

Autoregulation of Connexin43 Gap Junction Formation by Internally Translated Isoforms

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SUMMARY

During each heartbeat, intercellular electrical coupling via connexin43 (Cx43) gap junctions enables synchronous cardiac contraction. In failing hearts, impaired Cx43 trafficking reduces gap junction coupling, resulting in arrhythmias. Here we report that internal translation within Cx43 (*GJA1*) mRNA occurs, resulting in truncated isoforms that autoregulate Cx43 trafficking. We find that at least four truncated Cx43 isoforms occur in the human heart, with a 20 kDa isoform predominating. In-frame AUG codons within *GJA1* mRNA are the translation initiation sites and their ablation arrests trafficking of full-length Cx43. The 20 kDa isoform is sufficient to rescue this trafficking defect in *trans*, suggesting it as a trafficking chaperone for Cx43. Limiting cap-dependent translation through inhibition of mTOR enhances truncated isoform expression, increasing Cx43 gap junction size. The results suggest that internal translation is a mechanism of membrane protein autoregulation and a potent target for therapies aimed at restoring normal electrical coupling in diseased hearts.

INTRODUCTION

Gap junctions, which are comprised of connexin proteins, provide the intercellular coupling that is necessary for rapid action potential propagation through the myocardium, triggering synchronized heart contraction (Rohr, 2004; Shaw and Rudy, 1997). Connexin43 (Cx43) is the most commonly expressed of the 21 human connexins, occurs in all organ systems, and is particularly enriched in ventricular cardiomyocytes (Beyer et al., 1987). Six Cx43 molecules oligomerize to form transmembrane channels, termed connexons, that couple with apposing connexons on neighboring cells and coalesce into dense gap junction plaques (Unwin and Zampighi, 1980). Given the broad expression pattern of Cx43, it is unsurprising that alterations in Cx43 gap junction coupling are associated with diverse pathologies, including heart disease (Akar et al., 2007; Beardslee

et al., 2000; Luke and Saffitz, 1991; Smith et al., 1991), connective tissue disease (Paznekas et al., 2003), and cancer (Solan et al., 2012). In fact, altered Cx43 trafficking contributes to the arrhythmias of sudden cardiac death (Kalcheva et al., 2007; Peters et al., 1997; Remo et al., 2011; Shaw and Rudy, 1997; Smyth et al., 2010).

Gap junction turnover and the Cx43 half-life occur within several hours (Beardslee et al., 1998; Jordan et al., 1999; Shaw et al., 2007). Regulation of Cx43 trafficking is therefore a critical and continuous cellular need. Ion channel function and trafficking are usually regulated by related auxiliary protein subunits (Smyth and Shaw, 2010). To date, no such auxiliary molecules that are specific to connexins have been identified, and the mechanisms that regulate anterograde transport of Cx43 from the cell interior to the surface remain under investigation (Shaw et al., 2007; Smyth et al., 2010).

Human Cx43 is encoded by the *GJA1* gene, which comprises two exons, and the entire coding sequence of the Cx43 protein resides within the second exon (Fishman et al., 1991). Only the 5' UTR of *GJA1* mRNA has been reported to undergo alternative splicing events (Pfeifer et al., 2004). Recent data indicate that eukaryotic internal ribosome entry sites (IRES) exist as a form of translational regulation, and cap-independent translation events are now accepted as a powerful, but previously underappreciated, mechanism of posttranscriptional protein regulation (Candeias et al., 2006; Ingolia et al., 2011). Interestingly, an IRES element has been found within the 5' UTR of *GJA1* mRNA (Schiavi et al., 1999), but not within the coding sequence. We are not aware of internal translation sites being identified for Cx43 or any mammalian membrane channel.

We previously found that altered Cx43 intracellular trafficking contributes to losses in cell-cell coupling in diseased hearts (Smyth et al., 2010, 2012). In this study, we present the finding that Cx43 trafficking is genetically autoregulated by chaperone proteins generated through internal translation events within the coding sequence of Cx43 (*GJA1*) mRNA. In human heart and cell lines, several truncated isoforms of Cx43 that encompass the C terminus of Cx43 are detectable. Mutagenesis experiments reveal that these isoforms originate from internal translation initiation events, and that loss of their expression arrests full-length Cx43 trafficking. We also find that translation of these isoforms is regulated by the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway,

