GJA1-20k Arranges Actin to Guide Cx43 Delivery to Cardiac Intercalated Discs

Wassim A. Basheer, Shaohua Xiao, Irina Epifantseva, Ying Fu, Andre G. Kleber, TingTing Hong, Robin M. Shaw

Rationale: Delivery of Cx43 (connexin 43) to the intercalated disc is a continuous and rapid process critical for intercellular coupling. By a pathway of targeted delivery involving microtubule highways, vesicles of Cx43 hemichannels are efficiently trafficked to adherens junctions at intercalated discs. It has also been identified that actin provides rest stops for Cx43 forward trafficking and that Cx43 has a 20 kDa internally translated small C terminus isoform, GJA1-20k (Gap Junction Protein Alpha 1- 20 kDa), which is required for full-length Cx43 trafficking, but by an unknown mechanism.

Objective: We explored the mechanism by which the GJA1-20k isoform is required for full-length Cx43 forward trafficking to intercalated discs.

Methods and Results: Using an in vivo Adeno-associated virus serotype 9–mediated gene transfer system, we confirmed in whole animal that GJA1-20k markedly increases endogenous myocardial Cx43 gap junction plaque size at the intercalated discs. In micropatterned cell pairing systems, we found that exogenous GJA1-20k expression stabilizes filamentous actin without affecting actin protein expression and that GJA1-20k complexes with both actin and tubulin. We also found that filamentous actin regulates microtubule organization as inhibition of actin polymerization with a low dose of latrunculin A disrupts the targeting of microtubules to cell–cell junctions. GJA1-20k protects actin filament from latrunculin A disruption, preserving microtubule trajectory to the cell–cell border. For therapeutic implications, we found that prior in vivo Adeno-associated virus serotype 9–mediated gene delivery of GJA1-20k to the heart protects Cx43 localization to the intercalated discs against acute ischemic injury.

Conclusions: The internally translated GJA1-20k isoform stabilizes actin filaments, which guides growth trajectories of the Cx43 microtubule trafficking machinery, increasing delivery of Cx43 hemichannels to cardiac intercalated discs. Exogenous GJA1-20k helps to maintain cell–cell coupling in instances of anticipated myocardial ischemia.

Key Words: adherens junctions ■ connexin 43 ■ cytoskeleton ■ gap junctions ■ latrunculin A ■ trafficking ■ tubulin

The mechanism by which membrane proteins are localized to their respective subdomain remains largely unknown. Seminal trafficking studies in cell biology have uncovered the major organelles of the cell,1 zip codes which allow proteins to recognize their destination once they arrive,2 and the lipid transport machinery which performs the transport.1 However, we have yet to understand how prearrival delivery specificity of proteins is achieved.

Meet the First Author, see p 1022

The ventricular cardiomyocyte is a highly organized and polarized cell with distinct membrane subdomains and, thus, an excellent model for studying delivery specificity. Furthermore, Cx43 (connexin 43) gap junction proteins tend to cluster and have very high turnover rates, both are features which can be exploited in live cell, interventional, and in vivo studies to uncover mechanisms of membrane protein trafficking. Physiologically, Cx43 gap junctions comprise hexameric hemichannels from neighboring cells that form gap junctions at the intercalated discs which are essential for electric coupling in cardiac ventricles,4,5 synchronizing beat-to-beat heart contractions.6,7 Considering the specific localization of Cx43 and rapid protein turnover which is within several hours,8 the intracellular movement of Cx43 is highly regulated, disruptions of which are causal for lethal arrhythmias.9

Growing evidence supports a cytoskeletal-based trafficking mechanism for Cx43 delivery directly to the intercalated disc.10,11 It is understood that Cx43 hemichannels oligomerize in the trans-Golgi network12 and are subsequently packaged into vesicles transported by motor proteins along microtubules.13-15

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Novelty and Significance

What Is Known?

- Heart disease and heart failure cause a decrease of gap junction communication channels between heart cells, increasing the chance of sudden cardiac death.
- The intracellular cytoskeleton is the delivery highway for gap junction channels to arrive to their correct location between muscle cells, but specificity of delivery is still being determined.
- A small truncated isoform of Cx43 (connexin 43), called GJA1-20k, is created by ribosomal alternative translation and may help with delivery of full-length channel to cell–cell borders.

What New Information Does This Article Contribute?

- GJA1-20k contributes specificity to full-length protein delivery by promoting organization of the actin cytoskeleton, which directs the microtubule network to deliver cargo to the right location.
- Alternatively translated GJA1-20k is a critical link to targeted delivery of gap junction proteins, increasing the amount of native full-length protein at cell–cell borders.
- GJA1-20k could be a novel therapeutic to limit the reduction of gap junction coupling and limit arrhythmogenesis in situations of anticipated ischemia of the heart.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AAV9</td>
<td>Adeno-associated virus serotype 9</td>
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<tr>
<td>Cx43</td>
<td>connexin 43</td>
</tr>
<tr>
<td>EB1</td>
<td>end-binding protein 1</td>
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<tr>
<td>F-actin</td>
<td>filamentous actin</td>
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<td>LatA</td>
<td>latrunculin A</td>
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Our previous work identified a targeted delivery paradigm,16–18 by which the specificity of Cx43 trafficking is in part achieved by the interaction between microtubule plus-end-binding proteins and the membrane scaffolding proteins of the intercalated disc.17,18 We have also discovered that actin is a necessary component of the Cx43 forward trafficking machinery, serving as rest stops at which Cx43 vesicles pause and slow down before they are delivered to the cell surface along microtubules.19 However, the interrelationship between the actin and microtubule machineries for Cx43 trafficking is not understood. Adding to the complexity of regulation of Cx43 trafficking is the autoregulation of Cx43 localization by smaller accessory subunits20 generated through internal translation of the GJA1 mRNA.21 In hearts and cell lines, ≤6 N-terminal truncated isoforms containing the Cx43 C-terminal cytoplasmic tail have been identified,21–23 of which the 20-kDa isoform (GJA1-20k) is the most abundantly expressed. We have found that GJA1-20k aids in Cx43 gap junction localization,21 but how trafficking regulation is accomplished by this particular isoform and whether GJA1-20k contributes specificity to cytoskeletal-based Cx43 transport remain unknown.

In this study, we present the finding that GJA1-20k contributes to the specificity of Cx43 trafficking by stabilizing actin polymerization to organize the growing paths of microtubules toward the intercalated discs, onto which Cx43-containing vesicles are loaded for delivery. Using an in vivo Adeno-associated virus serotype 9 (AAV9)–mediated gene transfer system in adult mice, we find that exogenous GJA1-20k markedly increases endogenous Cx43 gap junction plaque size at the intercalated discs. In HeLa cells, exogenous GJA1-20k not only complexes with both actin and tubulin but also stabilizes filamentous actin (F-actin) in micropatterned cells. These results are further supported in micropatterned neonatal mouse ventricular cardiomyocytes, where GJA1-20k stabilizes F-actin fibers and preserves microtubule trajectory to the cell–cell border when cells are subjected to actin depolymerization by low-dose latrunculin A (LatA). Moreover, by maintaining the actin and microtubule cytoskeletal system, GJA1-20k protects Cx43 localization to the intercalated discs after acute ischemic injury in isolated mouse hearts. These findings reveal that delivery specificity of Cx43 gap junction is dependent on the internally translated GJA1-20k isoform that stabilizes F-actin, which in turn is required to organize microtubule directed trajectories to the cell–cell border for Cx43 transport. The results further indicate that exogenous GJA1-20k can serve to preserve cardiac gap junction coupling in the setting of anticipated ischemic insults.

Methods

An expanded Methods section is available in the Online Data Supplement.

