Dynamic UTR Usage Regulates Alternative Translation to Modulate Gap Junction Formation during Stress and Aging

Michael J. Zeitz¹, Patrick J. Calhoun¹,², Carissa C. James¹,³, Thomas Taetzsch¹, Kijana K. George¹,³, Stefanie Robe¹,²,⁴,⁵, Gregorio Valdez¹,²,⁴, James W. Smyth¹,²,⁴,⁶,*

¹Fralin Biomedical Research Institute, Roanoke, VA 24016, USA
²Department of Biological Sciences, Virginia State University and Polytechnic Institute, Blacksburg, VA 24060, USA
³Graduate Program in Translational Biology, Medicine, and Health, Virginia State University and Polytechnic Institute, Blacksburg, VA 24060, USA
⁴Virginia Tech Carilion School of Medicine, Roanoke, VA 24016, USA
⁵School of Neuroscience, Virginia State University and Polytechnic Institute, Blacksburg, VA 24060, USA
⁶Lead Contact

SUMMARY

Connexin43 (Cx43; gene name GJA1) is the most ubiquitously expressed gap junction protein, and understanding of its regulation largely falls under transcription and post-translational modification. In addition to Cx43, Gja1 mRNA encodes internally translated isoforms regulating gap junction formation, whose expression is modulated by TGF-β. Here, using RLM-RACE, we identify distinct Gja1 transcripts differing only in 5′ UTR length, of which two are upregulated during TGF-β exposure and hypoxia. Introduction of these transcripts into Gja1−/− cells phenocopies the response of Gja1 to TGF-β with reduced internal translation initiation. Inhibiting pathways downstream of TGF-β selectively regulates levels of Gja1 transcript isoforms and translation products. Reporter assays reveal enhanced translation of full-length Cx43 from shorter Gja1 5′ UTR isoforms. We also observe a correlation among UTR selection, translation, and reduced gap junction formation in aged heart tissue. These data elucidate a relationship between transcript isoform expression and translation initiation regulating intercellular communication.

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Correspondence: smythj@vtc.vt.edu.

AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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In Brief

Connexin43 gap junctions enable direct intercellular communication facilitating action potential propagation. Internal translation of connexin43 mRNA generates the truncated isoform GJA1–20k, which promotes gap junction formation. During aging, Zeitz et al. find that activation of stress-response pathways shortens connexin43 mRNA UTRs to limit GJA1–20k translation coincident with gap junction loss.

Graphical Abstract

INTRODUCTION

Connexin43 (Cx43; gene name GJA1) (Fishman et al., 1990) is the most ubiquitously expressed gap junction protein, affecting direct intercellular communication crucial to organ function, such as in the heart where its expression and localization are tightly regulated to enable electrical coupling and maintain rhythmic contractions (Peters et al., 1997). Additional functions of Cx43 encompass cytoskeletal, metabolic, developmental, and cell cycle regulation, so it is unsurprising that alterations in Cx43 expression are implicated in a multitude of human pathologies (Chen et al., 2015; Clasadonte et al., 2017; Crespin et al., 2010; Meyer et al., 1997; Moorby and Patel, 2001). GJA1 mRNA encodes N-terminally truncated protein isoforms arising from alternative translation initiation within its coding sequence (Salat-Canela et al., 2014; Smyth and Shaw, 2013; Ul-Hussain et al., 2014). Tight regulation of GJA1 protein expression extends to its internally translated isoforms, as experimentally eliminating four of these isoforms, namely, GJA1–32k, GJA1–29k, GJA1–26k, and GJA1–20k, impairs gap junction formation reviewed in Basheer and Shaw (2016). Interestingly, reintroduction of GJA1–20k is able to rescue gap junction formation (Smyth and Shaw, 2013). We have subsequently identified changes in Gja1 translation initiation site selection in response to transforming growth factor β (TGF-β)-induced epithelial-mesenchymal transition (EMT), demonstrating the role of alternative translation in a
physiological process that limits gap junction formation (James et al., 2018). Additionally, modulation of GJA1–20k expression has been reported during pathological stressors, including chemically induced hypoxia and cardiac ischemia (Basheer et al., 2018; Basheer et al., 2017; Ul-Hussain et al., 2014). The GJA1–20k fragment was also found in a model of fetal alcohol spectrum disorder (FASD) (Ramani et al., 2016). Although some signaling pathways have been implicated, the cellular mechanisms regulating changes in Gja1 translation are unknown.