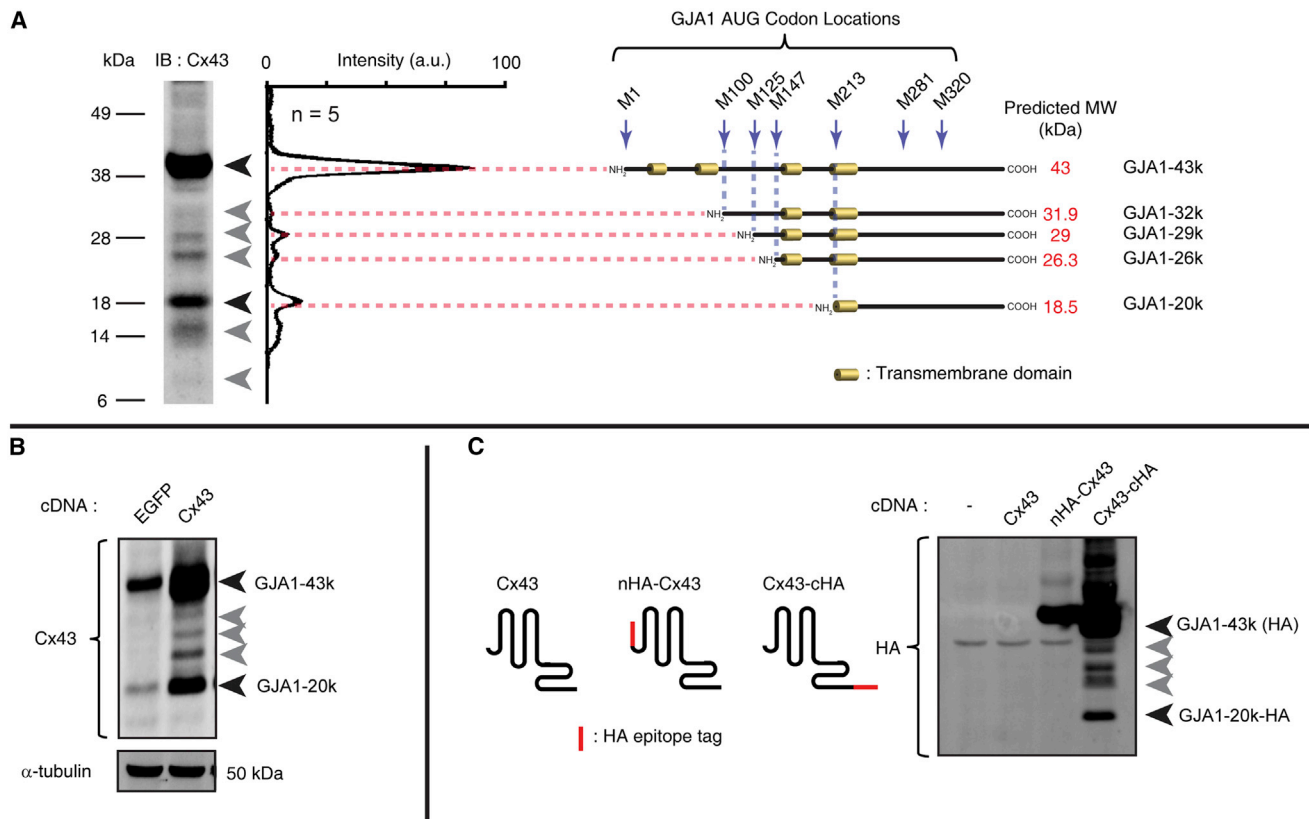


Figure 1. Multiple C-Terminal Isoforms of Cx43 Occur in Human Heart

(A) Western blot of nonfailing human left ventricle with monoclonal antibody against the Cx43 C terminus. Intensity profiles of five separate hearts were averaged and plotted in the graph, with peaks corresponding to major immunodetectable bands (arrows). Schematic: predicted methionine-initiated polypeptides and their molecular weights.

(B) Western blot of HaCaT cells transfected with cDNA encoding EGFP or Cx43.

(C) Cx43 N- and C-terminal HA-tagged fusion proteins expressed in 293T cells. Western blot probed with monoclonal HA antibody.

See also [Figures S1 and S2](#).

which promotes cap-dependent translation. Inhibition of mTOR kinase activity increases the Cx43 gap junction plaque size at cardiomyocyte cell-cell borders. Taken together, these findings introduce a paradigm of membrane protein autoregulation through internal translation and reveal a role for cap-independent translation in maintaining electrical coupling in the heart.

RESULTS AND DISCUSSION

We explored expression of Cx43 in the left ventricle of nondiseased human hearts. Western blot using a Cx43 C-terminus-directed antibody reveals multiple distinct bands ([Figure 1A](#)). The dominant band between 40 and 45 kDa is typically reported for the 43 kDa protein and has been separated into phosphospecific isoforms ([Solán and Lampe, 2007](#)). A second dominant band, which we term GJA1-20k, occurs at 18–20 kDa and has occasionally been observed in cell lines ([Joshi-Mukherjee et al., 2007](#)). There are as many as five other distinct, but previously unreported, immunoreactive bands detectable below the predicted 43 kDa full-length Cx43 protein (we use the term GJA1-43k) protein ([Figure 1A](#), gray arrows). We then investi-

gated, in different human cell lines, whether the Cx43 isoforms were also present ([Figure S1A](#)). Just as in the human hearts, the 43 kDa and 20 kDa bands typically predominate and three middle forms are detectable, with variation between each cell line. Of note, we find that the dominant GJA1-20k isoform migrates between 18 and 25 kDa and can occur as a doublet. We attribute this to altered phosphorylation status of the Cx43 C-terminal region ([Solán and Lampe, 2007](#)).