Mice

C57BL/6 mice used for heart preparations were maintained under sterile barrier conditions. All procedures were reviewed and approved by Cedars-Sinai Medical Center Institutional Animal Care and Use Committee.

Molecular Biology

Human GJA1 cDNAs encoding full-length Cx43 and smaller isoforms were cloned as previously described21 with internal methionine start sites in GJA1-43k, and GJA1-20k mutagenized to leucine...
(QuickChange Lightning Mutagenesis Kit; Agilent) to ensure single isoform expression. C-terminal V5-, HA (hemagglutinin)-, or GFP (green fluorescent protein)-tagged proteins were subsequently made.

AAV9 Gene Delivery
AAV9 vectors expressing GST (glutathione S-transferase)-GFP, GJA1-20k-GFP; or GJA1-43k-GFP driven by the cytomegalovirus (CMV) promoter were purchased and purified at Welgen Inc (Worcester, MA). Eight- to 12-week-old male C57BL/6 mice received 100 µL of 3x10⁸ vector genomes through retro-orbital injection.⁴²,²⁵ Four weeks post-injection, the hearts were excised and processed for immunofluorescence imaging, biochemical analysis, or RNA expression.

Langendorff-Perfused Mouse Heart Preparation
Four weeks post-AAV9 injection, a subset of mouse hearts was excised, ex vivo perfused on a Langendorff perfusion apparatus, and subjected to acute ischemic injury study as previously described.¹⁹ In brief, hearts were perfused using a Langendorff apparatus (ADInstruments) at a constant rate of 2.6 mL/min with Krebs–Henseleit (K–H) buffer, which was constantly gassed with 95% O₂/5% CO₂, and maintained at 37°C. Equilibration was performed for 20 minutes before hearts were subjected to 30 minutes of global ischemia. During no-flow ischemia, the heart was immersed in warm K–H buffer to maintain temperature. Control hearts were perfused continuously throughout the protocol. Immediately after the procedure, hearts were embedded in optimal cutting temperature compound (OCT) media (Sakura Finotek) and snap frozen for cryosectioning.

Western Blotting
Western blotting was performed as previously described.²¹ In brief, samples were subjected to SDS-PAGE electrophoresis using NuPAGE Bis-Tris gels and 2-(N-Morpholino)ethanesulfonic acid (MES) (Thermo Fisher Scientific) buffer according to the manufacturer’s instructions. Gels were transferred to FluorTrans polyvinylidene difluoride (PVDF) membranes (Pall) and subsequently fixed by soaking in 100% methanol and air-drying before rewetting with methanol and blocking for 1 hour at room temperature in 5% nonfat milk in Tris-NaCl-Tween (TNT) buffer (50 mMol/L Tris, pH 8.0, 150 mMol/L NaCl, 0.1% Tween-20). Membranes were probed overnight with primary antibodies in 5% milk in TNT buffer then incubated for 1 hour at room temperature with secondary antibodies in 5% milk in TNT. Membranes were then immersed in 100% methanol, air-dried, and imaged using the ChemiDoc MP fluorescent western detection system (BioRad).

Quantitative Real-Time PCR
Total RNA was isolated from mouse hearts 4 weeks post-AAV9 retro-orbital injection, using Trizol and PureLink RNA mini kit (Thermo Fisher Scientific) according to manufacturer’s instructions. The purified RNA was further treated for any DNA contamination using Turbo DNA-Free Kit (Thermo Fisher Scientific). cDNA was synthesized using SuperScript IV VILO Master Mix (Thermo Fisher Scientific). Taqman gene expression assays, using Taqman Universal Master Mix and Taqman probes (Thermo Fisher Scientific), were prepared for quantitative Real-Time Polymerase Chain Reaction (PCR) and ran using BioRad CFX Connect Real-Time System.

Micro patterning of Cell Pairs
We used soft microlithography⁴⁶ to pattern HeLa cells and neuronal mouse myocytes, as previously published.⁴⁷–⁴⁸

HeLa Cell Transfection
HeLa cells were transfected with pDEST-GST-GFP or pDEST-GJA1-20k-GFP using Lipofectamine 2000 according to manufacturer’s instructions (Thermo Fisher Scientific). Transfection efficiency was assessed by examining GFP fluorescence at 16 to 24 hours after transfection. The cells were detached using EDTA and seeded at a density of 10⁵ cells/mL on the micropatterned coverslips and cultured overnight in the 37°C incubator. The following day, the cells were washed with 1× PBS (3×5 min washes) and fixed in 4% paraformaldehyde (Electron Microscopy Services) and then incubated with Alexa Fluor secondary antibodies (Thermo Fisher Scientific) for 30 minutes at room temperature and mounted using ProLong gold-containing DAPI (Thermo Fisher Scientific) for image acquisition.

Electron Microscopy
For transmission electron microscopy, HeLa cells were transfected with either GST-GFP or GJA1-20k-GFP plasmids as described above. Cells were fixed in 2% glutaraldehyde in PBS, scraped, and centrifuged and the resulting pellet was post-fixed with 1% osmium tetroxide and incubated in 3% uranyl acetate. Samples were then dehydrated in ethanol, treated with propylene oxide, embedded in Spurr resin (Electron Microscopy Services), and sectioned using an ultramicrotome (Ultracut UC7, Leica). Sections were mounted on electron microscope grids and stained with uranyl acetate and lead citrate followed by imaging. All electron microscopy work was done by the core facility at the Electron Imaging Center of The California NanoSystems Institute, The University of California Los Angeles (UCLA).

Immunofluorescence Staining
Cells were fixed in either 4% paraformaldehyde (Electron Microscopy Services) or in ice-cold methanol. The cells were permeabilized and blocked at room temperature and incubated with primary antibody solution at 4°C overnight. The cells were then incubated at room temperature with antibody diluent containing Alexa Fluor secondary antibodies (Thermo Fisher Scientific) with phalloidin (Thermo Fisher Scientific) or with wheat germ agglutinin (Thermo Fisher Scientific). ProLong gold-containing DAPI (Thermo Fisher Scientific) was used to mount slides for image acquisition.

Fiber Quantification
Fiber number and length were quantified using imageJ (single z-plane with background subtracted).

Coimmunoprecipitation
Coimmunoprecipitation (Co-IP) was performed using transiently transfected HeLa cells expressing HA-tagged GJA1-20k.
Cells were lysed in lysis buffer (150 mmol/L KCl, 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) [pH 7.4], 2 mmol/L MgCl₂, 2 mmol/L K₂HPO₄, 1 mmol/L DTT, 25 μmol/L phalloidin, and Halt protease phosphatase inhibitors) containing 0.5% NP40. The cell lysate was precleared using Dynabeads protein G (Thermo Fisher Scientific), and immunoprecipitation was undertaken at 4°C overnight, using 5 μg of either mouse anti-HA (4C12, Abcam), or mouse anti-GST (B-14, Santa-Cruz Biotechnology) as negative isotype control. Dynabeads protein G were added to each reaction, and tubes were rotated for an additional hour at 4°C. Protein complexes were washed with lysis buffer containing 0.1% NP40 and pelleted using a Dynamag-2 magnet. Proteins were then eluted with 2X NuPAGE sample buffer supplemented with 100 mmol/L DTT, incubated at 37°C for 20 minutes and subjected to SDS-PAGE electrophoresis and Western blotting as described above.