Regulation of translation initiation can proceed through canonical and non-canonical mechanisms. Under normal physiological conditions, translation initiation typically involves eIF4F binding to the mRNA methyl 7-guanosine (m7G) cap, which is followed by formation and recruitment of the 43S pre-initiation complex (PIC). The PIC then scans for a start codon in a favorable context to initiate translation (Liu and Qian, 2014). Under conditions of cellular stress, traditional cap-dependent translation is suppressed, and many non-canonical modes of translation initiation are used to facilitate expression of key stress response and survival proteins (Holcik and Sonenberg, 2005). To date, it is unclear which mode of translation regulates the balance of Cx43 and its internally translated isoforms, but cap-dependency has been indicated (Salat-Canela et al., 2014). Regardless of the mechanism, a common feature of these modes of translation initiation is reliance on cis-acting elements within the 5′ UTR of mRNA.

Although the coding sequence of Gja1 is composed of an invariable single exon, one region of variability in Gja1 RNA composition is its 5′ UTR. Synthesis of this region was found to be regulated in a cell-type-specific manner through a combination of alternate promoter usage and splicing (Pfeifer et al., 2004). Gja1 5′ UTR variants were demonstrated to affect translation in vitro possibly through the use of an internal ribosome entry site (IRES) contained within the 5′ UTR (Pfeifer et al., 2004; Schiavi et al., 1999). Our previous work revealed upregulation of total Gja1 mRNA in response to TGF-β-induced EMT and identified downstream pathways responsible for subsequent translational changes (James et al., 2018). Here, we evaluate whether these signaling pathways play a role in Gja1 alternate promoter usage. Many of these pathways converge on activating protein 1 (AP-1), and this set of transcription factors has been demonstrated to regulate Gja1 expression (Geimonen et al., 1998; Geimonen et al., 1996; Hernandez et al., 2006). The presence of multiple transcription start sites (TSSs) for a given gene is common, and modulation of TSS selection by alternate promoter usage has been demonstrated to affect translational efficiency genome-wide (Cheng et al., 2018; Leenen et al., 2016; Rojas-Duran and Gilbert, 2012). This led us to hypothesize a role for TSS selection in regulating Gja1 alternative translation. We confirm this hypothesis in cell lines and in an in vivo model of cardiac aging, where remodeling of Cx43 at the intercalated disc has been demonstrated (Bonda et al., 2016). Here, we report the presence of stress responsive, dynamic alterations in TSS yielding Gja1 5′ UTR variants that regulate translation efficiency, alternative translation, and gap junction formation.
RESULTS

TGF-β Induces Translational and Transcriptional Changes to Gja1

In normal murine mammary gland (NMuMG) cells, we verify Cx43 induction with a decrease in the ratio of GJA1–20k to Cx43 by western blotting following stimulation with TGF-β (Figures 1A and 1B). Gja1 has been reported to express tissue-specific 5′ UTR isoforms capable of regulating translational efficiency (Pfeifer et al., 2004). We, therefore, performed RNA-ligase-mediated rapid amplification of cDNA ends (RLM-RACE) to determine whether TGF-β stimulation induces dynamic 5′ UTR alterations accompanying changes in alternative translation. RNA was isolated from NMuMG epithelial cells with or without 48 h of TGF-β treatment. Adaptor and gene-specific primers were used to amplify the entire Gja1 5′ UTR cDNA (Figure 1C). Interestingly, the prominent 5′ UTR isoforms from control and TGF-β stimulated RLM-RACE samples are unique, with the most intense bands from TGF-β-stimulated cells being shorter than those from untreated control cells (Figure 1D). DNA was gel extracted and Sanger sequenced with Gja1 5′ UTR sequences mapping to the most 5′ region of Gja1 exon 1, approximately 10.2 kb upstream of the start codon in the mouse genome (Figure 1E, green bar). These Gja1 5′ UTR isoforms labeled long (L), medium (M), and short (S) align with peaks of Cap analysis gene expression (CAGE) reads in the University of California, Santa Cruz (UCSC) genome browser, mouse genome build mm10 (Figure 1F), a readout from genome-wide cap analysis of gene expression representing TSSs from multiple cell types (Forrest et al., 2014; Lizio et al., 2015). This, therefore, suggests alternate promoter usage is responsible for generating truncated Gja1 5′ UTR isoforms arising from unique TSSs.

Gja1 5′ UTR Isoforms Regulate Canonical and Alternative Translation

We next performed qRT-PCR to quantify the abundance of each 5′ UTR because RLM-RACE is not quantitative. Because truncated Gja1 5′ UTR isoforms share the same sequence as longer isoforms, absolute quantitation was performed using non-overlapping primers and by comparison to a standard curve of amplification products from a cloned Gja1 5′ UTR template. Once absolute copy numbers were calculated, overlapping transcripts were corrected by subtraction, and fold difference relative to L was plotted (Figure 2A). In untreated cells, S has the highest abundance, followed by M and L (Figures 2A and 2B).

