Ultrastructure and Regulation of Lateralized Connexin43 in the Failing Heart


Rationale: Gap junctions mediate cell-to-cell electric coupling of cardiomyocytes. The primary gap junction protein in the working myocardium, connexin43 (Cx43), exhibits increased localization at the lateral membranes of cardiomyocytes in a variety of heart diseases, although the precise location and function of this population is unknown.

Objective: To define the subcellular location of lateralized gap junctions at the light and electron microscopic level, and further characterize the biochemical regulation of gap junction turnover.

Methods and Results: By electron microscopy, we characterized gap junctions formed between cardiomyocyte lateral membranes in failing canine ventricular myocardium. These gap junctions were varied in structure and appeared to be extensively internalizing. Internalized gap junctions were incorporated into multilamellar membrane structures, with features characteristic of autophagosomes. Intracellular Cx43 extensively colocalized with the autophagosome marker GFP-LC3 when both proteins were expressed in HeLa cells, and endogenous Cx43 colocalized with GFP-LC3 in neonatal rat ventricular myocytes. Furthermore, a distinct phosphorylated form of Cx43, as well as the autophagosome-targeted form of LC3 (microtubule-associated protein light chain 3) targeted to lipid rafts in cardiac tissue, and both were increased in heart failure.

Conclusions: Our data demonstrate a previously unrecognized pathway of gap junction internalization and degradation in the heart and identify a cellular pathway with potential therapeutic implications. (Circ Res. 2010; 106:00-00.)

Key Words: gap junctions • connexin43 • heart failure • autophagy • lipid rafts

Gap junctions (GJs) allow ions and small molecules to be exchanged between the cytoplasm of adjacent cells. Intercellular communication through GJs mediates coordinated cell behavior in nearly every mammalian tissue, contributing to diverse physiological processes. Efficient electric activation and action potential propagation in the heart requires current passage between cardiomyocytes, a function carried out by GJs. Thus, accurate targeting and maintenance of GJs in cardiomyocytes is essential for normal heart function. Consistent with this concept are studies that demonstrate impaired heart function on genetic disruption of connexin (Cx)43, the primary GJ channel protein in mammalian ventricular muscle.1–3 Furthermore, a variety of structural heart diseases in both humans and animal models are associated with remodeling of GJ proteins, including decreased expression and altered subcellular distribution of Cx43.4–10 Despite the critical role GJs play in the heart, and extensive evidence for GJ remodeling in cardiac disease, a mechanistic understanding of the GJ life cycle in cardiomyocytes is limited.

GJ channels are assembled from 2 hexameric hemichannels, or connexons, (formed by connexins) which dock together at contacts between cells. GJ channels cluster into tightly packed 2D arrays consisting of a few to thousands of channels, known as GJ plaques. In cardiac tissue GJs are formed primarily at the intercalated disk (ID), which is the site of contact between the ends of cardiomyocytes. This arrangement accounts for anisotropic current flow, with conduction progressing rapidly in the direction of tissue fiber orientation. GJ proteins have been shown to have a remarkably rapid rate of turnover, with half lives on the order of 1.5 to 5 hours both in vitro and in vivo.11–13 Such rapid turnover kinetics necessitates well coordinated and regulated trafficking. GJ plaques are internalized by a cellular process in which...
the plasma membranes of both coupled cells are internalized into one of the 2 cells, producing double membrane intracellular inclusions. Intracellular circular GJ membranes, termed annular GJs (AGJs), have been morphologically characterized in isolated cells and tissues by electron microscopy. Dynamic studies of GJs in cell systems have demonstrated internalization of entire plaques forming AGJs consisting of membranes from both coupled cells. Since their earliest description, annular GJs have been implicated in degradative pathways involving lysosomes or the proteasome. More recent studies have sought to characterize the cellular processes by which GJs are internalized and degraded, although many details remain unknown, especially in the physiological context.

Cx43 phosphorylation is widely implicated in the assembly and turnover of GJs. Cx43 can be phosphorylated on multiple amino acids on its carboxyl-terminal tail, with different phosphorylation sites having distinct effects on GJ function. It has been reported that the phosphorylation state of Cx43 is altered with cardiac disease and on pharmacological treatment of cardiac cells; however, the precise phosphorylation changes that occur, and the effect they have on GJ function, are unresolved.