Several mammalian genes have recently been identified to contain internal translation initiation start sites that permit cap-independent translation within their coding regions ([Ingolia et al., 2011](#)), and in zebrafish, Cx55.5 can generate distinct protein isoforms ([Ul-Hussain et al., 2008a, 2008b](#)). We hypothesized that human *GJA1* mRNA occurs as a polycistronic molecule and that different isoforms of Cx43 protein arise from internal translation events. Seven AUG (methionine) codons exist in frame within the coding sequence of Cx43. When we tested whether each methionine could be a separate start codon, in silico analysis of the *GJA1* coding mRNA revealed a striking correlation between the predicted protein size and that of five anti-Cx43 bands identified ([Figure 1A](#), schematic).

Transfection of cDNA containing the Cx43 coding sequence under transcriptional regulation of a cytomegalovirus (CMV) promoter is sufficient to increase expression of four smaller isoforms in addition to full-length GJA1-43k (Figure 1B). If the multiple isoforms arise from cleavage of the full-length protein, one would expect both C-terminal and N-terminal fragments to exist. However, recognition of truncated isoforms requires antibody against the C terminus, and not the N terminus, of Cx43 (Figure S1B, left panel, red arrow), indicating that the N terminus of Cx43 is contained only in the full-length protein, GJA1-43k (Figure S1B, right panel). To confirm C terminus specificity, we generated N- and C-terminal Cx43 fusion proteins with the hemagglutinin (HA) epitope tag. Only the C-terminally fused Cx43-HA construct generated detectable smaller HA-tagged isoforms dominated by the 20 kDa band as seen in human heart, in addition to full-length GJA1-43k-HA (Figure 1C, arrow). We then introduced previously described point mutations (R202E and F199L) that retard trafficking of full-length Cx43 (Olbina and Eckhart, 2003). Both mutations resulted in loss of gap junction plaque formation and redistribution of Cx43 to the cytoplasm (Figure S2A, arrows), yet the presence of truncated isoforms was not affected by the deficit in cytoplasmic trafficking (Figure S2B, arrows) suggesting their origin occurs prior to processing later in the vesicular transport pathway.

To confirm that all Cx43 isoforms detected in Figure 1 arise from the same mRNA molecule, we introduced three distinct siRNA duplexes into 293T cells. All three duplexes targeted regions of the mRNA sequence more 5' proximal than GJA1-20k, yet all three were capable of ablating expression of the smaller isoforms (Figure 2A). We then transfected *in vitro* transcribed GJA1 mRNA that is resistant to siRNA (duplex ii in Figure 2A) into 293T cells following knockdown of endogenous Cx43. Consistent with the possibility of internal translation initiation sites, the exogenously generated GJA1 mRNA was sufficient to encode the major full-length (43 kDa) and smaller (20 kDa) forms of Cx43 (Figure 2B). If the smaller Cx43 isoforms arise from internal translation initiation, then the initial start codon (M1) of the GJA1 gene should not be necessary for their expression. Indeed, ablation of the GJA1 start codon prevents expression of the full-length protein, but enhances expression of smaller isoforms (Figure 2C). The *in silico* studies shown in Figure 1A predict that in-frame internal AUG codons of GJA1 can be initiation sites for the truncated isoforms. We sequentially mutated each internal methionine codon (AUG) to aspartate (GAC). Each mutation resulted in loss of the corresponding predicted Cx43 isoform (Figure 2D, arrows). These data were confirmed by fusion of an HA epitope tag to the C terminus of Cx43, where substitution of all six internal methionines with the more innocuous mutation to leucine (Cx43-ML-HA) also ablated expression of the smaller isoforms (Figure S2C).

Internal translation within the coding sequence of GJA1 mRNA may arise from ribosomes that have read through previous start sites, or by internal ribosomal entry and initiation within the coding sequence proper. We introduced a potent triple-stop (TAGTAAATGA) signal by mutation at codons 177, 178, and 179 in the Cx43-HA construct (Cx43-180STOP-HA). These codons occur proximal to M213, so if GJA1-20k (HA tagged) is a result of ribosomal read-through, its expression should be prevented