The Gja1 5′ UTR encompasses multiple putative promoters (Pfeifer et al., 2004). Because alternate promoter usage is likely responsible for generating truncated Gja1 5′ UTRs in response to TGF-β (Figure 1F), we analyzed this genomic region using MatInspector software (Markoff, 2005) to identify transcription factor binding sites. We identified high-confidence transcription factor binding sites specific for AP-1, proximal to our putative TSSs within the 5′ UTR, consistent with published reports of its role in Gja1 expression (data not shown; Echetebu et al., 1999; Geimonen et al., 1998; Geimonen et al., 1996; Hernandez et al., 2006; Tacheau et al., 2008). TGF-β activates p38 mitogen-activated protein kinase (p38) through TAK1 upstream of AP-1 (Sorrentino et al., 2008; Tacheau et al., 2008; Watkins et al., 2006; Yamaguchi et al., 1995). To determine if this pathway contributes to the increase in TGF-β-induced Gja1 UTR isoforms, qRT-PCR was performed on RNA isolated from NMuMG cells following 24 h of TGF-β stimulation alone or in combination with the
p38 inhibitor SB202190. TGF-β stimulation significantly increases the M and S Gja1-5′ UTRs with no change in expression of L (Figures 2C–2E). Inhibition of p38 prevents the increase in truncated Gja1 5′ UTRs M and S following TGF-β treatment, implicating this pathway in regulating alternate Gja1 promoter usage to increase M and S Gja1 5′ UTR isoform abundance in response to TGF-β.

To determine if the TGF-β-activated pathways p38 and/or c-Jun N-terminal kinase (JNK) contribute to TGF-β-induced increases in Cx43 protein, the p38 inhibitor SB202190 or JNK inhibitor SP600125 was applied 15 min prior to TGF-β treatment and protein was harvested after 24 h. Cx43 significantly increases following 24 h of exposure to TGF-β, whereas inhibition of p38 prevents the TGF-β-dependent increase in Cx43 (Figures 2F and 2G). JNK inhibition with TGF-β does not significantly reduce the increase in Cx43, and combining SB202190 and SP600125 does not result in a synergistic effect. This finding highlights the necessity for p38-activated alternate promoter usage for the TGF-β-induced increase in full-length Cx43 expression.

Alternative translation is regulated by cis and trans-acting factors (Faye and Holcik, 2015; Hellen and Sarnow, 2001; Stoneley and Willis, 2004), leading us to investigate whether the expression of truncated Gja1 5′ UTR isoforms alone affect alternative translation initiation. Isoforms L, M, and S were cloned upstream of the Gja1 coding sequence, and plasmid constructs were transfected into Gja1−/− cells, which were generated using CRISPR/Cas9 to excise the Cx43 coding sequence from 293FT and NMuMG cell lines (Figure S1). Protein was harvested 24 h post-transfection for western blot analysis (Figure 2H). When transfected into Gja1−/− NMuMG cells, both M and S isoforms result in significantly reduced GJA1–20k to Cx43 translation, compared with the L isoform (Figure 2I). This change in protein isoform ratio is the result of decreased GJA1–20k levels for the M isoform and increased Cx43 levels for the S isoform. When transfected into GJA1−/− 293FT cells, the M isoform has significantly reduced GJA1–20k to Cx43 translation compared with the L isoform (Figure S2). This indicates that UTR truncation alone is sufficient to phenocopy the effects on Gja1 translation following stimulation with TGF-β, resulting in reduced GJA1–20k relative to full-length Cx43 expression, which we had observed during EMT (James et al., 2018).

Previously, we found that inhibition of Erk signaling in NMuMG cells results in a robust increase in GJA1–20k to Cx43 ratios, opposite to the effect observed with TGF-β stimulation (Figures 3A and 3B) (James et al., 2018). We hypothesized that Erk inhibition would result in differential expression levels of L, M, or S Gja1 5′ UTR isoforms. Following 24 h of treatment with Erk inhibitor SCH772984 or vehicle, RNA was isolated and UTRs quantified by qRT-PCR. The L UTR increases significantly following Erk inhibition, whereas M and S are significantly downregulated (Figures 3C–3E). This further demonstrates the active regulatory effect of Gja1-UTR isoform expression in modulating alternative translation.

