified a novel phenotype, broken hearted (bro), in which CCs lose adhesion to PCs (Yi et al., 2006). This screen identified HMG-CoA reductase (HMGCR) and downstream enzymes in the mevalonate pathway as key regulators of CC–PC adhesion. The mevalonate pathway ultimately mediates geranylgeranylation of the heterotrimeric G protein γ-subunit Gγ1, which is required to maintain CC–PC adhesion. In zebrafish, *hmgrc1b* mutants show abnormal heart morphology, defects in myocardial cell migration, and pericardial edema, suggesting that the role of the mevalonate pathway in heart tube morphogenesis is evolutionarily conserved (D’Amico et al., 2007). In light of the similarities in cardiogenesis between insects and vertebrates, it is likely that heterotrimeric G proteins are also targets for the mevalonate pathway during heart formation in zebrafish and other vertebrates.

Heterotrimeric G proteins regulate a multitude of developmental processes in metazoans by acting as intracellular effectors of G protein coupled receptors (GPCRs) (reviewed in Malbon [2005]). G proteins form heterotrimers with subunits designated as α, β, and γ. In the basal state, the G protein α subunit is bound to GDP and associates with the Gβγ subunits; binding of α to βγ prevents all three subunits from interacting with downstream effectors (e.g., adenyl cyclase). Ligand binding to a GPCR drives a conformational change in the G protein α subunit that stimulates the release of GDP. The nucleotide-free α subunit then binds GTP, which is present at a higher intracellular concentration than is GDP, and GTP binding decreases the affinity of the α subunit for βγ dimer and increases its affinity for downstream effectors. The α subunit also possesses intrinsic GTPase activity that hydrolyzes the bound GTP and returns the α subunit to the basal conformation. Regulators of G protein Signaling (RGS) proteins modulate α subunit GTPase activity. In addition, GPCR-independent activation of heterotrimeric G protein complexes has been reported (Malbon, 2005). Heterotrimeric G proteins are represented by three large protein families and these proteins specifically interact with a number of GPCRs (reviewed in Albert and Robillard [2002]). The available G protein-receptor combinations, in addition to GPCR-independent G protein activation, create a robust signal transduction system with the potential to carry out a multitude of cellular functions.

Septate junctions (SJs) are spoke and ladder-like septa that connect adjacent plasma membranes and function as diffusion barriers in the epithelia and nervous system of insects (Tepass
et al., 2001). The vertebrate nervous system has paranodal SJs, however insect epithelial SJs are functionally equivalent to chordate tight junctions (Hortsch and Margolis, 2003). Although SJs and vertebrate tight junctions have divergent morphologies and molecular components, the formation and function of both junctions requires members of the Claudin protein family, arguing SJs and tight junctions are indeed analogous (Wu et al., 2004). SJ proteins (i.e., the set of proteins required for SJ formation and function) were also identified in a screen for genes controlling tracheal tube size, highlighting a function for SJ proteins during organogenesis (Bettel and Krasnow, 2000). Interestingly, SJ proteins act in multiple pathways to control tracheal tube size and the role of at least one SJ protein, the Na,K-ATPase β-subunit Nervana2 (Nrv2), is independent of its role in regulating paracellular diffusion (Paul et al., 2003). Mechanistically, the secretion of extracellular matrix regulatory proteins, including Vermiform and Serperentine, into the tracheal lumen prevents tube overgrowth and requires the function of SJ proteins (Wang et al., 2006; Wu et al., 2007). Thus, SJ proteins fulfill both physiological and developmental functions and their regulation is indispensable for proper organ morphogenesis.

In the present study, we sought to identify both the mediators and the targets of Gγ1 signaling during embryonic cardiogenesis in Drosophila. We find that G-ox47A and Gβ13F are the Gα and Gβ subunits that function with Gγ1 to maintain CC-PC adhesion. Mutational analysis and overexpression studies indicate that crossregulation between the Gα and Gγ subunits, in concert with the RGS protein Loco, ensures proper heart morphology. We also identify eight SJ proteins that mediate CC-PC adhesion, including Neurexin-IV (Nrx-IV), Sinuous (Sinu), Coracle (Cora), and Nrv2. As SJs are absent from the embryonic heart, we conclude that these SJ proteins perform novel functions in the mature dorsal vessel. By double mutant analysis, Nrx-IV, Sinu, Cora, and Nrv2 are shown to function in a common pathway with Gγ1, and proper subcellular localization of these four proteins in the dorsal vessel is Gγ1-dependent. In addition, Nrx-IV mediates CC-CC adhesion and ensures formation of the cardiac lumen. Our results show that heterotrimeric G protein signaling maintains cardiac integrity, in part, by regulating the activities of SJ proteins.