In this article, we provide an ultrastructural characterization of GJs formed between cardiomyocyte lateral cell borders in failing canine cardiac tissue. Internalized GJs are incorporated into heterogeneous multilamellar membranes, reminiscent of autophagosomes. These data suggest that an autophagic pathway is enhanced in the failing heart, and involves a distinct phosphorylated form of Cx43 that targets to lipid rafts (LRs).

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and describes antibodies, plasmids, cell culture methods, biochemical fractionation and analysis of tissue, and both light and electron microscopic methods used in the study.

**Canine Heart Failure Model**

Dogs were rapidly paced into heart failure (HF) as previously described.

**Neonatal Rat Ventricular Myocyte Isolation and Culture**

Neonatal rat ventricular myocytes (NRVMs) were enzymatically dissociated from the ventricles of 2-day-old rats as previously described.

**Statistical Analysis**

For the frequency of lateralized GJs (normal versus failing hearts), statistical significance was determined by a proportion test of the mean frequencies. Statistical significance of Western blot data were determined using unpaired, 2-tailed Student’s t tests. Error bars represent SD of the mean.

All procedures involving animals were approved by the Johns Hopkins Animal Care and Use Committee.

**Results**

**Lateralized Cx43 Does Not Colocalize With Zonula Occludens-1 or Cadherin**

Lateralized Cx43 was first characterized in canine ventricular tissue by immunofluorescent confocal microscopy. Figure 1A and 1B shows representative failing cardiac tissue sections stained with antibodies to Cx43, zonula occludens (ZO)-1 or a pan-specific cadherin antibody, revealing extensive targeting of both Cx43 and mechanical junction proteins to the ID (boxed regions). Extensive Cx43 staining was also observed along cardiomyocyte lateral borders, a pattern previously shown to be exaggerated in a variety of structural heart diseases (arrowheads). The lateralized population of Cx43 exhibited diminished colocalization with ZO-1 and cadherin. ZO-1 staining was also present in spaces between cardiomyocytes, representing staining of noncardiomyocyte cell types including vascular endothelial cells within capillaries and fibroblasts.

**GJ Ultrastructure**

We used transmission electron microscopy (TEM) of canine cardiac tissue to examine the ultrastructure of GJs. By TEM, GJs appear as pentalaminar membranes. Immunogold labeling of Cx43 revealed extensive staining on pentalaminar membranes within the ID, representing GJs formed between cells (not shown). In both normal and failing hearts, Cx43-labeled pentalaminar membranes were also observed in circular structures emanating from the ID region, representing internalizing GJs destined to become AGJs (Figure 2A).

Conventional TEM was used to study the ultrastructure of GJs in greater detail. Figure 2B shows an electron micrograph of normal cardiac tissue demonstrating the orientation of GJs within the ID. The mechanical junctions formed between cardiomyocytes are formed by adherens junctions and desmosomes and run perpendicular to the direction of tissue fibers. GJs are located between intervening portions of mechanical junctions and run parallel to the fiber orientation (Figure 2B, boxed region). GJs located at the outer edge of the ID are not typically flanked by mechanical junctions at the edge which is continuous with lateral membranes (Figure 2B, 1).

Figure 2C and 2D shows electron micrographs of failing cardiac tissue. GJs are extensively formed between the lateral membranes of adjacent myocytes, in regions distant from the ID (Figure 2C [1 through 3] and 2D [1 and 2]).

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AGJ</td>
<td>annular gap junction</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>Cx43</td>
<td>connexin43</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GJ</td>
<td>gap junction</td>
</tr>
<tr>
<td>HF</td>
<td>heart failure</td>
</tr>
<tr>
<td>ID</td>
<td>intercalated disk</td>
</tr>
<tr>
<td>LC3</td>
<td>microtubule-associated protein light chain 3</td>
</tr>
<tr>
<td>LR</td>
<td>lipid raft</td>
</tr>
<tr>
<td>NRVM</td>
<td>neonatal rat ventricular myocyte</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
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<td>ZO-1</td>
<td>zonula occludens-1</td>
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lateralized GJs are not flanked by typical mechanical junctions. Lateralized GJs are atypical of GJs formed at IDs in that they exhibit complex membrane bending and are often in close association with mitochondria, and other indistinct cellular material. Lateralized GJs were more frequently observed in failing compared with normal tissue. A series of images of similar tissue orientations were acquired from 4 normal and 3 failing dog hearts, and the number of cell–cell pairs possessing lateralized GJs was counted (Figure 3A). In tissue sections from 4 normal dogs, 1 of 21, 1 of 22, 4 of 25, and 11 of 21 cell–cell pairs possessed lateralized GJs (average frequency=0.19), whereas in tissue sections from 3 failing dogs, 7 of 14, 14 of 21, and 9 of 12 cell–cell pairs possessed lateralized GJs (average frequency=0.64) (P=0.013) (Figure 3B).