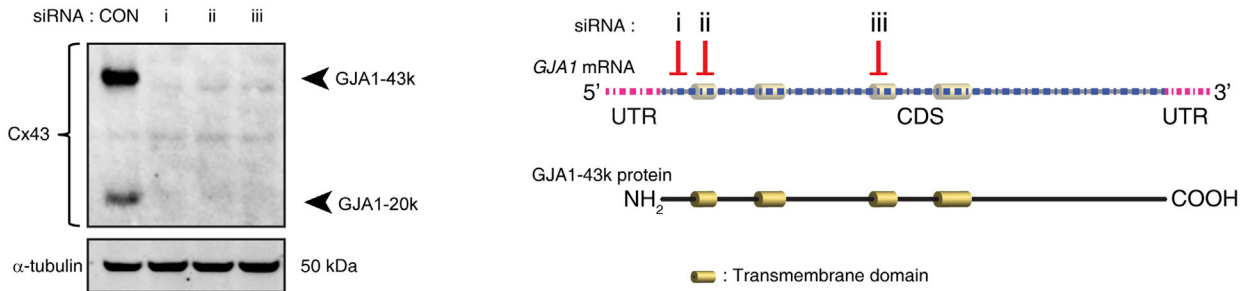
by the early stop codons. As expected, an 18 kDa N-terminal Cx43 fragment corresponding to residues 1–176 was detectable in cells transfected with the Cx43-180STOP-HA construct, and full-length GJA1-43k-HA protein was absent (Figure 2E, red bands). However, when we probed for HA, we found that the 177,178,179 stop codons failed to inhibit expression of GJA1-20k (Figure 2E, green bands). These data strongly support ribosomal entry and initiation of translation, as opposed to read-through or cleavage, as the mechanism of GJA1 mRNA internal translation.

Cx43 is a molecule with rapid turnover but no known regulatory subunits. We explored the relationship between internally translated Cx43 isoforms and full-length GJA1-43k trafficking. Immunofluorescence of 293T cells transfected with wild-type Cx43 reveals gap junction plaques at cell-cell borders in 78.7% of transfected cell pairs (Figure 3A). Introduction of sequential additive methionine-to-leucine mutations of their corresponding internal start codons (M100L, M125L, M147L, and M213L) reveals that the loss of all four internal start codons severely compromises GJA1-43k trafficking. The Cx43^{M4L} construct encodes a GJA1-43k protein with all four methionine-to-leucine mutations and is largely restricted to cytoplasmic reticular structures consistent with the endoplasmic reticulum (ER), with only 12% of cell pairs displaying gap junction plaque formation (Figures 3A and S3A). Given that GJA1-20k arises from M213 in the GJA1 coding sequence and is the most robustly expressed truncated Cx43 isoform, we explored whether it was sufficient to rescue transport of GJA1-43k^{M4L}. Indeed, when it is coexpressed with GJA1-20k-V5 (red), transport of full-length GJA1-43k^{M4L} (green) to gap junctions at the cell surface is rescued, whereas GJA1-20k-V5 remains cytoplasmic (Figures 3C and 3D). These data indicate that perturbed trafficking of GJA1-43k^{M4L} is not a result of mutation of internal methionines to leucines at the protein level, but rather is due to loss of the coexpressed truncated isoform(s).

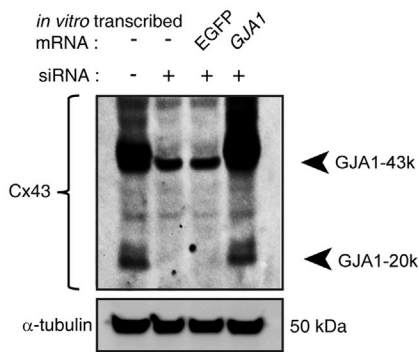
High-resolution immunofluorescence imaging of GJA1-20k-V5 reveals that this isoform is localized primarily to cytoplasmic reticular structures (Figure 3C). This compartment was confirmed as ER/Golgi by immunofluorescence colocalization studies (Figures S3B and S3C), suggesting a role for GJA1-20k early in the Cx43 vesicular transport pathway. Indeed, coimmunoprecipitation experiments reveal that full-length GJA1-43k complexes with GJA1-20k, and enrichment of GJA1-43k in the ER using Brefeldin A increases this interaction (Figure S3E). To further visualize this interaction and rule out any possible artifact of immunostaining, we cotransfected 293T cells with constructs encoding GJA1-20k-mCherry and GJA1-43k^{ML}-enhanced GFP (GJA1-43k^{ML}-EGFP). Data comparable to those shown in Figure 3C were obtained, indicating that GJA1-20k colocalizes with GJA1-43k^{ML}-EGFP in the perinuclear Golgi compartment, but is not incorporated into gap junction plaques at the cell surface with the full-length GJA1-43k^{ML}-EGFP protein (Figure S4, arrows). GJA1-20k may therefore act as a chaperone auxiliary protein, regulating trafficking of de novo GJA1-43k molecules through the ER/Golgi.