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**GJA1 Stress Responsive Transcript Isoforms Are Conserved in Humans and Primary Mouse Tissue**

To investigate whether alternate GJA1 5′ UTR usage occurs in human cells, RLM-RACE was performed in the human adult low calcium temperature (HaCaT) human keratinocyte cell line. RLM-RACE PCR products from control and TGF-β-stimulated HaCaT cells had a comparable banding pattern to NMuMG cells when analyzed by electrophoresis (Figure 4A). Also consistent with results from NMuMG cells, the second largest band also increases following 48 h of TGF-β. As was the case for the mouse UTRs, Sanger sequencing and mapping to human genome build hg38 reveal overlap with peaks of CAGE reads with both human and mouse UTRs mapping to homologous regions (Figure 4B). We next asked if alternate UTR usage occurs in vivo and turned to two primary cell types, astrocytes and neonatal mouse ventricular cardiomyocytes (NMVCMs), which are derived from tissues known to express high levels of Cx43. RLM-RACE products from both astrocytes and NMVCMs also had a similar banding pattern (Figures 4C and 4D). Gel-extracted and Sanger sequenced bands mapped proximal to L, M, and S UTR isoforms corresponding to blue, red, and black dotted lines, respectively (Figure 4E).

Previous studies have shown that GJA1–20k is responsive to hypoxic conditions (Basheer et al., 2017; Kotini et al., 2018; Ul-Hussain et al., 2014). We, therefore, exposed human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) to 48 h of 1% oxygen to assess the effect on UTR selection. Like TGF-β, hypoxic stress results in an increase in truncated 5′ UTRs (Figure 4F, top). This was accompanied by a reduction in GJA1–20k relative to Cx43 (Figure 4F, bottom) and a reduction in membrane-localized Cx43 (Figures 4G and 4H). Levels of GJA1–20k relative to Cx43 were also reduced following 48 h of hypoxic stress in NMuMG cells, and Cx43 membrane localization was similarly limited (Figures 4I–4K; Figure S3). Finally, we found lower GJA1–20k to Cx43 ratios in A549 human lung adenocarcinoma cells exposed to 48 h of hypoxic conditions (Figure S4). These data highlight conservation of UTR variants in mammalian cells, demonstrating their involvement in a common response to physiological stress.

**Gja1 5′ UTRs Have Distinct Translational Efficiency and Are TGF-β Responsive**

The 5′ UTR of Gja1 has been reported to possess IRES activity (Pfeifer et al., 2004; Schiavi et al., 1999). To determine whether these Gja1 5′ UTR isoforms are capable of IRES-mediated translation, we performed luciferase assays with in-vitro-transcribed RNA transfected into NMuMG cells, avoiding the contribution of alternate promoter activity that could lead to false positives (Figure 5A). We first compared translation of m7G-capped and A-capped RNA with the L, M, or S UTR (Figures 5B–5D). The m7G-capped L UTR was translated less efficiently than the M and S isoforms. Cap-independent translation of the L UTR is a greater percentage of its capped counterpart, and yet cap-independent translation activity is similar for all isoforms. None of the UTR sequences cloned in between renilla luciferase (Rluc) and firefly luciferase (Fluc) on a bicistronic transcript had appreciable Fluc signal, which argues against the presence of an IRES in these 5′ UTR constructs (Figures 5B–5D). When these m7G-capped transcripts are transfected into NMuMG following 48 h of TGF-β treatment, L translation decreases, whereas M and S translation significantly increases (Figures 5E–5G). This demonstrates the capability of these UTRs to dynamically
Regulate translational efficiency and taken together suggests that truncated Gja1 5′ UTRs promote full-length Cx43 translation over alternative translation of the N-terminally truncated GJA1–20k isoform.

**Truncation of the Gja1 mRNA-5′ UTR Occurs in Aged Hearts with Activation of p38 Correlating with Reduced Internal Translation**

Remodeling of the intercalated disc occurs during aging and is associated with an age-related decline in cardiac function (Bonda et al., 2016). Loss of Cx43 enrichment at the intercalated disc is associated with arrhythmogenesis in cardiomyopathy (Peters et al., 1997). To assess Cx43 localization, we performed immunofluorescence labeling on cryosections from young (Y; 3.8 month) and old (O; 29 month) mouse hearts (Figure 6A). In aged hearts, there is a significant decline in Cx43 intensity at N-cadherin-labeled intercalated discs (Figure 6B).