RESULTS

Gβ13F and Gγ1 Operate in a Single Pathway to Promote CC-PC Adhesion

At the end of Drosophila embryogenesis, cardiac and pericardial cells must adhere tightly to maintain the structural integrity of the dorsal vessel (Figures 1A and 1B). The bro phenotype is characterized by a failure in CC-PC adhesion and results in the loss of cardiac function and early larval lethality (Yi et al., 2006). The bro phenotype can be readily visualized as a perturbation in the ordered expression pattern of Hand-GFP in cardiac and pericardial cells; mutations in Gγ1 induce a fully penetrant bro heart phenotype (Figure 1C). In addition, a subset of Gγ1 embryos (<3%) show a severe loss of CC-CC adhesion (data not shown).

Since G proteins function as heterotrimers, we sought to identify the α, β, and γ subunits that mediate CC-PC adhesion in the embryonic dorsal vessel. The Drosophila genome encodes two Gγ subunits, Gγ1 and Gγ30A, and three Gβ subunits (see Table S1 available online). Gγ30A is known to mediate phototransduction in the adult eye (Schillo et al., 2004; Schulz et al., 1999); however, no lethal mutations have been reported for Gγ30A (Wilson et al., 2008). The two deficiencies uncovering Gγ30A, Df(2L)ED680 and Df(2L)N22-3, also uncover a known regulator of pericardial cell fate, numb, thus obscuring assessment of the bro phenotype. Based on the known role of Gγ30A in phototransduction and the lack of lethal mutations in the gene, we find it unlikely that Gγ30A functions during embryonic heart development.

Of the three Gβ subunits, two null mutations in Gβ13F induce the bro phenotype with full penetrance (Figures 1D and 1E), whereas deficiencies uncovering either Gβ5 or Gβ76C do not give rise to the bro phenotype (data not shown). To understand if Gβ13F and Gγ1 function in the same pathway, we performed double mutant analysis. Gβ13F; Gγ1 double mutant embryos show a bro heart phenotype comparable to that of either single mutant alone (Figure 1F), suggesting that Gβ13F and Gγ1 function in a common pathway during dorsal vessel development. Taken together, our genetic results identify Gγ1 and Gβ13F as the Gγ and Gβ subunits mediating CC-PC adhesion.

G-ox47A Mediates CC-PC Adhesion

Among the six Gα subunits encoded in the Drosophila genome (Table S1), only G-ox47A is known to be expressed in the embryonic heart (Fremion et al., 1999; Zaffran et al., 1995). In addition, embryos homozygous for Df(2R)47A, a deficiency uncovering G-ox47A, show a disrupted dorsal vessel and lack specific heart cell types (Fremion et al., 1999). To study the role of G-ox47A in CC-PC adhesion, we examined cardiac integrity in embryos
homozygous for two mutations in G-o47A; both mutations induce the bro phenotype with full penetrance (Figures 2A and 2B) and phenocopy Gj13F and GY1 mutants. In addition, the G-o47A mutation induces midline-positioning defects in a subset of embryos (A.N.J. and E.N.O. unpublished data). G-o47A; GY1 double mutant embryos show a bro phenotype comparable to either single mutant alone (Figure 2C), indicating that G-o47A and GY1 function in a common pathway to regulate cardiac integrity. We conclude that G-o47A is the Gx subunit that functions with Gj13F and GY1 to mediate CC-PC adhesion.

Regulation of G Protein Signaling during Cardiogenesis
In the basal state, Gx and GIY associate in an inactive trimeric complex; in response to upstream signals, the Gx subunit dissociates from GIY. The Gx subunit then activates a set of downstream pathways distinct from that of GIY. Deletion of GIY can inactivate GIY-dependent pathways and concomitantly hyperactivate Gx pathways (Clapham and Neer, 1997). When we overexpressed G-o47A in the heart using the Hand-Gal4 driver, we found the same bro phenotype observed in Gj13Fand GY1 mutants (Figure 2D), suggesting unregulated G protein signaling disrupts cardiac integrity.

The G-o47A gain-of-function phenotype could result from either hyperactivation of Gx pathways or the depletion of available Gj13F/GY1. To distinguish between these possibilities, we first overexpressed G-ix65A, a Gx known to couple to the same downstream signaling pathways as G-o47A (Katanave and Tomlinson, 2006). Overexpressing G-ix65A in the heart phenocopies embryos overexpressing G-o47A (Figure 2E), however G-ix65A mutant embryos do not show the bro phenotype (data not shown). G-ix65A can therefore activate G-o47A-dependent pathways in the dorsal vessel but G-ix65A is not required to maintain cardiac integrity.