Figure 1. GJA1-20k increases Cx43 (connexin 43) gap junction plaques at the intercalated discs in vivo. A, Schematic of the Adeno-associated virus serotype 9 (AAV9) gene delivery protocol. Mice received via retro-orbital injection 3×10¹⁰ vector genomes (vg) of AAV9 vectors expressing GST-GFP, GJA1-43k-GFP, or GJA1-20k-GFP. Localization of Cx43 at the intercalated discs was assessed at 4 wks post-injection. B, Immunofluorescence detection of N-cadherin (red, marking intercalated disc structures) and Cx43 (anti–N terminus, green) in heart sections from the different AAV9 treatment groups. Average intensity projections of confocal z-stacks (13.5 μm thickness), scale bar=50 μm. C, Quantification of Cx43 fluorescence intensity in intercalated disc regions. Data are presented as mean±SEM relative to GST group (n=3 hearts per group, 6–15 images analyzed per heart), *P<0.05, by 1-way ANOVA followed by Tukey post hoc test. D, Quantification of Cx43 fluorescence intensity normalized to N-cadherin signal. Data are presented as mean±SEM (n=3 hearts per group, 6–15 images analyzed per heart), *P<0.05, **<0.01, by 1-way ANOVA followed by Tukey post hoc test.
Cardiomyocyte Isolation, Transduction, and LatA Treatment

Primary neonatal mouse ventricular cardiomyocytes were isolated and maintained in culture medium as previously described.19 In brief, ventricles were dissected from postnatal hearts and digested with trypsin (Invitrogen) and type II collagenase (Worthington) at 37°C with constant gentle stirring. Cells were pre-plated on TC-treated culture dishes (Corning) to remove fibroblasts, before they are seeded on the micropatterned coverslips in culture media containing arabinofuranosyl cytidine and 5-bromo-20-deoxyuridine to inhibit rapidly proliferating nonmyocytes. Cardiomyocytes were then transduced overnight with adenovirus carrying GJA1-43k-V5, GJA1-20k-V5, or GFP-V5 (produced at the CURE Vector Core Facility at UCLA) and then treated with either 0.1% Dimethyl Sulfoxide (DMSO) control or 250 nmol/L LatA for 1 hour, and fixed in 100% ice-cold methanol (5 minutes at −20°C) or in 4% paraformaldehyde (Electron Microscopy Services) for 20 minutes at room temperature and stored in PBS at 4°C for immunofluorescence studies.

Quantification of α-Tubulin and EB1 (End-Binding Protein 1) Comets at the Cell–Cell Border

The cell–cell border was labeled using either N-cadherin or wheat germ agglutinin, and the cell counter plugin for ImageJ was used to quantify the number of EB1 (end-binding protein 1) comets or α-tubulin molecules reaching the cell–cell border. The number of EB1 comets and α-tubulin molecules at the border was normalized to the cell border length.

Statistical Analysis

All quantitative data were expressed as mean±SEM and analyzed using Prism 6 software (GraphPad). For comparison between 2 groups, unpaired 2-tailed Student t test was performed. For comparison among ≥3 treatment groups, 1-way ANOVA followed by either Bonferroni or Tukey post test was performed.

Results

GJA1-20k Increases Cx43 Gap Junction Plaque Size at the Intercalated Disc In Vivo

On the basis of our previous in vitro studies indicating that the GJA1-20k improves Cx43 gap junction plaque size,21 we tested whether GJA1-20k regulates Cx43 gap junction plaque size in vivo. We introduced GST-GFP control, GJA1-43k-GFP (lacking all internal methionines to generate only the full-length protein), or GJA1-20k-GFP (also lacking downstream internal methionines to generate only the 20k isoform) into adult mouse heart by recombinant AAV9-mediated gene transfer.34 Each mouse received 3×10^{10} vector genomes on experimental day 0 (Figure 1A). Four weeks after AAV9 delivery, hearts were harvested and assessed for Cx43 localization at the intercalated discs. Using an antibody specific to the Cx43 N terminus, thus only detecting full-length protein, we examined Cx43 localization in each AAV9 group by confocal imaging. N-terminal Cx43 immunofluorescence signal (green) is detected at intercalated discs which are demarcated by N-cadherin (red, Figure 1B). Introduction of full-length GJA1-43k (Figure 1B, middle) increased the overall intracellular accumulation of Cx43 signal, whereas GJA1-20k (Figure 1B, lower) robustly increased Cx43 immunofluorescence mainly at the intercalated discs. Quantification of Cx43 intensities at intercalated disc regions revealed that AAV9-mediated expression of GJA1-20k-GFP alone significantly increases Cx43 gap junction localization at the intercalated discs, when compared with GJA1-43k-GFP and GST-GFP groups (Figure 1C, all data normalized to the GST group). The increase in GJA1-20k holds when normalized to mean N-cadherin intensity (Figure 1D), which is not surprising given no significant difference in N-cadherin between the test groups (Online Figure I).

The Cx43 distribution was further examined using a Triton solubility assay to biochemically separate the heart tissue lysates into soluble (nonjunctional Cx43) versus insoluble (junctional Cx43) fractions.18,35 As seen in representative Western blots in Figure 2A (quantified in Figure 2B and 2C), GJA1-43k transduction led to full-length Cx43 protein accumulation primarily in the nonjunctional fraction (soluble Cx43), whereas GJA1-20k preferentially increased Cx43 amount in the junctional fraction (insoluble Cx43). This result supports the increase of Cx43 localization at plaques (Figure 1). To examine whether GJA1-20k is regulating Cx43 trafficking and not influencing overall expression of Cx43, we assessed the transcriptional level of Cx43 in the hearts of mice from each group. Cx43 mRNA levels were not significantly changed in the GJA1-20k-GFP animals when compared with the GST-GFP AAV9 control mice (Figure 2D).

Taken together, the imaging and biochemical data both indicate that exogenous GJA1-20k alone is sufficient to aid in targeted delivery of endogenous Cx43, increasing gap junction plaques at the intercalated discs in vivo. Interestingly, the increase in Cx43 plaques is significantly greater with exogenous GJA1-20k than with exogenous full-length GJA1-43k, highlighting the value of enhancing the trafficking apparatus rather than the protein substrate itself for its efficient localization into appropriate membrane subdomain.

GJA1-20k Stabilizes F-Actin Fibers

We have previously found that actin in general facilitates Cx43 forward trafficking.19 Actin is known to be responsive to the C-terminal tail of Cx43 which is homologous with GJA1-20k.18 We therefore used GJA1-20k overexpression to screen for the effects of GJA1-20k on the actin cytoskeleton. HeLa cells were used because they have low endogenous levels of Cx43 and are amendable to imaging experiments. Using transmission electron microscopy imaging in unpaired cells, we found that GJA1-20k overexpression results in a dramatic increase in both actin fiber length and number when compared with GST-transfected control cells (Figure 3A, top, arrows). The individual fibers are traced in red for better visualization (Figure 3A, bottom). On average, GJA1-20k increases average actin fiber length by 50% and doubles fiber number (Figure 3A, right).

Given that full-length Cx43 concentrates at cell–cell borders, the effect of GJA1-20k on actin fibers directed toward cell–cell borders was then studied in a micropatterned cell pairing system with controlled cell morphology and actin fiber orientation.33 Confocal images of micropatterned HeLa cells reveal that exogenous GJA1-20k expression can markedly promote the stability of F-actin fibers when compared with GST-transfected cells (Figure 3B) and untransfected cells (Online Figure II). GJA1-20k significantly increases actin fiber length and actin signal intensity as seen in the bar graphs (Figure 3B) without altering total actin protein levels (Figure 3C), indicating that GJA1-20k stabilizes polymerized actin fibers rather
than protein synthesis. These data suggest that GJA1-20k and actin fibers can be involved in making trafficking routes toward membrane subdomains. Using biochemical immunoprecipitation with HA-tagged GJA1-20k expressing HeLa cells, we find that GJA1-20k complexes with both actin and tubulin (Figure 3D), further indicating that GJA1-20k is a critical component of the actin–microtubule trafficking machinery.