In failing cardiac tissue, internalized GJs were often observed as concentric rings, suggesting extensive involution of GJ membranes during internalization. (Figure 2D, 2, arrowhead; Figure 4A through 4D). Internalized GJs are typically large (∼0.1 to 1 μm in diameter), highly heterogeneous structures with undefined cellular debris in their lumens (Figure 4A). Internalized GJ membranes also
formed multilamellar membrane structures (Figure 4B and 4C) reminiscent of autophagosomes. Further morphological support for autophagic sequestration of internalized GJs were crescent-shaped, putative isolation membranes that appeared to envelope internalized GJs (Figure 4D, arrowheads), as well as multiple putative isolation membranes in close proximity to the ID (Figure 4E).

To test the hypothesis that internalized GJs associate with autophagosomes, we transiently transfected HeLa cells with both Cx43 and a specific marker of autophagosomes, LC3 (microtubule-associated protein light chain 3) fused to enhanced green fluorescent protein (GFP-LC3). Figure 5A shows 2 HeLa cells, each expressing Cx43 and GFP-LC3. GJs are formed at sites of contact between the 2 cells (arrowhead in Figure 5A, merge) and exhibit strong Cx43 staining. A significant fraction of Cx43 staining is intracellular and punctate, representing trafficking intermediates of Cx43. GFP-LC3 is expressed as a cytoplasmic protein, resulting in diffuse green signal throughout the cell. However, GFP-LC3 is also concentrated on maturing autophagosomes by a covalent lipid modification, resulting in punctate green staining of autophagosomes. A proportion of intracellular Cx43 signal colocalized with GFP-LC3 (Figure 5A, merge), suggesting an association of internalized GJs with autophagosomes. To determine whether endogenous cardiac Cx43 associates with autophagosomes we cultured primary NRVMs that were transiently transfected with GFP-LC3. Under baseline culture conditions cytoplasmic Cx43 and GFP-LC3 only sporadically colocalized (data not shown). We hypothesized that internalized GJs may be rapidly cleared from the cytoplasm through lysosomal degradation. We therefore treated cells with a lysosomal inhibitor (10 μmol/L chloroquine) for 2 hours before fixing, immunostaining and
imaging the cells. Inhibiting lysosomal activity resulted in significant intracellular colocalization between endogenous Cx43 and expressed GFP-LC3 (Figure 5B).

LR Targeting of Cx43 and LC3-II
Cx43 has been reported to target to buoyant, cholesterol and sphingolipid-rich membrane domains termed LRs,\textsuperscript{31–33} although the significance of LR targeting is not known. Our observation that internalized GJs associate with multilamellar membranes led us to hypothesize these structures might represent detergent resistant, buoyant populations of Cx43. We prepared LRs from cardiac tissue by cold Triton X-100 extraction and sucrose density centrifugation. Western blotting revealed 2 populations of Cx43 (Figure 6A): a buoyant population (fraction 5) that cofractionated (but is not necessarily associated) with the muscle-specific caveolar protein caveolin-3 and the glycosylated sphingolipid G\textsubscript{M}1 (markers of LR domains) and a dense population (fractions 9 to 11). Cx43 is well known to separate into multiple bands by SDS-PAGE, representing distinct phosphorylated species of the protein.\textsuperscript{34} To better separate Cx43 into its distinct phosphorylated forms, we analyzed the sucrose fractions by large format SDS-PAGE (Figure 6B, top). The slowly migrating form of Cx43 (designated P2) was uniquely targeted to the LR fraction, whereas intermediate and fast migrating bands (designated P1 and P0 respectively) remained predominantly in the dense non-LR fractions. To confirm that the slow migration of LR targeted Cx43 was attributable to phosphorylation and not an artifact of the LR isolation procedure, we treated proteins isolated from fraction 5 with alkaline phosphatase (AP) (Figure 6C). AP treatment increased the mobility of Cx43, indicating that LR-targeted Cx43 constitutes a highly phosphorylated form of the protein.