Overexpressing a constitutively active form of G-ix65A that does not hydrolyze GTP and cannot bind GIY induced the bro heart phenotype (Figure 2F). However, overexpressing a dominant negative G-o47A, that constitutively binds GIY in both the GTP- and GDP-bound state (Hatley et al., 2003), did not cause the bro defect (Figure 2G). Thus, the G-o47A gain-of-function phenotype is caused by hyperactivating G-o47A downstream pathways rather than by sequestering GIY. We conclude that GIY prevents ectopic activation of G-o47A and its downstream pathways in the embryonic heart and that an appropriate level of Gx signaling maintains cardiac integrity.

To further test this hypothesis, we generated GY1/vv; G-o47A/vv embryos and found that reducing one copy of G-o47A rescued the bro phenotype in 8.3% of GY1 homozygous embryos (n = 3/36, Figure 2H). Conversely, the bro phenotype is fully penetrant in GY1/vv; G-o47A/vv embryos (n = 27; Figure 2I). These experiments strengthen the conclusion that GY1 regulates G-o47A in the dorsal vessel and demonstrate that G-o47A is epistatic to GY1.

RGS proteins enhance Gx GTPase activity and function as negative regulators of Gx signaling (Dohman and Thomer, 1997). Accordingly, mutations in the RGS protein loco induced the bro phenotype with full penetrance (Figure 2J). Therefore, G-o47A, Gj13F, GY1, and loco constitute a molecular pathway that mediates CC-PC adhesion, and maintenance of cardiac integrity requires appropriate regulation of Gx downstream pathways.

Figure 2. Regulated Gx Signaling Maintains CC-PC Adhesion
Hand-GFP expression in St17 embryos.
(A and B) Two null mutations G-o47A with G-o47A (B) induce a bro heart phenotype similar to that of Gj13F and GY1 mutations.
(C) The bro phenotype in GY1 G-o47A embryos is comparable to that of either single mutant.
(D) Embryos overexpressing G-o47A in the heart, via Hand-Gal4, phenocopy G-o47A mutant embryos.
(E and F) Overexpressing either WT (E) or constitutively active G-ix65A (Q205L) (F) in the heart induces the bro phenotype.
(G–I) Embryos overexpressing inactive G-o47A(A203T) in the heart do not show the bro phenotype. The bro phenotype can be rescued in GY1 bro embryos (H), but not in GY1 bro embryos (I).

Nrx-IV Mediates CC-PC Adhesion and Operates in a Common Pathway with GY1
One unmapped bro mutant in our collection, bro6, is Df(3L) Exel6116 that uncovers 25 genes. Embryos homozygous for Df(3L) Exel6116 display the bro phenotype with 100% penetrance (Figure 3B). To positionally clone the gene within this region that regulates CC-PC adhesion, we generated transheterozygotes for Df(3L) Exel6116 and four overlapping deficiencies within the region. This analysis identified a 55 kb region, housing 11 genes, that contains the bro6 gene (Figure 3A). Among the 11 genes in this region, only Neuromin-IV (Nrx-IV) and Est-6 have reported lethal alleles, however a UAS-RhoGAP68FstopRna line is available. Two Nrx-IV mutants showed a bro phenotype comparable to that of Df(3L) Exel6116 (Figures 3B, 3E, and 3F), whereas neither the Est-6 mutant nor the RhoGAP68FstopRna expressing embryos showed a heart phenotype (data not shown). Moreover, embryos transheterozygous for Nrx-IV/Df(3L) Exel6116 displayed a bro defect indistinguishable from that of embryos homozygous for Df(3L) Exel6116 (Figures 3C and 3D), confirming that Nrx-IV is the bro gene uncovered by Df(3L) Exel6116. In
addition to the bro phenotype, Nrx-IV mutations occasionally induce mesoderm closure defects (Figure S1), which we observed in embryos homozygous for sinuous (sinu), coracle (cora), nervana2 (nrv2), and contactin (cont) mutations (Figures 4A1–C1, and 4D–4E). Similar to Nrx-IV mutants, a subset of cora mutants also showed mesoderm closure defects (Figure S1), presumably due to known dorsal closure defects in cora embryos (Lamb et al., 1998). The bro phenotype was also observed at low penetrance (20%–30%) in embryos homozygous for mutations in Lachesin (Lac) (Figure 4F), Gliotactin (Gli) (Figure 4G), and Neuroglian (Nrg) (Figure 4H), which also encode SJ components. Since SJs themselves are absent from the embryonic heart (Rugendorff et al., 1994), we conclude that SJ proteins fulfill a noncanonical function outside of SJs to maintain CC-PC adhesion.