GJA1-20k Stabilizes β-Actin Microfilaments in Cardiomyocytes

Our previous findings indicate that Cx43 complexes with nonsarcomeric β-actin which is a central component of intracellular microfilament fibers responsible for cellular roles other than β-actin thin filament mediated contraction. Using biochemical immunoprecipitation with HA-tagged GJA1-20k expressing HeLa cells, we find that GJA1-20k complexes with both actin and tubulin (Figure 3D), further indicating that GJA1-20k is a critical component of the actin–microtubule trafficking machinery.

F-Actin Stabilization Is Required to Orient Microtubule Growth Toward the Cell–Cell Junctions

To reach adherens junctions at cell–cell borders, Cx43 hemi-channels undergo targeted delivery, whereby Cx43-containing vesicles are trafficked along microtubules which anchor at N-cadherin–containing membrane for offloading. Using high-resolution confocal imaging and micropatterned neonatal mouse ventricular cardiomyocyte pairing, we explored the role of GJA1-20k and actin cytoskeleton in regulating directionality and attachment of the microtubule trafficking machinery (Figure 5). Inhibition of actin polymerization with a low dose of LatA greatly decreased the number of microtubules reaching the fascia adherens at the cell–cell junctions (Figure 5A through 5D). Microtubules were marked with either the plus-end tracking protein (+TIP) EB1 (Figure 5A and 5B) or α-tubulin (Figure 5C and 5D). EB1 marks rapidly growing tips of microtubules and is essential for Cx43 forward trafficking. Quantification of EB1 and α-tubulin (green) labeled microtubules reaching the N-cadherin defined

Figure 2. GJA1-20k increases junctional Cx43 (connexin 43) protein levels without affecting Cx43 transcription. A, Adult mice received via retro-orbital injection 3×10¹⁰ vector genomes (vg) of Adeno-associated virus serotype 9 (AAV9) vectors expressing either GST-GFP, GJA1-43k-GFP, or GJA1-20k-GFP. Four weeks after AAV9 gene expression, mouse hearts were subjected to TritonX-100-based tissue fractionation of soluble (nonjunctional) and insoluble (junctional) proteins and probed for Cx43, α-tubulin, and N-cadherin using Western blot analysis. B and C, Quantification of the amount of Cx43 in the soluble (B) and insoluble (C) fractions, normalized to Cx43 input and expressed as fold change relative to GST control. Data are presented as the mean±SEM (n=3 hearts per group) *P<0.05, **P<0.01, by 1-way ANOVA followed by Tukey post hoc test. D, Four weeks after AAV9 gene expression of GST-GFP or GJA1-20k-GFP (retro-orbital injection 3×10¹⁰ vg), Cx43 mRNA level was assessed in the mouse heart using Taqman gene expression assay and Real-Time PCR. Data are presented as mean±SEM relative to GST group (n=3 hearts per group), no significance was shown by Student t test.

with GJA1-20k-V5 resulted in partial rescue (protection) of β-actin filaments from impairment induced by LatA treatment (Figure 4A, bottom, and 4B), indicating stabilization of polymerized actin fibers by GJA1-20k, which is consistent with results from HeLa cells (Figure 3).
cell–cell border (red) is shown in Figure 5B and 5D, respectively. Strikingly, in these micropatterned cardiomyocyte pairs (Figure 5E), only exogenous GJA1-20k but not GFP or GJA1-43k is protective of microtubule trajectories from LatA effects, normalizing microtubule targeting (α-tubulin labeling, arrows in the skeleton image) to the cell–cell borders (defined by wheat germ agglutinin labeling, dotted line). Quantification of microtubule number reaching the cell–cell border in the different treatment groups is shown in Figure 5F. These results indicate that GJA1-20k organizes the actin cytoskeleton which in turn is required for the specific targeting of microtubules to the cellular junctions, placing the GJA1-20k arranged actin cytoskeleton upstream of the microtubule-based Cx43 trafficking machinery.

**GJA1-20k Maintains Cx43 Gap Junction Localization at the Intercalated Discs After Acute Ischemia**

Our previous findings indicate that the acute ischemia in Langendorff-perfused mouse hearts or actin disruption with...
LatA treatment, decreased Cx43 localization at the intercalated discs by disrupting Cx43/β-actin interaction. The quantified level of disruption was similar between ischemia and actin disruption.19 Given that GJA1-20k maintains the Cx43 trafficking machinery in the setting of actin disruption (Figure 5), we tested whether GJA1-20k is protective against Cx43 plaque disruption after acute ischemia. For these experiments, we again used the recombinant AAV9-mediated gene transfer system to deliver GST-GFP control, GJA1-43k-GFP, or GJA1-20k-GFP into the mouse heart in vivo. Each mouse received $3 \times 10^{10}$ vector genomes on experimental day 0. Four weeks later, hearts were rapidly excised and perfused ex vivo on a Langendorff system. Hearts were then subjected to either 30 minutes of no-flow ischemia or were continually perfused as a control (Figure 6A). Immediately after ischemia, hearts were mounted in OCT medium and snap frozen for later cryosection preparation. Using confocal imaging of immunofluorescence labeled fixed slices, we assessed Cx43 (N terminus antibody, green signal) localization at the intercalated discs marked with N-cadherin (red; Figure 6B). Results are that the Cx43 signal detected at intercalated discs is reduced in hearts transduced with GST-GFP and GJA1-43k-GFP after ischemia (Figure 6B, middle 2) when compared with the no ischemia control hearts (Figure 6A, left). Strikingly, in hearts transduced with GJA1-20k AAV9, robust Cx43 immunofluorescence is still detected at the intercalated discs after ischemia (Figure 6A, right). Quantification of Cx43 intensities at intercalated disc regions revealed that only AAV9-mediated expression of GJA1-20k-GFP maintained Cx43 localization to the intercalated discs after ischemia, to a level not significantly different from that is observed in the control nonischemic hearts (Figure 6C and 6D; Cx43 plaque fluorescence normalized to N-cadherin signal at the intercalated discs). Together these data indicate that in an acute ischemic injury setting, full-length GJA1-43k protein by itself is not sufficient to maintain Cx43 localization at the cell–cell junctions, whereas the GJA1-20k isoform is protective of Cx43 forward delivery to the intercalated discs, thus preserving the gap junction plaques.

**Discussion**

Targeted delivery of ion channels to the appropriate subdomain requires 3 components: a cytoskeleton delivery apparatus, a membrane anchor, and the channel itself.11,38 Previously, we identified that key components of targeted delivery of Cx43 hemichannels to the ventricular intercalated disc include microtubules which are captured by the membrane anchor adherens junctions complex including N-cadherin, β-catenin, and p150 (Glued).17,18 Although these trafficking components serve as the highways to the destination of intercalated disc, it has not been clarified how the channel itself is involved in specificity of delivery. In this study, we find that GJA1-20k, an alternatively translated isoform of Cx43,21 organizes the actin cytoskeleton (Figures 3A, 3B, 4A, and 4B; Online Figure II) which in turn is required to orient microtubule trajectories to the cell–cell border (Figure 5) for Cx43 delivery. These findings indicate that the specificity of Cx43 in delivery is derived from one of its smaller alternatively translated isoforms.