Given the variable expression of truncated Cx43 isoforms in human cell lines (Figure S1A), we wondered whether their expression is regulated by signaling pathways associated with

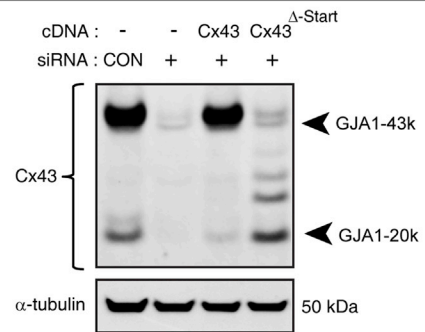
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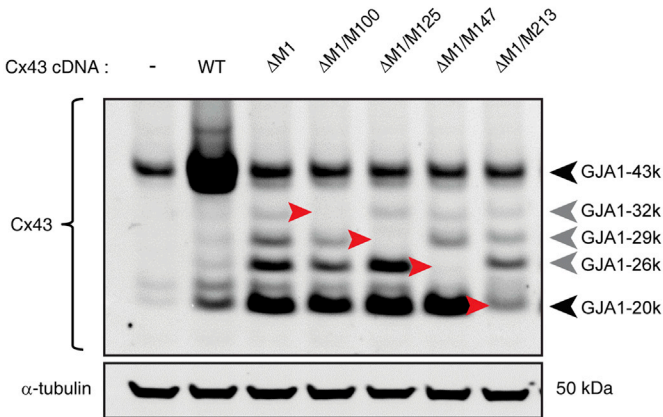
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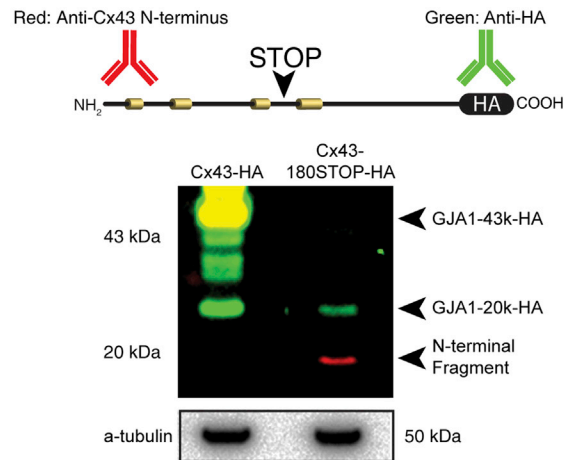


Figure 2. Truncated Cx43 Isoforms Arise from Internal Translation Initiation Events

(A) Western blot of endogenous Cx43 knockdown in 293T cells using three distinct siRNA duplexes. The schematic illustrates the location of each siRNA targeting sequence within *GJA1* mRNA.

(B) Cx43 western blot of siRNA-resistant *in vitro* transcribed mRNA transfection of 293T cells with endogenous Cx43 knockdown.

(C) Cx43 western blot following siRNA-resistant Cx43 cDNA transfection with knockdown of endogenous Cx43 in 293T cells. Cx43^{Δ-Start} contains the AUG-to-AUA point mutation to ablate the initial M1 start codon.

(D) Cx43 western blot of transfected Cx43 cDNA containing sequential AUG-to-GAC mutations for internal methionines M100, M125, M147, and M213 in addition to M1. Bands corresponding to internal translation products are highlighted by red arrows.

(E) Multiplex western blot of 293T cells transfected with siRNA-resistant wild-type Cx43-HA (left lane) or Cx43-180STOP-HA (right lane), which contains a triplet of stop codons at codons 177, 178, and 179 (schematic). Blot probed for HA (green) and Cx43 N terminus (red).

See also [Figure S2](#).