We next tested, by double mutant analysis, whether heterotrimeric G proteins and SJ proteins function in a common pathway. Heart morphology in Gγ1/Sinus, Gγ1/Cora, and Gγ1/Nrv2 double mutant embryos was comparable to that of each single mutant, except that Gγ1/Sinus and Gγ1/Cora double mutant embryos showed a higher frequency of mesoderm closure defects (Figures 4A2–4C2, 4J, and S1). We conclude that Gγ1, Sinus, and Nrv2 operate in a single pathway to regulate CC-PC adhesion, but Gγ1, Sinus, and Cora function in separate pathways during dorsal closure.

The functional relationship between SJ proteins and the ECM in tracheal tube size control (Wang et al., 2006) prompted us to ask if the ECM protein Pericardin (Prc) maintains CC-PC adhesion. Prc mediates the attachment of the dorsal mesoderm to the ectoderm and prc null mutants do not complete mesoderm closure (Chartier et al., 2002). Since hypomorphic prc alleles have not been identified, we investigated the requirement of prc for CC-PC adhesion by knocking down prc expression with double-stranded RNA. As shown in Figure S1, injecting blastoderm embryos with 5 μM prc dsRNA recapitulated the mesoderm closure phenotype prc null mutants (Chartier et al., 2002). However, injecting 0.5 μM prc dsRNA induced the bro phenotype (Figure 4I). As a control, injecting white dsRNA did not affect heart morphology (data not shown). These findings support the conclusion that Prc functions, at least in part, to mediate CC-PC adhesion.

To understand the effect of the bro phenotype on cardiac function, we assessed heart rate in Stage 17 embryos homozygous for mutations in Gγ1 and loco, as well as the SJ components Nrx-IV, Cont, and sinu. We found that heart rate was dramatically reduced in Gγ1 and loco embryos and that the SJ mutants had a similar reduction in heart rate (Figure 4K). These results indicate that both heterotrimeric G protein signaling and SJ proteins are indispensable for proper cardiac performance.
Correct Localization of Nrx-IV in the Dorsal Vessel Requires G_{Y1}

To further investigate the noncanonical, G protein-associated function of SJ proteins in the embryonic heart, we characterized the expression of SJ proteins in the dorsal mesoderm. Based on our genetic results, we predicted SJ proteins would be expressed in the embryonic heart and that G_{Y1} would regulate the expression or subcellular localization of SJ proteins. By whole mount immunostaining, we found that Nrx-IV localizes to the cell membrane of both CCs and PCs (Figure 5A). In both G_{Y1} and G-α_{4}47A embryos, Nrx-IV does not correctly localize in CCs or PCs, and Nrx-IV expression is largely undetectable in cells that have lost PC-CC adhesion (Figures 5B and 5C; white arrows). In addition, Prc does not accumulate at wide-type levels in either G_{Y1} or G-α_{4}47A mutants (Figures 5B and 5C). To further characterize Nrx-IV localization in the dorsal vessel, we also made transverse sections through wild-type embryos and found that Nrx-IV localizes to all membrane domains of PC and CCs (Figure 5D). Thus, G protein-mediated localization of Nrx-IV and Prc is required to maintain cardiac integrity and the Nrx-IV expression pattern suggests it may promote cell-cell adhesion throughout the dorsal vessel.

The dramatic loss of Nrx-IV expression in G_{Y1} embryos opened the possibility that G_{Y1} signaling may transcriptionally regulate Nrx-IV. However, by in situ hybridization, we found that Nrx-IV mRNA expression in G_{Y1} embryos is comparable to wild-type embryos (Figures 5E and 5F), even in those PCs that have lost CC adhesion. Therefore, G_{Y1} signaling provides posttranscriptional regulation of Nrx-IV in the embryonic heart.

The localization of Nrx-IV to the CC luminal membrane prompted us to ask if Nrx-IV also mediates CC-CC adhesion. By EM, we find that in Nrx-IV embryos, the heart lumen is collapsed (n = 8/8; Figures 5G and 5H), the CCs are often misaligned, and the distance between adjoining CC membranes is increased (n = 6/6; Figures 5I and 5J). The Nrx-IV CC phenotypes we observe are similar to those reported for Stil mutants, a known regulator of CC-CC adhesion (MacMullin and Jacobs, 2006). These results further demonstrate the essential role of Nrx-IV in maintaining not only PC-CC but also CC-CC adhesion.

Correct Localization of the SJ Proteins Cora, Sinu, and Nrv1/2 in the Dorsal Vessel is G_{Y1}-Dependent

Our genetic studies of Cora, Sinu, and Nrv2 suggested these SJ proteins also carry out novel functions essential for CC-PC adhesion. Similar to Nrx-IV, both Cora and Sinu localize to CC and PC membranes (Figures 6A and 6B). Two Nrv isoforms, Nrv1 and Nrv2, are recognized by the Nrv antibody (Sun and Salavaterra, 1995), and Nrv1/2 also localize to CC and PC membranes (Figure 6C). Since Nrv1 and Nrv2 have nonoverlapping subcellular localizations in other epithelial tissues (Paul et al., 2007), the expression pattern of the SJ specific isoform, Nrv2, may be more restricted than shown.