LatA binds with 1:1 stoichiometry to the monomeric globular actin (G-actin) near its nucleotide binding cleft and shifts the dynamic equilibrium to F-actin depolymerization.39 It is worth noting that our treatment of cardiomyocytes with a low dose of LatA (250 nmol/L) only partially inhibits actin polymerization, which might explain why actin fibers (Figure 4) and microtubule growth toward the cell–cell border (Figure 5) are not completely abolished in the LatA-treated GST-transduced

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**Figure 4.** GJA1-20k promotes β-actin polymerization, preserving and protecting the filamentous actin (F-actin) structure in cardiomyocytes. A, Confocal images of micropatterned neonatal mouse ventricular cardiomyocytes transduced with GFP-V5, GJA1-43k-V5, or GJA1-20k-V5 adenovirus and treated with DMSO or 250 nmol/L of Latrunculin A (LatA) and labeled for β-actin by immunofluorescence. Box insets show zoomed in areas of the cardiomyocytes. Scale bar=5 µm. β-actin fiber number is quantified in B, and data are presented as means±SEM (number of cells quantified for each group shown on the bars). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by 1-way ANOVA (Kruskal–Wallis test) followed by Dunn multiple comparisons test.
and GJA1-43k–transduced cardiomyocytes. GJA1-20k interacts with actin (Figure 3D) and stabilizes polymerized actin fibers (Figures 3A, 3B, 4A, and 4B; Online Figure II), shifting the equilibrium toward formation of F-actin opposing the effect of LatA. We previously identified the actin cytoskeleton as a necessary component of forward trafficking.19 Because Cx43 is relatively stationary when associated with actin, our paradigm from the earlier work was that actin serves as rest stops along the microtubule highway at which Cx43 vesicles slow down and dwell. The rest stops collect channels in an ever-ready reservoir able to be rapidly mobilized to ensure continuous supply en route to the intercalated disc. Our findings in this study indicate that GJA1-20k serves as an upstream stabilizer of these actin rest stops which in turn act as active organizers of the microtubule highways by orienting microtubule trajectories toward the cell–cell border for cargo delivery.

Precedent exists, in plants and neurites, for actin laying the scaffolds by which microtubules are patterned. 40,41 Our finding that GJA1-20k interacts with both actin and tubulin (Figure 3D) and that it stabilizes the actin cytoskeleton (Figures 3 and 4), thus regulating the microtubule network (Figure 5), provides a powerful tool to manipulate Cx43

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**Figure 5.** Actin polymerization as regulated by GJA1-20k is required to orient microtubule growth trajectories toward the cell–cell border. A, Confocal images of micropatterned neonatal mouse ventricular cardiomyocyte pairs treated with DMSO or 250 nmol/L of latrunculin A (LatA) and labeled for EB1 (end-binding protein 1; green, marking rapidly growing tips of microtubules) and N-cadherin (red, marking cell–cell border) by immunofluorescence. B, Number of EB1 molecules touching the cell–cell border is quantified and normalized to border length as shown in the graph. Data are presented as mean±SEM, n=25 cell pairs analyzed for each group from 2 dishes (10–15 images per dish).****P<0.0001 by Students t test. C, Confocal images of micropatterned neonatal mouse ventricular cardiomyocytes treated with DMSO or 250 nmol/L of LatA and labeled for α-tubulin (green, marking microtubule structures) and N-cadherin (red) by immunofluorescence. D, Number of α-tubulin molecules touching the cell–cell border is quantified and normalized to border length as shown in the graph. Data are presented as mean±SEM, n=19 cell pairs for each group from 2 dishes (9–10 images per dish).****P<0.0001 by Student t test. E, Confocal images of micropatterned neonatal mouse cardiomyocyte pairs transduced with GFP-V5, GJA1-43k-V5, or GJA1-20k-V5 adenovirus and treated with DMSO or 250 nmol/L of LatA and labeled for α-tubulin (green) by immunofluorescence. The cell–cell border is labeled with wheat germ agglutinin (WGA) and shown as a traced dotted line. The microtubules reaching the border are traced with arrows (right). F, Quantification of microtubules number that are touching the cell–cell border, normalized to border length. Data are presented as mean±SEM (n=10 cells per group); *P<0.05, ***P<0.001 by 1-way ANOVA followed by Tukey post hoc test.
trafficking. Using an in vivo AAV9 delivery system, we find that GJA1-20k offers high potency of facilitating the Cx43 trafficking pathway, as exogenous GJA1-20k alone increases endogenous Cx43 gap junction localization at the intercalated discs even greater than overexpression of full-length GJA1-43k itself (Figure 1).

We have previously found that in mouse heart, either acute ischemia or LatA treatment resulted in disruption of the actin cytoskeleton and disruption of Cx43/β-actin complexing, limiting localization of Cx43 to the intercalated discs. In this study, we extend these findings with evidence that in vivo AAV9 mediated introduction of GJA1-20k maintains Cx43 gap junction coupling after acute ischemia (Figure 6).

![Figure 6](image)

**Figure 6.** GJA1-20k maintains Cx43 (connexin 43) gap junction localization to the intercalated discs after acute ischemia. A, Adult mice received via retro-orbital injection 3×10¹⁰ vector genomes (vg) of Adeno-associated virus serotype 9 (AAV9) vectors expressing GST-GFP, GJA1-43k-GFP, or GJA1-20k-GFP. Four weeks post-injection, the hearts were excised and maintained using Langendorff perfusion apparatus for 20 min followed by 30 min of no-flow ischemia or continuous perfusion for control hearts. B, Immunofluorescence detection of N-cadherin (red, marking intercalated disc structures) and Cx43 (anti-N terminus, green) in heart sections from the different AAV9 treatment groups after ischemia or control perfusion. Average intensity projections of confocal z-stacks (13.5 µm thickness), scale bar=50 µm. C, Quantification of Cx43 fluorescence intensity at intercalated disc regions. Data are presented as mean±SEM relative to GST ischemia group (number of hearts analyzed per group is shown on bars, 5–10 images analyzed per heart), *P<0.05, **P<0.01, ***<0.001, ****<0.0001, by 1-way ANOVA followed by Tukey post hoc test. D, Quantification of Cx43/N-cadherin signal at intercalated disc regions. Data are presented as mean±SEM (number of hearts analyzed per group in shown on bars, 5–10 images analyzed per heart), ***P<0.001, ****P<0.0001, by 1-way ANOVA followed by Tukey post hoc test.
of anticipated cardiac ischemia such as cardiac surgery or planned percutaneous interventions.

It should be noted that although the focus of this study has been on forward trafficking, ischemia will also induce changes in Cx43 phosphorylation of the C terminus,\(^2\) including dephosphorylation of the casein kinase serines\(^3\) and phosphorylation of 14-3-3 binding motif including serine 373,\(^4\) both of which will accelerate internalization. It is possible that the protection of GJA1-20k against ischemia may occur not only in the acute conditions studied but is also involved in protecting the Cx43 C terminus against pathological post-translational modification. Future studies will examine the effects of GJA1-20k in the setting of subacute and chronic ischemia.

Cx43 has been implicated in the forward trafficking of other cardiac channels and junctional proteins to the cell–cell border, including Nav1.5 and N-cadherin.\(^45\)–\(^48\) It remains unclear where, between the Golgi and intercalated disc membrane, Cx43 facilitates their trafficking. It may be that rather than Cx43, GJA1-20k is a common facilitator of forward trafficking by stabilizing actin to organize microtubule growth trajectories for cargo delivery or by mediating actin-to-microtubule package handoff to aid in the delivery of multiple ion channels and membrane proteins. Although Cx43 is not known to contain a direct actin-binding domain, it interacts with F-actin indirectly via binding to zonula occludens-1 (ZO-1) at the last 4 amino acid residues of the C terminus.\(^39\) In an elegant study by Lübkeemeier et al,\(^46\) the last 5 amino acids were removed in a (Cx43D378stop) knock-in mouse. Cx43D378stop leads to reduction in sodium and potassium current densities and decreased Nav1.5 protein from intercalated discs. The impaired sodium and potassium trafficking in the Cx43D378stop mouse could be because of a truncated GJA1-20k isoform as discussed above. An additional F-actin interaction site downstream of the microtubule-binding domain in Cx43 has also been reported, which directly interacts with the actin-binding protein drebrin.\(^50\) This proximal F-actin interacting site is retained in GJA1-20k but this still requires further study as does mechanism by which GJA1-20k regulates actin-to-microtubule transfer of Cx43 vesicles.