During aging, p38 protein kinase activity increases, which can result in a state of chronic stress signaling (Hsieh and Papaconstantinou, 2002; Hsieh et al., 2003). To determine levels of phosphorylated p38 (p38\(^{pT180/Y182}\)) in cardiac ventricular tissue during aging, we performed western blots on protein lysates from young and old mouse hearts. There is a significant increase in p38\(^{pT180/Y182}\) at 29 months (Figures 6C and 6D). We next assessed whether increased p38\(^{pT180/Y182}\) correlates with the expression of shortened UTRs, as we found in cell lines (Figures 6E–6G). In agreement with these data, we find significantly elevated levels of M UTR isoform by qRT-PCR in aged hearts (Figure 6D). Importantly, our cell-based studies also identify the M isoform as least permissive to internal translation (Figures 2H and 2I). We performed western blotting for Cx43 on protein lysates from young and old mouse hearts (Figure 6H). Quantification by densitometry reveals significantly lower levels of Gja1 internal translation product GJA1–20k relative to Cx43 in aged hearts (Figure 6I). Importantly, this reduction in internal translation also correlates with increasing levels of p38\(^{pT180/Y182}\) independent of age (Figure 6J).

**DISCUSSION**

Pfeifer et al. (2004) reported the presence of a total of nine different Gja1 5′ UTR isoforms in mice. Subsets of these isoforms are expressed with tissue specificity and proposed to arise from a combination of alternate promoter usage and alternative splicing. Here, we find that alternate promoter usage gives rise to truncated Gja1 UTR isoforms, and yet we do not detect the presence of alternative splicing. This may be due to differences in the cell types used. Previously, we identified Smad and non-Smad signaling pathways responsible for TGF-β-induced alterations in Gja1 alternative translation (James et al., 2018). Here, we demonstrate that these signaling pathways act on alternate Gja1 promoters to dynamically regulate the level of UTR isoforms. Importantly, we reveal the activation of this mechanism in response to cellular stress and cardiac aging and demonstrate conservation of these UTR isoforms in humans.

Our data illustrate the complex regulation of Gja1 translation by UTR selection. We observe reduced ratios of GJA1–20k to Cx43 in response to both TGF-β and hypoxic stress. We reveal that Erk and p38, components of non-Smad signaling downstream of TGF-β, can...
modulate UTR selection, with Erk functioning to limit GJA1–20k translation, and p38 promoting translation of Cx43. The mechanism by which p38 and Erk modulate Gja1 translation may also involve their known role in activation of Mnk½, as it has been shown that inhibiting Mnk½ significantly increases levels of GJA1–20k (Salat-Canela et al., 2014). These findings are consistent with the fact that hypoxia also activates p38.

The Gja1 5′ UTR has been reported to possess IRES activity capable of enhancing translational efficiency (Schiavi et al., 1999). To assess IRES activity, we performed luciferase assays by using transfection of in-vitro-transcribed RNA as opposed to plasmid-based constructs to avoid confounding factors, such as cryptic promoter activity or splicing, as reviewed in Terenin et al. (2017). In our hands, we did not detect any IRES activity for these UTRs. This discrepancy may be due the lack of trans-acting nuclear factors binding to an in vitro-transcribed RNA. For instance, factors such as hnRNP A1 have been demonstrated to regulate IRES activity (James and Smyth, 2018; Jo et al., 2008).

Cx43 gap junction formation and maintenance consists of multiple levels of regulation from transcription to post-translational modifications. Previously, we revealed the necessity for the internal translated isoform GJA1–20k in Cx43 trafficking, with a suggested role in Cx43 hexamer formation, adding another layer of regulation to this process (James et al., 2018). We further demonstrate GJA1–20k’s role in Cx43 trafficking in response to hypoxic stress in epithelial cells and iPSC-CMs, where reduced GJA1–20k translation correlates with a reduction in levels of membrane-localized Cx43. Other functions have also been ascribed to GJA1–20k, including stabilization of actin filaments to guide Cx43 trafficking (Basheer et al., 2017) and promotion of mitochondrial transport (Fu et al., 2017). Recently, upregulation of GJA1–20k was shown to occur in response to ischemic injury, and experimental overexpression prior to ischemic injury therapeutically regulates cardiac mitochondrial function and biogenesis (Basheer et al., 2018). Here, we find that endogenous GJA1–20k levels are reduced in response to extended exposure to hypoxic conditions in a range of cells lines, including NMuMG, A549, and iPSC-CMs. Reported differences appear to stem from the duration of hypoxia, as we do detect a trend of rising GJA1–20k levels at earlier time points (4 h) before a reduction in expression at 24 h (Figure S3). Together, these data highlight the complexity of Cx43 versus GJA1–20k translation, and indeed our work herein uncovers independent pathways discretely regulating translation of each. Finally, another recent article has described a direct role for GJA1–20k in the transcriptional regulation of N-cadherin (Kotini et al., 2018), opening the possibility of feedback loops from products of alternative translation initiation influencing gene expression at a more global level.