In G_{Y1} mutant embryos, subcellular localization of Cora to the CC luminal membrane is lost and localization to sites of CC-PC adhesion is often disrupted (Figure 6D). CC localization of Sinu in G_{Y1} mutants is largely unaffected, however the punctate organization of Sinu along the cell membrane is compromised (Figure 6E). In addition, proper subcellular localization of Cora and Sinu in PCs is disrupted in G_{Y1} embryos. Accordingly, Nrv1/2 fails to restrict to CC and PC membranes in G_{Y1} embryos (Figure 6F). Thus, correct subcellular localization of Cora, Sinu, and perhaps Nrv2 in the dorsal vessel requires G_{Y1} signaling.

G_{Y1} Does Not Regulate CC Polarity

Since heterotrimERIC G proteins activate the planar cell polarity pathway downstream of Frizzled receptors (Katanaev et al.,

Figure 4. SJ Proteins Maintain Cardiac Integrity and Support Cardiac Function

Hand-GFP expression in St17 embryos. (A–E) The bro phenotype is observed in embryos homozygous for sinu^{null} (A1), cora^{4} (B1), nrv2^{273.I1645} (C1), nrv1^{21}315 (D), and cora^{4} (E). The severity of the bro phenotype in Gy^{+}/G^{-} sinu^{null} (A2), Gy^{+}/G^{-} cora^{4} (B2), and Gy^{+}/G^{-} nrv2^{273.I1645} (C2) embryos is comparable to that of each single mutant. (F–H) The bro phenotype is observed at low penetrance (~20%–30%) in embryos homozygous for Lac^{593.1462} (F), Gli^{7} (G), and Nrg^{204.1480} (H). (I) Injection of 0.5 μM dsRNA against prc induces the bro phenotype. Arrows denote the bro phenotype.

The SJ proteins Cora, Sinu, and Nrv1/2 in the dorsal vessel are G_{Y1}-dependent. Our genetic studies of Cora, Sinu, and Nrv2 suggested these SJ proteins also carry out novel functions essential for CC-PC adhesion. Similar to Nrx-IV, both Cora and Sinu localize to CC and PC membranes (Figures 6A and 6B). Two Nrv isoforms, Nrv1 and Nrv2, are recognized by the Nrv antibody (Sun and Salavaterra, 1995), and Nrv1/2 also localize to CC and PC membranes (Figure 6C). Since Nrv1 and Nrv2 have nonoverlapping subcellular localizations in other epithelial tissues (Paul et al., 2007), the expression pattern of the SJ specific isoform, Nrv2, may be more restricted than shown.

In G_{Y1} mutant embryos, subcellular localization of Cora to the CC luminal membrane is lost and localization to sites of CC-PC adhesion is often disrupted (Figure 6D). CC localization of Sinu in G_{Y1} mutants is largely unaffected, however the punctate organization of Sinu along the cell membrane is compromised (Figure 6E). In addition, proper subcellular localization of Cora and Sinu in PCs is disrupted in G_{Y1} embryos. Accordingly, Nrv1/2 fails to restrict to CC and PC membranes in G_{Y1} embryos (Figure 6F). Thus, correct subcellular localization of Cora, Sinu, and perhaps Nrv2 in the dorsal vessel requires G_{Y1} signaling.

G_{Y1} Does Not Regulate CC Polarity

Since heterotrimeric G proteins activate the planar cell polarity pathway downstream of Frizzled receptors (Katanaev et al.,
2005), we asked if these G proteins regulate cardiac cell polarity such that SJ protein mislocalization could be a secondary effect to polarity loss. However, by immunostaining, heterotrimeric G protein mutants do not show mislocalization of the CC polarity markers α-spectrin or Fasll (Figure S2). We conclude that CC polarity is not regulated by heterotrimeric G protein signaling and that this signaling pathway specifically regulates the localization of SJ proteins in CCs.