We examined the distribution of the autophagosome marker LC3 in LR fractions (Figure 6B, bottom). LC3 migrates as 2 forms, LC3-I and LC3-II. LC3-I is cytoplasmic and migrates at \(\sim 18\) kDa. During autophagy LC3-I is partially cleaved and covalently linked to maturing autophagosomes, resulting in the LC3-II form that migrates at \(\sim 15\) kDa. The dense, non-LR fractions contained both LC3-I and LC3-II. The buoyant fraction 5 contained LC3-II, indicating that a subpopulation of autophagosomes

\[ ... \]
possess the physicochemical properties of LRs. The LR fraction, as expected, is devoid of ZO-1, which was confined to the pellet (Figure 6D).

We then examined the ultrastructure of membranous material from fraction 5 (Figure 6E). By TEM, fraction 5 consists primarily of multilamellar membrane structures, with no discernable structure or morphology. However, interspersed in this material we observed pentalaminar membranes. The edges of these pentalaminar membranes were not associated with structures consistent with mechanical junctions (fascia adherens and desmosomes). This observation is consistent with a population of Cx43 possessing LR properties after incorporation into GJs, but not associated with mechanical junctions, which may include the internalized, multilamellar membranes associated with GJs we observed in cardiac tissue.

**Figure 4. Ultrastructure of internalized GJs from failing myocardium.** Representative micrographs of failing myocardium showing internalized GJs with concentric pentalaminar membranes and cellular debris in their lumens (A), as well as pentalaminar membranes incorporated into multilamellar membrane structures (B and C). D, Internalized and/or internalizing concentric GJ in close association with a putative isolation membrane shown at higher resolution in 1 (arrowheads). E, Multiple double membrane structures with the appearance of isolation membranes in close proximity to the ID. The insets show the detail of pentalaminar membranes from boxed regions. Scale bars: 100 nm (A through C); 2 μm (D); 500 nm (E).

**Cx43 and LC3 Expression in HF**

We examined LR fractions from both normal and pacing-induced HF dog ventricles (Figure 7A). LR domains isolated from failing hearts exhibited an approximately 3.5-fold greater Cx43 signal than LR domains from normal hearts ($P<0.011$). In these same samples, the total Cx43 levels decreased by approximately 2-fold ($P<0.01$). We examined the expression levels of LC3-I and LC3-II in normal and HF hearts (Figure 7B). The levels of LC3-I were not statistically different, whereas the levels of LC3-II increased by approximately 2-fold in HF hearts as
compared to normal hearts ($P=0.016$), indicative of increased autophagosome formation in failing hearts.

**Discussion**

In cardiac tissue, Cx43 forms GJs primarily at the ID. A prominent feature of structural heart disease is a redistribution of Cx43 to the lateral cell borders and a decrease in Cx43 expression levels.\(^6,7,29\) Despite the consistency of altered Cx43 expression and distribution in structural heart disease, the functional status and structural nature of lateralized Cx43 is incompletely understood. In this study, we demonstrate that GJs are formed between the lateral membranes of cardiomyocytes with increased frequency in a canine model of pacing induced HF. However, the lack of colocalization with ZO-1 and cadherin, and the heterogeneous structure of lateralized GJs demonstrate that they are structurally and likely functionally distinct from those formed at the ID.

Cx43 has been shown to be directly bound by ZO-1\(^35\) and it is thought that ZO-1 in part regulates the assembly of Cx43 into the periphery of GJ plaques.\(^36\) Changes in the relationship between ZO-1 and Cx43 have been reported in cardiac disease\(^17,38\) and is emerging as a potentially critical interaction for the maintenance of proper GJ-mediated intercellular communication. Furthermore, Cx43-containing vesicles have been shown to be directly delivered to cadherin-containing adherens junctions via microtubules.\(^39\) The formation of apparently atypical GJs at cardiomyocyte lateral membranes, devoid of ZO-1 and cadherin colocalization, suggests impairment in the mechanisms responsible for direct delivery of Cx43 to sites of mechanical junction formation.