Together our results indicate that the internally translated GJA1-20k isoform contributes specificity to Cx43 delivery by stabilizing polymerized F-actin to organize the growth trajectories of microtubules, guiding full-length Cx43 protein traffic toward the cell–cell junctions. Given its ability to maintain Cx43 localization at intercalated discs in the setting of acute ischemia, GJA1-20k is a potential therapy to modulate the cytoskeletal trafficking machinery in the setting of anticipated ischemic cardiac injury.

Acknowledgments

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Disclosures

None.

References

GJA1-20k Arranges Actin to Guide Cx43 Delivery to Cardiac Intercalated Discs
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SUPPLEMENTAL MATERIAL

Online Detailed Methods

Mice
C57BL/6 mice used for heart preparations were maintained under sterile barrier conditions. All procedures were reviewed and approved by Cedars-Sinai Medical Center Institutional Animal Care and Use Committee.

Molecular Biology
Human GJA1 cDNAs (Open Biosystems) encoding full-length Cx43 and smaller isoforms were cloned into pDONR/221 to generate Gateway entry clones (Thermo Fisher Scientific) as previously described. Using these clones, destination vectors (pDEST) encoding C-terminal V5-, HA- or GFP-tagged proteins were subsequently made. All constructs are driven by the cytomegalovirus (CMV) promoter. Internal methionine start sites in GJA1-43k, and GJA1-20k were mutagenized to leucine (QuickChange Lightning Mutagenesis Kit, Agilent) to ensure single isoform expression as described. Plasmids from this study are available at the Addgene repository (http://www.addgene.org/Robin_Shaw/).

AAV9 gene delivery
AAV9 vectors expressing GST-GFP, GJA1-20k-GFP, or GJA1-43k-GFP driven by the CMV promoter were produced in human embryonic kidney 293 cells by a virus-free triple-transfection method (Welgen Inc.). Cells were collected at 72 hours post transfection, lysed and used to purify AAV9 viruses by two cycles of cesium chloride gradient centrifugation as previously described. The final viral preparations were kept in phosphate-buffered saline and glycerol at -80 °C. All AAV9 viruses were purified by Welgen, Inc. (Worcester, MA). 8-12 week old Male C57BL/6 mice received 100 microliters of 3 x 10^{10} vector genomes (vg) through retro-orbital injection. Vector encoded GFP expression in heart tissue was confirmed by immunofluorescence detection at 4 weeks post injection. For acquiring the data in Figure 1, whole ventricles were collected at 4 weeks post AAV9 injection and embedded in OCT (Sakura Finotek) and snap-frozen by immersing in dry ice-isopentane bath before storage at −80°C for immunofluorescence. For Figure 2, the ventricular
samples were placed in cryovials and snap-frozen in dry ice-isopentane for biochemical studies or homogenized in Trizol for RNA analysis. For acquiring the data in Figure 6, mouse hearts were isolated at 4 weeks post AAV9 injection and subjected to acute ischemic injury using a Langendorff perfusion system as previously reported. The hearts were then embedded in OCT (Sakura Finotek) and snap-frozen by immersing in dry ice-isopentane bath before storage at −80°C for immunofluorescence.

**Langendorff-perfused mouse heart preparation**

C57BL/6 mice were maintained under sterile barrier conditions. All procedures were reviewed and approved by Cedars-Sinai Medical Center Institutional Animal Care and Use Committee. 4 weeks post AAV9 injection, a subset of mouse hearts was excised, ex vivo perfused on a Langendorff-perfusion apparatus, and subjected to acute ischemic injury study as previously described. Briefly, hearts from 12-14 week old mice were dissected in ice-cold oxygenated Krebs-Henseleit (K-H) solution, pH 7.4, containing (mM): 118 NaCl, 4.7 KCl, 2.5 CaCl2.H2O, 1.2 MgCl2, 24 NaHCO3, 1.2 KH2PO4, 11 glucose, and 0.5 EDTA. The hearts were attached to a Langendorff apparatus (ADInstruments) and retrogradely perfused at a constant rate of 2.6 ml/min with the K-H buffer, which was constantly gassed with 95% O2/5% CO2, and maintained at 37°C. Equilibration was carried out for 20 minutes before hearts were subjected to 30 minutes of global ischemia. During no-flow ischemia, the heart was immersed in warm K-H buffer to maintain warmth and moisture. Control hearts were perfused continuously throughout the protocol. Immediately after the procedure, hearts were embedded in OCT media (Sakura Finotek) and snap frozen for cryosectioning.

**Tissue immunofluorescence**

For tissue immunofluorescence, cryosections (10 µm) were fixed in 4% PFA for 20 minutes at room temperature, blocked and permeabilized at room temperature for 1 hour with 10% normal goat serum (NGS) and 0.5% TritonX-100 in PBS. The tissue sections were incubated with primary antibodies diluted in 5% NGS and 0.1% TritonX-100 in PBS (antibody diluent) at 4°C overnight. The primary antibodies used are: rabbit anti-N-cadherin (1:100, Santa Cruz Biotechnology) and mouse anti-Cx43 (N-terminus epitope, 1:200, Acris AM32558SU-N). Tissue slides were then incubated with Alexa fluor secondary antibodies (Thermo Fisher Scientific) in antibody diluent (1:500) for 1 hour at room temperature. ProLong gold-containing DAPI (Thermo Fisher Scientific)
was used to mount slides for image acquisition using our Nikon Eclipse Ti imaging system with a x60/1.49 Apo objective or x20/0.75 Plan Apo objective, a spinning disk confocal unit (Yokogowa CSU-X1) with 486, 561, and 647-nm diode-pumped solid state lasers, and an ORCA-Flash 4.0 Hamamatsu camera (C11440), controlled by NIS Elements software.

Quantification of Cx43 at the intercalated disc
For Figures 1 and 6, quantification of Cx43 expression at N-Cadherin-containing intercalated disc regions was carried out as previously described\(^5,6\) using average intensity projections of 13.5 \(\mu\)m confocal z-stacks. To isolate intercalated discs, ImageJ was used to subtract the background signal from N-Cadherin images, to which equal thresholding was applied to generate binary masks. Within this mask image, intercalated disc areas had a value of 1, while pixels outside of these regions had a value of 0. Masks were image-multiplied by the corresponding Cx43 image (background subtracted), thus excluding all Cx43 signal except those at the intercalated disc. Cx43 plaque fluorescence intensity was subsequently measured and normalized to GST control (Figure 1C and Figure 6C) or to N-Cadherin signal at the intercalated disc regions for each of the treatment groups (Figure 1D and Figure 6D). For each sample, 5-12 randomly selected images with comparable numbers of intercalated discs were quantified from three separate hearts for each condition.

Triton Solubility Assay
Snap-frozen mouse ventricular tissue samples were weighed and added to 1% Triton X-100 buffer (50 mM Tris pH 7.4, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 250 mM NaCl, 1 mM NaF, 0.1 mM Na\(\text{3VO}_4\) and 1x Halt Protease-Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific)) at 100 mg tissue/ml. Samples were homogenized and nutated for 1 hour at 4°C. Subsequently, 100\(\mu\)l of lysate was added to an equal volume of 1% Triton X-100 buffer containing 8 M urea and 2 M thiourea (Sigma-Aldrich) to solubilize junctional proteins and generate the total protein fraction. The remaining lysate was centrifuged at 10,000 \(xg\) for 20 minutes in pre-weighed tubes. The supernatant was removed and added to an equal volume of 1% Triton X-100 buffer containing 8 M urea and 2 M thiourea for the soluble fraction. Pellets were weighed and suspended in 1% Triton X-100 buffer containing 4 M urea and 1 M thiourea to a final concentration of 30 mg/ml. A motorized pestle (Argos Technologies) was used to dissolve the pellet and generate the insoluble protein fraction. NuPAGE sample buffer (ThermoFisher Scientific) containing 100mM DTT was added to
all fractions prior to sonication and the samples were reduced for 45 minutes at room temperature before Western blotting. After quantification of band intensities, all values were normalized to Cx43 in 10mg of total cell lysate. The amount of Cx43 in each fraction was expressed at fold change relative to GST control.