Figure 5. Go_1-Dependent Localization of Nrx-IV in the Dorsal Vessel Maintains Cardiac Integrity
(A–C) St16 WT, G_1^T159S, and Go_47A^D611T mutant embryos labeled with α-Prc, α-Nrx-IV, and Hoechst. (A) In WT embryos, Nrx-IV localizes to CC and PC membranes. (B and C) Nrx-IV does not correctly localize to CC and PC membranes in G_1^T159S and Go_47A^D611T embryos, particularly in PCs that have lost adhesion with CCs (white arrows). Prc accumulation at the PC-CC boundary is also reduced in G_1^T159S and Go_47A^D611T mutants.
(D) Transverse section of a St17 WT embryo labeled with α-Nrx-IV and Hoechst. (D1) Low magnification view showing the entire embryo oriented with dorsal to the top. Dashed box indicates position of high magnification scans. (D2) High magnification view. Nrx-IV localizes to all CC and PC membrane domains. Nrx-IV signal is highest at sites of CC-PC contact. (D3) DIC/Nrx-IV overlay shows Nrx-IV is indeed membrane localized.
(E and F) Nrx-IV in situ hybridization in St16 embryos colabeled with α-Mef2. (E) WT embryos express Nrx-IV in Mef2-positive CCs and in neighboring Mef2-negative PCs. (F) Nrx-IV expression in G_1^T159S embryos is comparable to WT, including the PCs that lose CC adhesion (white arrowheads).
(G–J) Transverse sections of Nrx-IV^EP694/+ and Nrx-IV^EP694 homozygous embryos at St17. (G and H) Low magnification view, and accompanying schematics, depicts the position of CCs, PCs, and the cardiac lumen. The lumen is collapsed and CCs are often misaligned in Nrx-IV^EP694 mutants. (I and J) High magnification scans reveal gaps (black arrows) between CC membranes in Nrx-IV^EP694 embryos. CC, cardiac cell; PC, pericardial cell; EC, ectoderm; DIC, differential interference contrast microscopy.

Figure 6. Go_1-Dependent Localization of SJ Proteins in the Dorsal Vessel
(A–F) St16 embryos labeled for Cora (A and D), Sinu (B and E), and Nrv1/2 (C and F) costained with α-Mef2 or Hoechst. In WT embryos (A–C), Cora, Sinu, and Nrv1/2 are detected in all CC and PC membrane domains. Cora and Sinu prominently localize to sites of CC-CC contact (arrowhead) and along the CC luminal domain (arrow); localization of both proteins along the membrane joining ipsilateral CCs is less pronounced. (D) In CCs of G_1^T159S mutants, Cora fails to localize to the luminal domain and localization to sites of PC-CC contact is often interrupted. (E) Sinu localizes to the correct CC membrane domains in G_1^T159S mutants, although the punctate nature of Sinu localization is compromised. Correct localization of Cora and Sinu in PCs is also lost in G_1^T159S embryos. (F) In G_1^T159S embryos Nrv1/2 localization is diffuse, particularly in misaligned CCs (open arrowhead). CC, cardiac cell; PC, pericardial cell.
DISCUSSION

The results of this study show that the heterotrimeric G proteins G-oa47A, Gb13F, and G-γ1 function together to maintain CC-PC adhesion during the late stage of heart formation in Drosophila. By mapping a new bro mutant (Nrx-IV) and characterizing additional candidate genes, we discovered a noncanonical role for SJ proteins in mediating CC-PC and CC-CC adhesion outside SJs. We found four SJ proteins, Nrx-IV, Sinu, Cora, and Nrv2, that operate in a common pathway with G-γ1 to maintain cardiac integrity and that require G-γ1 for proper subcellular localization in the heart. Mechanistically, the presence of SJ proteins in both CCs and PCs suggests that these proteins act in trans to maintain cell-cell adhesion in the dorsal vessel (Figure 7). We favor a model in which the extracellular domain of Nrx-IV engages in heterophilic interactions with SJ-proteins such as Neuroglian or Contactin (reviewed in Hortsch and Margolis [2003]), and that these interactions would be stabilized by ECM proteins such as Prc. Alternatively, the SJ proteins may directly interact with ECM proteins to provide a structural basis for cardiac integrity.

G Protein Signaling and SJ Proteins

G protein signaling regulates SJ formation in Drosophila and tight junction formation in mammalian cells. Even though SJs are analogous to vertebrate tight junctions, it is striking that G protein signaling components colocalize with both SJ and tight junction proteins (Denker et al., 1996; Saha et al., 2001; Schwabe et al., 2005). In addition, Gαs interacts with the tight junction protein ZO-1 throughout junction formation, suggesting that Gαs subunits physically regulate tight junction assembly (Denker et al., 1996). Thus, sepalate/tight junction proteins appear to be direct targets of G proteins in both flies and vertebrates.