AGJs have been described in a number of cells and tissues including isolated cardiomyocytes\(^15,40\) and cardiac tissue subjected to stress.\(^29,41\) AGJs have been suggested to be endocytosed GJ plaques destined for lysosomal or proteasomal degradation.\(^14,15,19,21,23\) Direct evidence for the origin of AGJs comes from the work of Jordan et al\(^17\) using time lapse studies of fluorescently tagged Cx43, and Piehl et al\(^18\) using dye injection studies to demonstrate internalization of intercellular GJ plaques into 1 of 2 coupled cells. We suggest that intracellular uni- and multilamellar GJ-containing membrane rings observed in cardiac tissue represent AGJs in progressively advanced stages of processing for degradation.

The incorporation of internalized GJs into multilamellar membranes, their close association with cellular debris, and the colocalization of Cx43 with LC3 is suggestive of autophagy playing a role in GJ clearance. The association of internalized GJs with autophagosomes has been suggested in the literature but is not an accepted mechanism of GJ degradation. Autophagosomes are dynamic organelles which
sequester cell contents for delivery to lysosomes. In post-mitotic cells, such as cardiomyocytes, autophagy is constitutively active to help maintain cell size and integrity. Under stressful conditions, the rate of autophagy is increased, where it serves an adaptive function by providing a source of nutrients through catabolism of “unnecessary” cellular contents, as well as clearing damaged organelles. Autophagy has been implicated in the pathogenesis of many diseases.
including ischemia/reperfusion injury and HF,45,46 as a mechanism of cell survival. Internalized GJs are quite large and structurally distinct relative to typical endocytic organelles, and therefore connexins are relatively nonstandard endocytic cargo. The cellular machinery involved in GJ internalization and degradation is therefore likely to be distinct from that involved in more conventional endocytic processes. Thus, it is plausible that GJs would be sequestered by autophagic machinery for delivery to lysosomes.

The signaling mechanisms regulating the internalization and degradation of Cx43 GJs are poorly understood, but likely involve post-translational modification of Cx43. Cx43 is phosphorylated extensively on its carboxyl terminus, regulating both the trafficking and permeability of Cx43 GJ channels.47,48 By SDS-PAGE, the 3 major bands observed for Cx43 are typically referred to as P0 (fastest migrating, least phosphorylated), P1, and P2 (slowest migrating, most phosphorylated), and the P2 form only appears after Cx43 has reached the plasma membrane and formed GJs.34 Changes in the phosphorylation state of cardiac Cx43 have been reported altered in disease.31–33 Consistent with this hypothesis are studies which demonstrate that connexins can target to LRs, but that intercellular GJs themselves are not LRs.31 Musil and Goodenough34 have described the relationship between Cx43 Triton solubility, phosphorylation state, and assembly into GJs in vitro. The authors suggest that Cx43 begins as a Triton soluble, hypophosphorylated form during its progression toward the plasma membrane. On arrival at the plasma membrane Cx43 acquires Triton resistance and matures into a hyperphosphorylated form. The authors further suggest that the acquisition of Triton resistance and maturation to the P2 phosphorylated form corresponds to the formation of mature GJ plaques. Based on our ultrastructural characterization of fraction 5 (Figure 6E), which contains P2 phosphorylated Cx43, we do not believe these buoyant pentalaminar membranes represent GI plaques coupling 2 cells at IDs, but rather internalized GJs incorporated into multilamellar membranes. We therefore hypothesize that maturation of the P2 form in fact corresponds to the degradation of GJs through autophagic processing, although further experiments will be required to confirm this hypothesis.

Our data demonstrate that GJs with highly variable morphology are formed between cardiomyocyte lateral membranes with increased frequency in HF and that GJ turnover likely occurs via an autophagic pathway. The mechanisms by which Cx43 GJs are targeted for internalization and degradation by autophagy appears to involve changes in phosphorylation, as well as processing through membranes with physicochemical properties of LRs. The regulated process of...
GI turnover via autophagy suggests a novel pathway for the regulation of cardiac GJs and therefore conduction in the mammalian ventricle. These findings offer insight into the mechanisms regulating electric conduction in mammalian myocardium, and may in part contribute to the arrhythmogenic electric remodeling associated with HF.