**Western blotting**

Protein concentration determination (Bio-Rad DC Protein Assay) and Western blotting were performed as previously described\(^1\). Briefly, samples were subjected to SDS-PAGE electrophoresis using NuPAGE Bis-Tris gels and MES (Thermo Fisher Scientific) buffer according to the manufacturer’s instructions. Gels were transferred to FluoroTrans PVDF membranes (Pall) and subsequently fixed by soaking in 100% methanol and air drying before rewetting with methanol and blocking for 1 hour at room temperature in 5% nonfat milk in TNT buffer (0.1% Tween 20, 150 mM NaCl, 50 mM Tris pH 8.0). Membranes were probed overnight with the following primary antibodies in 5% milk in TNT: rabbit anti-GST (1:2000, Santa Cruz), rabbit anti-Cx43 (1:2000, Sigma-Aldrich), mouse anti-Cx43 CT1 (C-terminus, 1:500, Fred Hutchinson Cancer Research Center), mouse anti-HA (1:1000, Sigma-Aldrich), rabbit anti-N-term actin (1:3000, Sigma-Aldrich), mouse anti-GAPDH (1:2000, Sigma-Aldrich), mouse anti-α-tubulin (1:1000; BD Biosciences), rabbit anti-α-tubulin (1:1000, Abcam) and rabbit anti-N-Cadherin (1:200, Santa Cruz Biotechnology). Membranes were washed 5 times and incubated for 1 hour at room temperature with secondary antibodies in 5% milk TNT. Goat secondary antibodies conjugated to Alexa Fluor 555 and 647 were used (1:1000). Membranes were washed 5 times, immersed in 100% methanol and air dried. Images were obtained using the ChemiDoc MP fluorescent western detection system (BioRad).

**Quantitative Real Time PCR**

Total RNA was isolated from mouse hearts, 4 weeks post AAV9 retro orbital injection, using Trizol and PureLink RNA mini kit (Thermo Fisher Scientific) according to manufacturer’s instructions. The purified RNA was further treated for any DNA contamination using Turbo DNA-Free Kit (Thermo Fisher Scientific) and five micrograms of total RNA were used for cDNA synthesis using the SuperScript IV VILO Master Mix (Thermo Fisher Scientific). Taqman gene expression assays were prepared using 0.5ug of cDNA per well, Taqman Universal Master Mix (Thermo Fisher Scientific) and Taqman probes (Gja1 (Mm00439105_M1) and Hprt (Mm01545399_M1) (Thermo
Fisher Scientific). Quantitative Real Time PCR reactions were run using Biorad CFX Connect Real-Time System using the following protocol: UNG incubation 50°C for 2 min, Polymerase activation 95°C for 10 minutes, then 40 cycles of denaturing 95°C for 15 seconds and annealing/extending 60°C for 1 minute.

**Micropatterning of cell pairs**

We used soft-microlithography \(^7\) to pattern HeLa cells and neonatal mouse myocytes, as previously published\(^8\)–\(^{13}\). Micropatterned coverslips were stored at 4 °C in the dark prior to cell seeding. On the day of seeding, micropatterned glass coverslips (placed in 24 well plates) were washed 3x with PBS (with 0.49 mM Mg\(^{2+}\) and 0.9 mM Ca\(^{2+}\)) and then incubated for at least 30 min with culture medium.

**Hela cell transfection**

HeLa cells (ATCC, CCL-2) were seeded at a concentration of 2 x10\(^6\) cells on 10cm culture dishes and the cells were maintained in culture medium (DMEM media, antibiotic free, containing 10% FBS, non-essential amino acids and sodium pyruvate) (Thermo Fisher Scientific) at 37°C in a humidified atmosphere of 5% CO\(_2\) for 16-24 hours. The cells were then transfected with pDEST-GST-GFP or pDEST-GJA1-20k-GFP using Lipofectamine 2000 according to manufacturer’s instructions (Thermo Fisher Scientific). 16-24 hours after transfection, the cells were washed 2x with culture medium and then incubated for an additional 1hr in the 37°C incubator, after which the transfection efficiency was assessed by examining GFP fluorescence. The cells were detached with 0.02% EDTA in PBS for 15 minutes in the 37°C incubator, and seeded at a density of 10\(^5\) cells/ml on the micropatterned coverslips. Cells were allowed to adhere for 2 hours and then washed 3x with culture medium and cultured overnight in the 37°C incubator. The day following seeding on the micropatterned coverslips, Hela cell pairs were washed with 1x PBS (3x5 min washes), fixed in 4% PFA (Electron Microscopy Services) for 20 minutes at room temperature before immunofluorescence studies.

**Electron microscopy**

For transmission electron microscopy (TEM), HeLa cells were transfected with either GST-GFP or GJA1-20k-GFP plasmids as described above. Cells were fixed in 2% glutaraldehyde in PBS (10 minutes at room temperature), scraped, and centrifuged at 4200 x g, followed by 16,000 x g. Pellets were fixed for 2 hours, and post-fixed with 1% osmium tetroxide and incubated in 3% uranyl
acetate. Samples were then dehydrated in ethanol, treated with propylene oxide, embedded in Spurr resin (Electron Microscopy Services), and sectioned using an ultramicrotome (UCT, Leica). Sections were mounted on EM grids and stained with uranyl acetate and lead citrate. Images were acquired using the JEM1200-EX, JEOL microscope equipped with a digital camera (BioScan 600W, Gatan). All electron microscopy work was done by the core facility at the Electron Imaging Center of The California NanoSystems Institute, UCLA.

**Hela cells and neonatal cardiomyocytes immunofluorescence**

Transfected Hela cells were fixed in 4% paraformaldehyde (PFA, Electron Microscopy Services) for 20 minutes at room temperature and then blocked and permeabilized using 0.2% Triton X-100 and 5% normal goat serum (NGS) (Thermo Fisher Scientific) for 1 hour at room temperature. The cells were incubated with primary antibodies in diluent (2.5% NGS and 0.1% TritonX-100 in PBS) at 4°C overnight. The primary antibodies used are: chick anti-GFP (1:500, Abcam) and rabbit anti-Cx43 (1:500, Sigma-Aldrich). Hela cells were then incubated for 2.5 hours at room temperature with antibody diluent containing Alexa fluor secondary antibodies (1:500) (Themro Fisher Scientific) and phalloidin Alexa555 (1:30) (Thermo Fisher Scientific). ProLong gold-containing DAPI (Thermo Fisher Scientific) was used to mount slides for image acquisition.

Neonatal mouse ventricular cardiomyocytes were either fixed with ice cold methanol for 5 minutes at -20°C (for EB1 and α-tubulin staining) or with 4% PFA (Electron Microscopy Services) for 20-30 minutes at room temperature (for β-actin staining). The cardiomyocytes were permeabilized with 0.1% TritonX-100 in PBS for 10 minutes at room temperature then incubated with blocking solution (5% NGS in PBS) for 2 hours at room temperature. The cardiomyocytes were then incubated with primary antibodies diluted in 1% NGS in PBS (antibody diluent) at 4°C overnight. The primary antibodies used are: mouse anti-N-Cadherin (1:100, BD Biosciences), rabbit anti-Cx43 (1:120, Sigma-Aldrich), rat anti-EB1 (1:25, Abcam), rat anti-α-tubulin (1:50, abcam), rabbit anti-tubulin (1:100 abcam), mouse anti-β-actin (1:500, sigma). The cardiomyocytes were then incubated for 1 hour at room temperature with antibody diluent containing Alexa fluor secondary antibodies (1:500) (Themro Fisher Scientific) and wheat germ agglutinin (WGA) AlexaFluor-647 (Thermo Fisher Scientific) to label the cell membrane. The cardiomyocyte coverslips were then mounted with ProLong gold-containing DAPI (Thermo Fisher Scientific) for image acquisition. Hela cells
and the neonatal cardiomyocytes were imaged using our Nikon Eclipse Ti imaging system with a ×100/1.49 Apo TIRF objective.