Cardiac function declines with age independent of extrinsic factors (Bonda et al., 2016; Fannin et al., 2014; Sandstede et al., 2000). During aging, the heart undergoes pathological remodeling at multiple levels ranging from whole heart size to its molecular components (Bonda et al., 2016; Cheng et al., 2009; Cohn et al., 2000; Fannin et al., 2014). Gap junctions encompassing Cx43 facilitate well-orchestrated and rapid electrical coupling in the heart. Disruption of Cx43 localization at the intercalated disk of cardiomyocytes results in decreased gap junction intercellular communication (GJIC) and increased susceptibility to arrhythmias of sudden cardiac death (Peters et al., 1997; Poelzing and Rosenbaum, 2004;
Smith et al., 1991). Here, we observe a role for alternate promoter usage and reduction of internal translation in limiting Cx43 gap junction formation during aging.

Variation in TSSs due to alternate promoter usage is widespread, with most genes having more than one TSS (Forrest et al., 2014). It is common for TSSs to cluster at multiple promoters within the same gene, resulting in multimodal peaks of transcription initiation (Carninci et al., 2006). This allows cell-type- and cell-state-specific transcription factors to modulate 5′ UTR composition and regulate translational efficiency (Tamarkin-Ben-Harush et al., 2017). Our transcript isoform data from cell lines and primary cells when combined with CAGE reads suggest a multimodal-based promoter region occupying the most 5′ exon of the Gja1 5′ UTR, which is dynamically regulated in response to stress signaling. Through transfection, we demonstrate the capability of cis-acting elements in the Gja1 5′ UTR to affect translation. Future work to dissect the mechanism of Gja1 alternative translation, including the contribution of trans-acting factors, will be necessary to fully understand the complex regulation of Cx43 gap junctions.

More broadly, altered translation initiation may also contribute to pathological phenotypes from genetic disorders involving Cx43, such as oculodentodigital dysplasia (Churko et al., 2011; Shibayama et al., 2005). It is interesting to speculate how mutations may not necessarily have deleterious impacts on the encoded full-length protein per se but rather alter RNA structure and/or affect trans-acting factor binding to modulate the translational readout from a given mRNA. Although this likely would not impact 5′ UTR usage, responsiveness to trans-acting factors may be altered and deserves further investigation in this context.

Together, our data reveal dynamic regulation of Gja1 transcript isoforms and uncover a cellular mechanism to balance the translation of Cx43 and its alternative translation product GJA1–20k (Figure 7). This process is similar to the regulatory relationship between 5′ UTR splicing and N-terminally truncated isoform translation reported for the glucocorticoid receptor (Turner et al., 2014), suggesting it is a more widespread cellular mechanism of regulating alternative translation. Genome-wide mapping of translation initiation site activity identifies multiple upstream open reading frames (uORFs) in the Gja1 5′ UTR (Lee et al., 2012). Selective inclusion of these uORFs in 5′ UTR variants by alternate promoter usage is, therefore, a candidate mechanism to explain the differences in translation efficiency and initiation site selection. This may be analogous to examples of leaky ribosome scanning, where uORFs play a role in regulating translation under normal and stress conditions (Barbosa et al., 2013; Brubaker et al., 2014). Future work using deep sequencing is necessary to define the totality of Gja1 TSSs under conditions of stress to define the exact sequence elements regulating Gja1 translation.

**STAR★METHODS**

Detailed methods are provided in the online version of this paper and include the following:

**CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, James W. Smyth, PhD. (smythj@vtc.vt.edu).
EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Cell Culture**—NMuMG cells, female, a kind gift from Prof. Rik Derynck (University of California San Francisco), and 293 FT cells, female, (Thermo Fisher Scientific) were grown as in James et al. (2018). Human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs), male, and culture media were obtained from Axol BioSciences and maintained according to manufacturer’s instructions. Cells were maintained in a humidified atmosphere of 5% CO2 at 37°C, for hypoxic studies O2 was dropped to 1% in a tri-gas incubator. Cell treatments: TGF-β1 (2 ng/ml; Humanzyme), p38 inhibitor SB 202190 (20 μM; Cayman), JNK inhibitor SP 600125 (20 μM; Cayman), Erk½ inhibitor SCH 772984 (0.1 μM; Cayman), or vehicle (DMSO; Sigma).