Although the embryonic heart lacks SJs, our results are consistent with the idea that SJ proteins are direct targets of G proteins in the dorsal vessel. G protein mutants phenocopy SJ-protein mutants and G proteins operate in a common pathway with SJ proteins to maintain cardiac integrity. In addition, proper localization of SJ proteins in the embryonic heart requires G protein signaling, and G proteins regulate at least one SJ protein at the posttranscriptional level. Finally, loss of G-oα47A signaling (G-oα47A mutants) and hyperactivation of G-oα47A signaling (overexpressing G-oα47A) both result in the bro phenotype; thus Gα signaling is localized to specific foci in cells of the dorsal vessel. We propose that an appropriate level of Gα signaling mediates SJ-protein localization, whereas loss or hyperactivation of
Gs signaling mislocalizes SJ proteins leading to a loss in cardiac integrity.

**Cell Adhesion during Drosophila Heart Morphogenesis**

Cell-cell adhesion plays an essential role during organ morphogenesis. In the *Drosophila* heart, cell-cell adhesion along three distinct CC membrane domains is required to maintain cardiac integrity. Medioni et al. (2008) provide a detailed description of two CC domains participating in cell-cell adhesion: the *adherent domain*, positioned immediately dorsal and ventral to the cardiac lumen, promotes cell-cell adhesion between CCs on opposing sides of the heart, and the *basal-lateral adherent domain*, positioned along the lateral CC membrane, promotes cell-cell adhesion between neighboring CCs on one side of the heart. Our studies suggest that a third CC membrane domain, which we refer to as the *pericardial adherent domain*, is positioned opposite to the luminal domain and promotes PC-CC adhesion. The loss of cell-cell adhesion along each of the three CC domains gives rise to a unique phenotype: luminal collapse (referred to hereafter as type-1), breaks between neighboring cardiac cells (type-2), and loss of PC-CC adhesion (type-3), respectively. The unique nature of these three phenotypes can provide insight into the molecular pathways regulating cardiac integrity.

Loss of heterotrimeric G proteins or SJ proteins induces the type-3 (*bro*) phenotype, and mutations in at least one SJ-protein gene, Nrx-IV, leads to the type-1 phenotype. In addition, Sinu, Cora, and Nrv2 localize to the luminal and perhaps the adherent domains, suggesting that loss of these proteins will also cause the type 1 phenotype. We do observe the type 2 phenotype in a subset of *Gγ1* embryos, but not in any other heterotrimeric G protein or SJ-protein mutants. Thus, the pathways regulating cell-cell adhesion along the CC basal-lateral membrane may be distinct from those identified in this study.

The guidance ligand Slt regulates multiple aspects of cardiogenesis in *Drosophila*, and mutations in *slt* induce type-1, type-2, and likely type-3 phenotypes (MacMullin and Jacobs, 2006; Qian et al., 2005). In addition, *slt* mutant embryos show mesoderm migration and CC polarity defects (Qian et al., 2005); however these defects are genetically separable from cardiac integrity defects (MacMullin and Jacobs, 2006). Slt signals through the Robo receptors and mutations in genes encoding downstream components of the Robo signaling pathway do not dominantly enhance *slt* mutations. On the other hand, mutations in genes encoding integrins or integrin ligands, such as *scab*, *mys*, and *Lan-A*, dominantly enhance *slt* mutations and transheterozygous embryos show the type-2 phenotype (MacMullin and Jacobs, 2006). This study suggests that Slt activates two pathways during cardiogenesis: one pathway utilizes typical Robo signaling to regulate mesoderm migration and CC polarity while a second pathway uses atypical, or Robo-independent, signaling to regulate cell adhesion between neighboring CCs and likely between opposing CCs to promote lumen formation. Although the role of Slt in regulating PC-CC adhesion has not been studied in detail, one possibility is that Slt signals through G-ox47A/ G13F/G171 to regulate CC-CC and PC-CC adhesion.

### The Cellular Function of SJ Proteins in the Dorsal Vessel

SJ proteins are functionally interdependent and localization of Sinu to SJs requires Nrx-IV, Cora, and Nrv2 (Wu et al., 2004), while Nrx-IV, Cora, Cont, and Nrg are equally interdependent for localization to SJs (Baumgartner et al., 1996; Faivre-Sarrailh et al., 2004). In addition, both Nrv2 and Nrx-IV are transmembrane proteins, and the extracellular domain of Nrv2 at least is required for SJ function (Baumgartner et al., 1996; Paul et al., 2007). Since every SJ-protein mutant we examined showed PC-CC adhesion defects, SJ proteins likely form interdependent complexes in PCs and CCs. The extracellular domains of SJ proteins may act in *trans*, either through direct interactions with SJ proteins along opposing membranes or through indirect interactions with ECM proteins such as Prc, to maintain cardiac integrity. A search of the *Drosophila* protein interaction map reveals an interaction between Prc and Sinu, supporting the latter possibility. Alternatively, SJ proteins could be required for the formation or function of adherens junctions in the dorsal vessel.