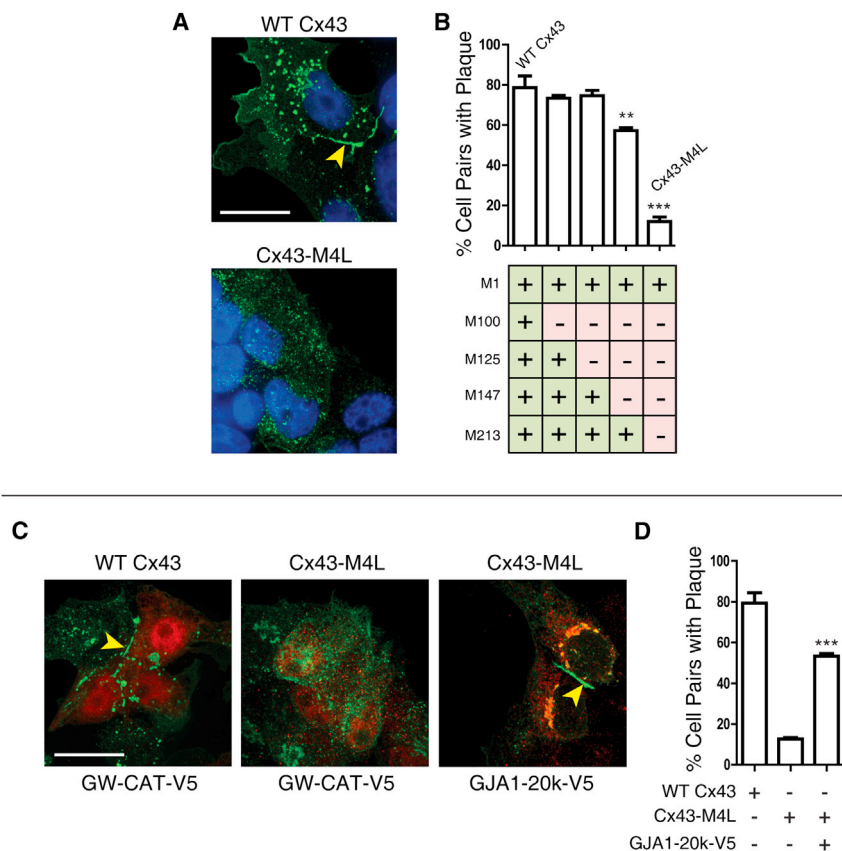


Figure 3. Truncated Cx43 Isoforms Are Necessary for Trafficking of Full-Length Cx43 to Gap Junction Plaques

(A) Fixed-cell immunofluorescence of 293T cells expressing siRNA-resistant Cx43 (green) with knockdown of endogenous Cx43. Nuclei were detected with DAPI (blue); arrow: gap junction plaque at the cell-cell border. Cell pairs were randomly selected and assessed for the presence of gap junction plaque for Cx43 constructs containing additive methionine-to-leucine mutations at M100, M125, M147, and M213. Cx43^{M4L} encompasses all four mutations.

(B) Percentage of cell pairs with plaque.

(C) Fixed-cell immunofluorescence of 293T cells coexpressing the control GW-CAT-V5 construct or the GJA1-20k-V5 isoform (red) and siRNA-resistant Cx43 (green). Nuclei were counterstained with DAPI (blue); arrow: gap junction plaque at the cell-cell border.

(D) Percentage of cell pairs from (C) assessed for the presence of gap junction plaque. Original magnification $\times 100$. Scale bar, 25 μm . Data are presented as mean \pm SEM. *** $p < 0.001$, ** $p < 0.01$. See also Figures S3 and S4.

translation. Using primary neonatal mouse ventricular cardiomyocytes (NMVMs), we found that inhibition of PI3K/AKT signaling using the PI3K inhibitor GDC-0941 (Folkes et al., 2008) increases expression of GJA1-20k (Figure S5). The mTOR pathway is an established regulator of cap-dependent protein translation, which is integral to PI3K/AKT signaling. Inhibition of mTOR kinase activity using the inhibitor PP242 (Feldman et al., 2009) significantly augments expression of internally translated isoforms in 293T cells transfected with Cx43^{ΔSTART}, revealing that this pathway acts independently of the cap and first mRNA start codon, and directly inhibits internal or cap-independent translation (Figure 4A). Just as we observed with P13K inhibition, PP242 also increased expression of GJA1-20k in NMVMs (Figure 4B). The early formation of additional Cx43 isoforms (Figure S2) and their regulation by the PI3K/AKT/mTOR pathway (Figures 4A, 4B, and S4) indicate that truncated GJA1 isoforms originate from internal, cap-independent translation events. Fixed-cell immunofluorescence of NMVMs incubated for 16 hr with DMSO (left panels) or PP242 (right panels) is presented in Figure 4C. An increase in Cx43 (green) gap junction density is apparent in cells treated with PP242, with N-cadherin (red) identifying cell-cell borders. Quantification of the Cx43 fluorescence intensity profiles of 10 μm vectors bisecting cell-cell borders is presented in Figure 4D. Consistent with data presented in Figure 3, these findings implicate internally translated Cx43 isoforms in positive regulation of gap junction formation.

2003). It is possible that alternatively translated gene products could be responsible for some of these observations. In recent years, regulation of translation has emerged as a potentially major component of cellular protein control, and the mTOR pathway has been shown to play a central role in promoting cap-dependent translation. The number of identified eukaryotic IRES elements is increasing, with the majority occurring within the 5' UTR of mRNAs to allow cap-independent translation during mitosis and stress (Komar and Hatzoglou, 2011). Initiation of translation within the mRNA coding sequence has also been reported for proteins such as ECSIT, Myc, and NANOG, yielding truncated gene products that can regulate full-length protein function (Ingolia et al., 2011). Many ion channels require subunits to chaperone their journey from the ER to the plasma membrane (Smyth and Shaw, 2010). In this study, we present the finding that GJA1 mRNA is in fact polycistronic, and several N-terminally truncated Cx43 isoforms are generated at the translational level. These alternatively translated Cx43 "subunits" are necessary for successful transport of the full-length protein to the cell surface, and the subunit GJA1-20k in particular is sufficient to affect this process. It is also possible that non-Cx43 ion channels and other proteins without known chaperones may undergo similar autoregulation. Cap-independent translation events therefore represent a potential target for protein regulation and therapeutic restoration of normal electrical coupling in diseased hearts.