**Animals**—All mice were C57BL/6N for primary cell isolation and adult aged (3.8 month old, male) were obtained from Charles River Laboratories with aged (29 month old, male) C57BL/6N obtained from the National Institute on Aging. All experiments were carried out under NIH guidelines and animal protocols were approved by the Virginia Tech Institutional Animal Care and Use Committee.

METHOD DETAILS

**Primary cell isolation**—**Neonatal mouse ventricular myocytes** (NMVCM) were isolated from C57BL/6 P7 mice as previously described (Smyth et al., 2012). Briefly, pups were decapitated and hearts removed and washed in Hank’s balanced salt solution without calcium or magnesium (HBSS; Thermo Fisher Scientific). Atria were removed and quartered ventricles placed in 5 mL HBSS with 1.5 mg/ml collagenase II (Worthington Biochemical) warmed to 37°C. Tissue was stirred for 5 min at 37°C and gently triturated 20 times with a transfer pipette prior to a 2 min rest allowing tissue to settle. Supernatant was removed and added to 5 mL ice-cold FBS (Thermo Fisher Scientific), and 5 mL warm HBSS with collagenase added to remaining tissue. This process was repeated three times, after which the cell/FBS suspension was centrifuged for 5 min at 300 × g. The supernatant was discarded and cell pellet resuspended in 10 mL F12/DMEM 50/50 (Thermo Fisher Scientific) supplemented with 5% FBS, insulin-transferrin sodium selenite media supplement (Thermo Fisher Scientific), and Mycozap-PR (Lonza). Cells were pipetted through a 70 μM cell strainer (BD Biosciences) and pre-plated for 30 min at 37°C on 100 mm dishes (Genesee Scientific). Cell suspension was removed and pre-plating repeated on a fresh 100 mm dish to further enrich for cardiomyocytes. Adherent cells were discarded and cell suspension centrifuged for 5 min at 500 × g. Supernatant was discarded and cell pellet snap frozen for RNA isolation.

**Astrocyte isolation**, Mice were decapitated, brains were removed and the cortical gray matter of eight C57BL/6 P7 mice was dissected in carbogenated (95% O2 5% CO2) ice cold artificial cerebral spinal fluid (ACSF, 120mM NaCl, 3.0 mM KCl, 2mM MgCl, 0.2 mM CaCl, 26.2 mM NaHCO3, 11.1 mM glucose, 5.0 mM HEPES). Meninges were removed. The tissue was minced into 1 mm³ pieces and dissociated into a single cell suspension using a Papain Dissociation kit (Worthington Biochemical). Magnetic cell sorting was used to remove myelin and microglia before positive selection for astrocytes. In short, a debris removal step using modified protocols from Miltenyi Biotec’s Myelin Removal Kit with
anti-myelin Microbeads was performed first followed by removal of microglia using anti-Cd11b+ Microbeads. Astrocytes were selected using a modified protocol from Miltenyi Biotec’s Anti-ACSA-2 kit. A detailed protocol is provided in Holt and Olsen (2016).

RNA Extraction and RT-qPCR—RNA was extracted from NMuMG cells using Purelink RNA Mini Kit (Thermo Fisher Scientific) and homogenized by passage 10 times through an 18 gauge needle. Mouse hearts were homogenized in Trizol, and chloroform extracted prior to Purelink column purification. DNA was digested on column using PureLink DNase (Thermo Fisher Scientific). 1 μg of RNA was reverse transcribed with iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). Quantitative PCR was performed using SYBR Select Master Mix for CFX (Thermo Fisher Scientific) on a Quant Studio 6 Flex cycler (Thermo Fisher Scientific) using PrimeTime qPCR primers (Integrated DNA Technologies). UTR primers were designed using primer3 and ordered from Integrated DNA Technologies. GAPDH was used as an internal control. Cycling parameters consisted of 50°C 2 min; 95°C 2 min; 39 cycles of 95°C 15 s, 55°C 15 s, 72°C 1 min. To calculate baseline levels of UTR isoform abundance in untreated NMuMG cells, qPCR was performed on a dilution series made with plasmids containing cloned Gja1-UTR sequence, and a standard curve was used to calculate absolute quantity. Absolute quantities of overlapping UTRs were then subtracted from one another. Primer efficiencies were calculated from standard curves and were included in calculations of relative fold change by the Pfaffl equation (Pfaffl, 2001). For mouse experiments, primer efficiencies were calculated using RT miner software (Zhao and Fernald, 2005). Data were log transformed prior to statistical analysis.