There are limitations to the present study. We did not perform serial sectioning and/or 3D reconstruction of TEM sections. It is therefore possible that apparently internalized GJs are in reality extensively convoluted GJs still attached to the plasma membrane. Regardless of this possibility, the presence of GJs at lateral membranes is nonetheless more frequent in failing hearts, and lateral GJs are more convoluted, if not more extensively internalized, than GJs at the ID.

These data were obtained in a canine model of HF that in many respects mimics human HF but is clearly different from the most common forms of HF occurring in humans. The detailed regulation of Cx43 and GJ localization may not be identical with human disease. Reassuringly, many of the features of GJ remodeling characterized in this model are similar to those previously observed in diseased human hearts.

Although we have provided ultrastructural, microscopic, and biochemical evidence in support of GJ association with autophagosomes, we have not demonstrated connexin degradation via autophagy per se. The precise contribution of autophagy to connexin turnover will require a more quantitative analysis.

**Acknowledgments**

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**Disclosures**

None.

**References**


39. Shaw RM, Fay AJ, Puthenveedu MA, von Zastrow M, Jan YN, Jan LY. Internalized GJs are incorporated into multilamellar membrane structures characteristic of autophagosomes, and Cx43 colocalizes with an autophagosome marker (LC3-II) in both HeLa cells and primary cultured neonatal rat ventricular myocytes.


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Antibodies and plasmids
The following antibodies were used in this study: Anti-Cx43 (Chemicon, MAB3068), Anti-ZO-1 (Zymed, 40-2200), Anti-Pan-Cadherin (Sigma, C1821), Anti-Caveolin-3 (BD-Transduction Laboratories, 610421), Anti-LC3 (Sigma, L8918), HRP conjugated Cholera Toxin B (Molecular Probes, C-34780). Human Cx43 was PCR amplified from plasmid DNA (a gift from Steven Taffet at SUNY Upstate, Syracuse, NY) and subcloned into the mammalian expression pcDNA3.1 vector (Invitrogen, V790-20). The EGFP-LC3 plasmid was created by Karla Kirkegaard (Stanford University) and obtained through the Addgene plasmid repository (Addgene plasmid 11546, www.addgene.org).

Cell Culture
HeLa cells were maintained in DMEM supplemented with 10% FBS in a humidified incubator at 37°C and 5% CO2. Cells plated on coverslips were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to manufacturer’s specifications.

Canine Heart Failure Model
Mongrel dogs were rapidly paced into heart failure as previously described. All procedures were approved by the Johns Hopkins Animal Care and Use Committee.

Neonatal rat ventricular myocyte (NRVM) isolation and culture
NRVMs were enzymatically dissociated from the ventricles of 2-day-old Sprague-Dawley rats (Harlan) with trypsin and collagenase (Worthington) as previously described. NRVMs were maintained in a humidified incubator at 37°C and 5% CO2 and transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to manufacturer’s specifications.

Immunofluorescent confocal microscopy
For immunohistochemistry the tissue was cut into 5-6 mm³ cubes and fixed with 4% formaldehyde in PBS for 1 hour at room temperature. The tissue was infiltrated with 15% sucrose in PBS until it sank, followed by infiltration with 30% sucrose in PBS overnight at 4°C. The tissue was placed in cryomolds with OCT freezing medium and floated on isopentane cooled with dry ice. Ten micron sections were cut with a cryostat and adhered to microscope slides. For immunocytochemistry, and confocal microscopy HeLa cells or NRVMs were grown on coverslips, then fixed with 4% formaldehyde in PBS for 30 minutes. Fixed samples were washed with TBS followed by permeabilization with TBS and 0.1% Triton X-100 for 30 minutes. The samples were blocked with 5% non-fat dry milk in TBS-T for 2 hours at RT, incubated with primary antibodies, then washed and incubated with fluorescent secondary antibodies. The samples were washed extensively with TBS-T, and mounted with Pro-Long Gold Fluorescent Mounting Medium (Invitrogen), sealed with nail polish and imaged with an LSM-510 META confocal microscope system (Carl Zeiss). All confocal image analysis was carried out using ImageJ software. Control experiments were performed with secondary antibody only to confirm absence of non-specific secondary antibody binding (not shown).