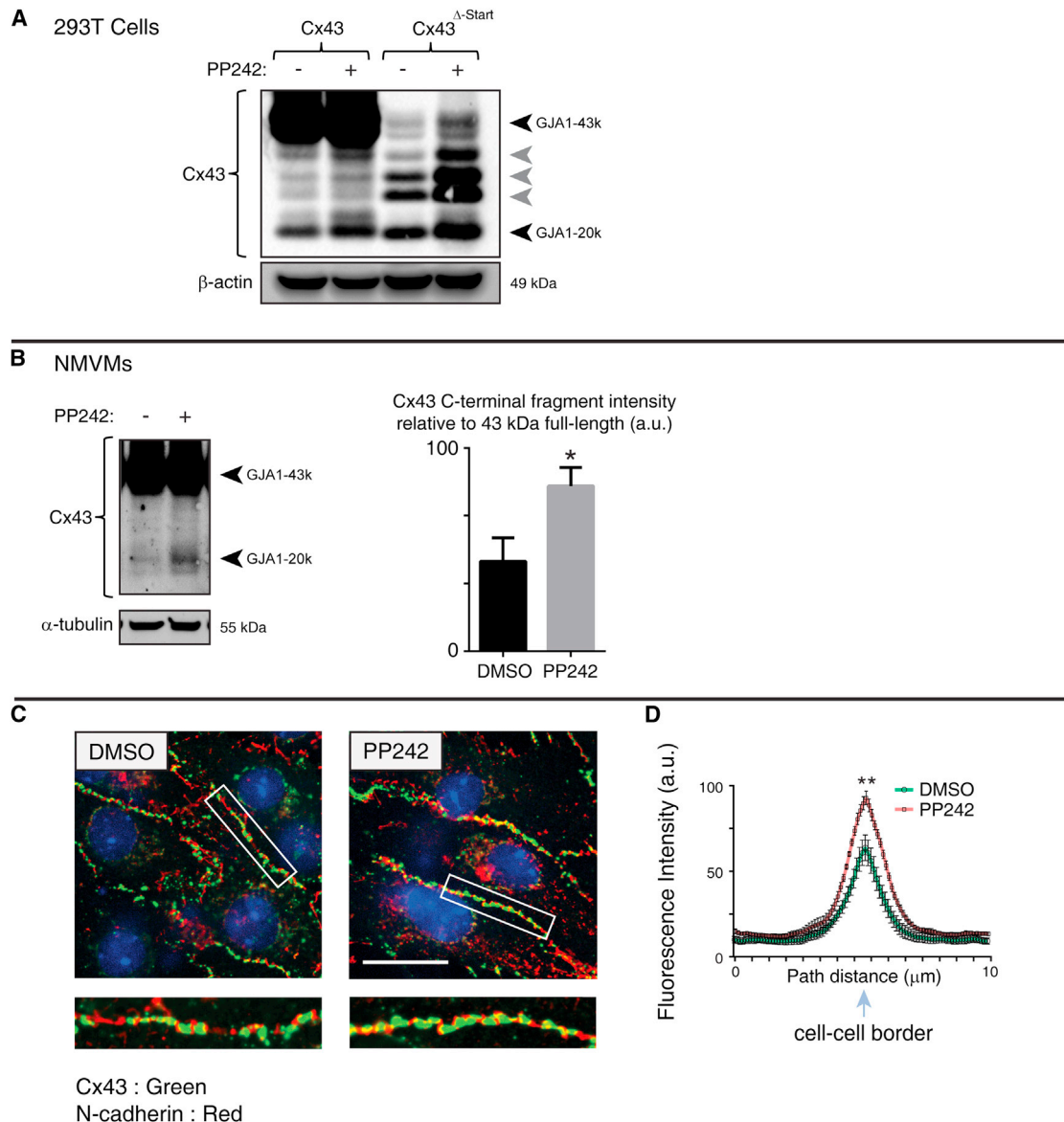


Figure 4. Expression of Cx43 Truncated Isoforms Is Regulated by the mTOR Pathway

(A) 293T cells transfected with siRNA-resistant Cx43 or Cx43^{ΔSTART} with knockdown of endogenous Cx43 incubated for 8 hr in the presence or absence of the mTOR kinase inhibitor PP242. Cx43 was detected by western blot.

(B) Primary NMVMs were incubated for 16 hr with DMSO or PP242. Cx43 was detected by western blot, with quantitation of the C-terminal fragment relative to full-length Cx43 (43 kDa) in the graph.

(C) Fixed-cell immunofluorescence of cells from (B). Cell borders are identified with N-cadherin (red) for quantification of Cx43 (green) gap junction density. Original magnification $\times 60$. Scale bar, 25 μm .