**Fiber quantification:**
For the TEM data (Figure 3A), fiber number and length were quantified using the segmented line tool in ImageJ, from TEM images of Hela cells that were transfected with either GST-GFP or GJA1-20k-GFP plasmids as described above. 13 images with similar regions of interest were selected in total from each genotype, acquired from 13 different cells at 50,000× magnification, and were used for the quantification of average fiber length and number. Fibers that were less than 0.1um in length or didn’t have clear end points were excluded from the quantification.

For Figure 3B, average actin fiber length and average mean intensity in the micropatterned Hela cell images were measured using ImageJ. Actin fiber length was measured from 10-12 cells per group (single z-plane with background subtracted) and fibers that were less than 0.5 um in length or didn’t have clear end points were excluded from the quantification. Actin signal intensity was quantified using the whole cell as a region of interest (single z-plane, background subtracted) with 23-25 cells quantified per group.

For Figures 4A-4B, average β-actin fiber number in the micropatterned neonatal mouse cardiomyocytes was quantified using cell counter plugin for ImageJ with 25-41 cell images quantified per group (single z-plane, background subtracted). β-actin fibers that were less than 5 um in length and that were outlining the sides of the cells were excluded from the quantification.

**Co-immunoprecipitation**
Co-IP was carried out using transiently transfected HeLa cells expressing HA-tagged GJA1-20k. Cytoskeletal-protein interactions were stabilized by the addition of phalloidin (25µM) during cell lysis in 0.5% NP40 buffer (in mM, 150 KCl, 20 HEPES, 2 MgCl2, 2KHPO4, 1 DTT, 1 NaF, 0.1 Na3VO4, 0.5% NP40, pH7.4 with halt protease inhibitor). Cells were lysed for 30 minutes with gentle rotation and spun down at 10,000 x g for 20 minutes at 4°C to remove insoluble debris. Following protein normalization, the cell lysate was precleared using Dynabeads protein G (Thermo Fisher Scientific) for 30 minutes with gentle rotation at 4°C. Beads were discarded and 2mg of precleared lysate was used per reaction. Immunoprecipitation was undertaken using 5µg of either mouse anti-HA (4C12, Abcam), or mouse anti-GST (B-14, Santa-Cruz Biotechnology) as negative isotype control at 4°C for 4 hours with gentle rotation. Dynabeads protein G (20 µl) was added to
each reaction, and tubes were rotated for 1 hour at 4°C. Protein complexes were washed 3 times with lysis buffer containing 0.1% NP40 on ice using a Dynamag-2 magnet. Proteins were then eluted with 30 μl 2X NuPAGE sample buffer supplemented with 100 mM DTT, incubated at 37 degrees for 20 minutes and subjected to SDS-PAGE electrophoresis and Western blotting as described above.

**Cardiomyocyte isolation, transduction and latrunculin A treatment.**

Primary neonatal mouse ventricular cardiomyocytes were isolated and maintained in culture medium (DMEM: F12 media (Thermo Fisher Scientific) supplemented with 5% fetal bovine serum (FBS), 1X ITS (insulin- transferrin-selenium) (Thermo Fisher Scientific), 10nM 5-bromo-20-deoxyuridine (BrdU), 20nM arabinofuranosyl cytidine (AraC) and 1x Mycozap-PR (Lonza)) as previously described\(^{14}\). Briefly, ventricles were dissected from postnatal day 1 to 3 hearts and digested with 0.2 mg/ml trypsin (Invitrogen) and 50 U/ml type II collagenase (Worthington) in Hank’s Balanced Salt Solution (HBSS, Life Technologies) at 37 °C with constant gentle stirring. Dispersed cells were collected and stored in 5% FBS on ice, while fresh pre-warmed isolation buffer was added every 10 min until tissues were completely digested. Cells were pre-plated on TC-treated culture dishes (Corning) for 25 min to remove fibroblasts, before they are seeded on the micropatterned coverslips in culture media containing arabinofuranosyl cytidine (AraC) and 5-bromo-20-deoxyuridine (BrdU) to inhibit rapidly proliferating non-myocytes. After 16-24 hours, cells were transduced overnight with adenovirus encoding GJA1-43k-V5, GJA1-20k-V5 or GFP-V5 at the multiplicity of infection (M.O.I.) of 4 plaque-forming unit (pfu)/cell. Adenoviruses were generated at the University of California Los Angeles CURE Vector Core Facility. At 24 hours post transduction, the cardiomyocytes were treated with either 0.1% DMSO control or 250 nM latrunculin A for 1 hour, and fixed in 100% ice-cold methanol (5 minutes at -20°C) or in 4% PFA (Electron Microscopy Services) for 20 minutes at room temperature and stored in PBS at 4 °C for immunofluorescence studies.

**Quantification of α-tubulin and EB1 comets at the cell-cell border.**

For Figures 5A-5D, N-Cadherin staining (red) was used to identify the cell-cell border. The cell counter plugin for imageJ was used to quantify the number of EB1 comets (from 25 cell images, single z-plane, background subtracted) or α-tubulin molecules (from 19 cell images, single z-plane, background subtracted) that were partially overlapping with the N-Cadherin signal. The number of
EB1 comets and α-tubulin molecules were then normalized to the cell border length measured in µm. Partial overlap in fluorescence signal between EB1 or α-tubulin and N-Cadherin indicates that they are in the same z-plane and they were therefore included in the quantification. EB1 and α-tubulin molecules that were completely overlapping with the N-Cadherin signal were excluded from the quantification. For Figures 5E-5F, WGA was used to label the cell-cell border, shown as a white dotted line in the images. The number of α-tubulin molecules touching the cell-cell border was quantified using the cell counter plugin for ImageJ and then normalized to the cell border length in µm as measured using imageJ (10 cell images were quantified per group, single z-plane, background subtracted).

**Statistical analysis**

All quantitative data were expressed as mean +/- s.e.m. and analyzed using Prism 6 software (GraphPad). For comparison between two groups, unpaired two-tailed student’s t-test was performed. For comparison among three and more treatment groups, one-way ANOVA followed by either Bonferroni’s or Tukey’s post-test was performed.
**Online Figures**

**Online Figure I**

**Online Figure I. GJA1-20k does not affect N-Cadherin expression level at the intercalated discs.**
Quantification of N-Cadherin fluorescence intensity at intercalated disc regions in heart sections from mice injected with AAV9 vectors expressing GST-GFP, GJA1-43k-GFP, or GJA1-20k-GFP. Data are presented as mean ±SEM (n = 3 hearts per group, 6-15 images analyzed per heart), no statistical significance shown by one-way ANOVA followed by Tukey’s post-hoc-test.
Online Figure II

20k-GFP

Transfected cell

Non-transfected cell

Actin

Merge

Online Figure II. GJA1-20k isoform promotes actin polymerization and stabilizes F-actin. Confocal image of a micropatterned non-transfected Hela cell (bottom cell) or a transfected cell with GJA1-20k-GFP (top cell) with both cells found in the same field of view. F-actin is labeled with phalloidin (red) and exogenous GJA1-20k expression is confirmed using immunofluorescence labeling of GFP (green). Scale bar = 10µm
Online References