#### Broken hearted Genes Are Evolutionarily Conserved

All of the *bro* genes have close vertebrate orthologs (Table S2). Since the function of mevalonate pathway genes in heart development is conserved from *Drosophila* to vertebrates (D’Amico et al., 2007; Edison and Muenke, 2005; Yi et al., 2006), we speculate that G protein-mediated regulation of SJ proteins is also evolutionarily conserved. To date, the role of heterotrimeric G proteins in regulating vertebrate heart development has not been identified, but heterotrimeric G proteins do play a role in heart disease (Zolk et al., 2000). On the other hand, Sinu is a member of the Claudin protein family and even though this protein family is rather divergent (Wu et al., 2004), vertebrate Claudin-1 is required for normal heart looping in the chick (Simard et al., 2008). In addition, Claudin-5 localizes to the lateral membrane of cardiomyocytes and is associated with human cardiomyopathy (Sanford et al., 2005). Lastly, mutations in the *prc* ortholog, collagen alpha-1(IV), cause vascular defects in mice and humans (Gould et al., 2005). Taken together, our studies raise the possibility that heterotrimeric G proteins and tight junction proteins ensure proper vertebrate cardiovascular morphogenesis.

### Experimental Procedures

#### Drosophila Strains

The following fly stocks were used: Hand-GFP (Han et al., 2006), Gγ14N759 (Izumi et al., 2004); Gγ13F315 and Gγ13E261 (Fuse et al., 2003); G-ox47A007 (Fremion et al., 1999); G-ox47A061 (Kataanev et al., 2005); loco413 (Grandersmith et al., 1999); Contivos (Faivre-Sarrailh et al., 2004); sinu12F007 (Wu et al., 2004); Gγ14M001, Nrv-IV54G064, Nrx-IV54P040, nrv213315, nrv22C1164g, cora14, Gi+/31, LacZ14G123G003, Nrg23B8; Df(3L)Xele1616, Df(3L)v165, Df(3L)v164, Df(3L)BkJ9 and Df(3L)F10 (Bloomington Stock Center). Overexpression studies used the following fly lines: Hand-GAL4 (Han et al., 2006); UAS-G-ox47A and UAS-G-ox47A(G203T) (Kataanev et al., 2005); UAS-G-G165A and UAS-G-G165A(G205L) (Schaefer et al., 2001).

#### Double-Stranded RNA Injection

dsRNA synthesis and injection was performed as described in Kennerdell and Carthew, 1998). dsRNAs were generated for *prc* and *white*. Hand-GFP blastoderm embryos were injected and assessed for cardiac phenotypes. Primers used to generate the templates for in vitro transcription reactions are available upon request.

#### Immunohistochemistry

Immunostaining of *Drosophila* embryos was performed as described (Yi et al., 2006), except that embryos were heat-fixed as described (Wu et al., 2004) for...
α-Sinu immunostaining. The following primary antibodies were used: α-Nrx-IV (gift from H. Bellen), α-Sinu (gift from G. Beitel), α-Mef2 (gift from B. Paterson), α-Cora (gift from R. Feihon), α-Prc, α-Nrv, α-Fasll, and α-alpha-Spectrin (Developmental Studies Hybridoma Bank). Primary antibodies were detected with Alexa488 and Alexa633 conjugated secondary antibodies (Molecular Probes, Carlsbad, CA) except α-Nrx-IV, α-Sinu, and α-Cora which were detected with Tyramide Signal Amplification ( TSA; Molecular Probes). Zenon647 (Molecular Probes) was used to detect α-Mef2 for the Cora/Mef2 double label. To image Hand-GFP, St17 embryos were dechorionated and mounted in halocarbon oil. Images were obtained with a Zeiss LSM510-Meta confocal microscope.

In Situ Hybridization

In situ hybridization was performed as described in (Johnson et al., 2007) except that TSA was used to detect labeled RNA probes. Nrx-IV probes were generated from the DGRC clone RE18634.

Electron Microscopy

Embryos were collected in grape agar plates for 30 min and aged to St17. Homozygous mutant embryos were identified by the absence of GFP expressed from balancer chromosomes. Heterozygous embryos were used as control. Embryos were dechorionated and manually devitelinated in 4% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer, postfixed in 1% osmium tetroxide, and embedded in Epon–Spurr resin. Sections were stained with uranyl acetate and lead citrate. Images were gathered on a Phillips CM-100 transmission electron microscope.

Heart Rate Quantification

St17 WT or homozygous mutant embryos bearing the hand-GFP transgene were collected and manually dechorionated. Heart rate was counted under a fluorescent microscope. For each genotype, three embryos were selected and counted. For each embryo, heart rate was measured for 2 min.

SUPPLEMENTAL DATA

Supplemental Data include two tables and two figures and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/15/5/704/DC1/.

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REFERENCES