(D) Averaged fluorescence intensity profiles of Cx43 expression at cell-cell borders. Data are presented as mean \pm SEM. ** $p < 0.01$, * $p < 0.05$.

See also Figure S5.

EXPERIMENTAL PROCEDURES

Human Tissue Acquisition

With the approval of the UCSF Committee for Human Research, we obtained tissue from the hearts of organ donors whose hearts had not been transplanted for technical reasons. The California Transplant Donor Network provided the unused donor hearts and obtained informed consent for their use from the next of kin.

Mice

All procedures were reviewed and approved by the University of California Institutional Animal Care and Use Committee.

Molecular Biology

Human GJA1 was obtained from Open Biosystems and cloned into pDONR/221 and pcDNA3.2/V5-Dest using Gateway technology (Life Technologies). Constructs encoding N- and C-terminal HA-epitope-tagged Cx43, and

C-terminal EGFP and mCherry fusion proteins were generated as previously described (Smyth et al., 2012). Mutagenesis was performed using the QuikChange Lightning Mutagenesis Kit (Agilent) according to the manufacturer's instructions.

In vitro transcription was undertaken using the T7 mScript Standard mRNA Production System (CellsScript) and pcDNA3.2-huCx43 or pcDNA3.2-EGFP as the template. Stealth siRNA duplexes targeting human *GJA1* were obtained from Life Technologies. Plasmids are available at the nonprofit repository Addgene (http://www.addgene.org/Robin_Shaw/).

NMVM Isolation and Culture

P1 NMVMs were isolated and maintained in culture as previously described (Smyth et al., 2012). For inhibitor experiments, cells were insulin starved and fetal bovine serum dropped to 1% in Dulbecco's modified Eagle's medium/F12 for 8 hr prior to addition of GDC-0941 (2.5 μ M), PP242 (5 μ M), or DMSO. Following 30 min of incubation, insulin was added to a final concentration of 10 μ g/ml and cells were fixed for immunofluorescence or harvested for biochemistry 1 hr and 16 hr after stimulation.

Western Blotting

Lysates were prepared in RIPA buffer and western blotting was performed as previously described (Smyth et al., 2010; Smyth et al., 2012). Mouse monoclonal anti-Cx43 directed against C-terminal (1/3000) and N-terminal (1/2,000) regions was obtained from the Fred Hutchinson Cancer Research Center. Mouse monoclonal anti- α -tubulin (1/3,000), anti-HA (1/5,000), and rabbit polyclonal anti-Cx43 (1/3,000) were obtained from Sigma-Aldrich. Rabbit polyclonal antibodies against AKT (1/1,000) and phospho-AKT^{Ser473} (1/1,000) were obtained from Cell Signaling Technology.

Immunofluorescence

Cells were cotransfected with pcDNA3.2-GW-CAT-V5 as a transfection control, or pcDNA3.2-GJA1²¹³⁻³⁸²-V5, together with either wild-type or mutated pcDNA3.2-huCx43-Stop. Eighteen hours after transfection, the cells were fixed with 4% paraformaldehyde and immunostained for Cx43 (rabbit polyclonal 1/3,000; Sigma) and V5 tag (mouse monoclonal 1/1,000; Sigma) with AlexaFluor488 and AlexaFluor555 (Life Technologies) as previously described (Smyth et al., 2010). For mouse monoclonal Golgi apparatus marker 58K (1/200; Abcam) and ER marker PDI (1/200; Abcam) immunodetection, cells were fixed in ice-cold methanol for 5 min. Monoclonal N-cadherin (1/250; BD Biosciences) was used to detect cell-cell borders in NMVMs.

Slides were imaged using a Nikon Ti microscope with a \times 100/1.49 Apo TIRF objective, Yokogawa CSU-X1 spinning-disk confocal unit with 486 and 561 nm laser sources, and CoolSnap HQ2 camera controlled by NIS Elements software.

Quantification of Cx43 Gap Junction Plaque Formation

Cell pairs were screened in blinded fashion for comparable levels of V5-tag and then assessed for the presence of Cx43 gap junction plaque. A total of 75 cell pairs were assessed for each condition.

Quantification of Cx43 Gap Junction Plaque Density

For quantification of Cx43 expression at cell-cell borders (Figure 4C), we generated maximum intensity projections of 10 μ m confocal z stacks for N-cadherin (to identify cell-cell borders) and Cx43. We then implemented a MATLAB routine that generates 10 μ m fluorescence intensity profiles bisecting traced cell-cell borders, automating a previously described quantification technique (Smyth et al., 2012). This routine is freely available upon request.

Statistical Analysis

All data are presented as mean \pm SEM, and Student's t test or one-way ANOVA (with Bonferroni post test) was used accordingly. A p value < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.10.009>.

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