Immuno-gold Transmission Electron Microscopy (TEM)
In preparation for immunogold TEM the tissue was minced into 1-2mm³ cubes and fixed in 4% formaldehyde and 0.1% glutaraldehyde in PBS at room temperature for 1 hour. Fixed tissue was then infiltrated with 10% bovine gelatin and placed at 4°C to solidify. The tissue was then cryo-preserved in 2.3M sucrose with 20% polyvinylpyrrolidone and frozen in liquid nitrogen. Thin cryosections were cut with a Leica Ultracut UCT ultramicrotome and placed on formvar film coated, glow discharged nickel grids. The cryosections were labeled with anti-Cx43 primary antibody followed by goat anti-mouse colloidal gold conjugated secondary antibody. The grids were then contrasted and stained by the PVA-embedding method. Grids were imaged with a
Hitachi 7600 transmission electron microscope operated at 80kV. Control experiments were performed with secondary antibody only to confirm absence of non-specific secondary antibody binding (not shown).

**Conventional TEM**

Conventional TEM was performed with tissue that was minced into 1-2 mm$^3$ cubes and fixed with 2% glutaraldehyde in 0.1M cacodylate buffer for 1 hour at RT. The tissue was postfixed with 2% osmium tetroxide (OsO$_4$) for 1 hour at 4°C then stained with 2% uranyl acetate for 30 minutes at RT. The tissue was progressively dehydrated with a series of ethanol washes (50%, 70%, 90%, and 100% EtOH) followed by dehydration with propylene oxide followed by infiltration with 1:1 propylene oxide:Epon with 1.5% DMP-30 for 1 hour. The tissue was then infiltrated with Epon overnight, followed by 3 more incubations with fresh Epon for 2 hours each, then placed in fresh Epon and baked at 60°C for 2 days. The tissue was cut into 70 nm sections with an ultramicrotome, placed on TEM grids, stained with lead citrate, and imaged with either a Hitachi 7600 or Philips CM120 transmission electron microscope operated at 80kV.

**Lipid raft fractionation**

Frozen cardiac tissue was homogenized in 10 volumes (vol/wt) of ice cold MES buffered saline (MBS) with 1% Triton X-100 plus phosphatase, kinase, and protease inhibitors. Homogenates were maintained on ice for one hour then mixed 1:1 (vol:vol) with 80% sucrose in MBS (final sucrose concentration is 40%) and 1 mL was placed at the bottom of an ultracentrifuge tube with 6 mL of 38% sucrose in MBS, followed by 4 mL of 5% sucrose in MBS layered on top. The samples were centrifuged at 39,000 rpm in an SW41 rotor for 18 hours with no braking. Serial 1 mL fractions were collected.

**Western and dot blotting**

For dot blotting, aliquots of sucrose fractions were mixed 1:1 (vol:vol) with 2X TBS-Tween20 and incubated at 37°C for 30 min. Equal volumes of each fraction were blotted onto nitrocellulose membranes using a BioRad BioDot Filtration System (Bio-Rad). For western blotting, aliquots of sucrose fractions were precipitated by TCA/acetone precipitation. Precipitates were pelleted and resuspended in 2% SDS and protein concentrations were determined by the BCA assay (Pierce). Dephosphorylation was performed by resuspending the precipitate in RIPA buffer (50mM Tris pH8, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and incubating with or without alkaline phosphatase (AP) (New England Biolabs) overnight at 37°C. Samples were prepared in Laemmeli buffer for SDS-PAGE. Proteins were transferred onto 0.45 μm nitrocellulose membranes using an iBLOT apparatus (Invitrogen). Equal protein loads were confirmed by Ponceau-S stain. Western blotting was performed using standard procedures and visualized with chemiluminescent substrate and X-ray film. Signals were quantified with Progenesis software (Non-Linear Dynamics). Control experiments were performed with secondary antibody only to confirm absence of non-specific secondary antibody binding (not shown).

**Statistical analysis**

For comparison of the frequency of lateralized gap junctions between normal and failing hearts, statistical significance was determined by a proportion test of the mean frequencies. Statistical significance of Western blot data was determined using unpaired, two-tailed Student’s T-tests. Error bars represent standard deviation of the mean.