Western blotting—Cells were lysed in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 2 mM NaF, 200 μM Na3VO4, 0.1% SDS, 5.6 mM NEM) supplemented with HALT Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) and western blotting conducted as previously described (Smyth et al., 2010; Smyth et al., 2012). Briefly, cells were scraped into RIPA buffer and sonicated prior to centrifugation at 10,000 x g for 20 min at 4°C. Protein concentration was quantified using the Bio-Rad DC Protein Assay and lysates normalized to the same concentration. 4X Bolt LDS sample buffer (Thermo Fisher Scientific) supplemented with DTT (400 mM, final concentration 100 mM) was added and samples heated for 10 min at 70°C prior to SDS-PAGE and western blotting with the following primary antibodies: rabbit anti-Cx43 (1:5000; Sigma), mouse anti-α-tubulin (1:5000; Sigma). Goat secondary antibodies conjugated to Alexa Fluor 555 and Alexa Fluor 647 (Thermo Fisher Scientific) were used at 1:1000, or secondary antibodies conjugated to HRP (1:5000) (Abcam) and detected with clarity western ECL substrate (BioRad), and membranes imaged on a ChemiDoc MP (Bio-Rad). Mouse heart tissue was homogenized in RIPA buffer with an omni tissue homogenizer (Omni international) and processed as above. Primary antibodies used were phospho-p38 MAPK Thr180/Tyr182 (1:1000, Cell Signaling Technology) and p38 MAPK (1:1000, Cell Signaling Technology). Mouse anti-GAPDH (1:5000, Fitzgerald) was used as loading control.
**RLM-RACE**—RLM-RACE was performed with the GeneRacer Kit (Thermo Fisher Scientific) according to manufacturer instructions. Briefly, RNA was isolated from NMUMG cells untreated or treated with TGF-β for 48 h. RNA integrity was verified by denaturing gel electrophoresis. 4 μg of RNA from each sample was dephosphorylated to prevent ligation of the RNA oligo adaptor to truncated mRNA and other non-mRNA. The 5′ cap was then removed and the GeneRacer RNA oligonucleotide was ligated to 5′ end of the RNA. Reverse transcription was performed with a *Gja1* specific primer to generate cDNA. The FWD Adaptor specific GeneRacer alternative primer and a *Gja1* specific Rev primer were used to amplify the entire 5′ UTR. A second round of nested PCR was performed. PCR products were run on an agarose gel followed by gel extraction of bands and cloning into the PCR 4 Blunt TOPO vector (Thermo Fisher Scientific) for sequencing. UTR sequences from RACE were aligned to the *Gja1* 5′ UTR using Snapgene software. Sequences were then mapped to the appropriate genome (mouse: mm10; human: hg38) using the UCSC genome browser and correlated with Cap Analysis of Gene Expression (CAGE) reads from the FANTOM5 consortium, RIKEN. RACE was performed in HaCaT as above.

**CRISPR/Cas9 gene editing**—Generation of 293FT GJA1−/− cell line: Single guide RNA targeting 5′ and 3′ flanking regions of GJA1 exon 2, respectively were cloned into pSpCas9(BB)-2A-GFP (PX458) at Bbs1 site. PX458 was a gift from Feng Zhang (Addgene) (Ran et al., 2013). Cas9 transfected 293FT cells were selected for single cell colonies following serial dilution into 96 well plates. Clonal populations were screened by PCR using primers flanking GJA1 exon 2. Colonies positive for biallelic deletion of *GJA1* exon 2 were further screened by Cx43 immunoblotting and immunofluorescence. Generation of NMuMG *Gja1*−/− cell line was done as above.

**Cloning and expression of Gja1–5′UTR isoforms**—The 5′UTR isoforms identified from RACE were cloned in front of *Gja1* into expression vector pcDNA3.2/V5-DEST using in-fusion HD cloning (TAKARA). Vector pcDNA3.2/V5-DEST was modified to exclude sequence downstream of the CMV promoter in order to exclude sequences that could influence translation. The ratio of GJA1–20k to Cx43 expression of each UTR isoform was analyzed by transfection of plasmids into *GJA1*−/− 239FT and *Gja1*−/− NMuMG cell lines. DNA Transfections were performed with Lipofectamine 3000 reagent (Thermo Fisher Scientific) and 500 ng of each UTR construct per well of a 24 well dish.

**Luciferase assays**—UTR isoforms were cloned into plasmid pcDNA3 RLUC POLIRES FLUC which was gift from Nahum Sonenberg (Addgene) (Poulin et al., 1998), in front of Fluc using infusion cloning. T7 tailed primers were used to PCR amplify luciferase constructs for *in vitro* transcription. The mMESSAGE mMACHINE T7 Transcription Kit (Thermo Fisher Scientific) was used for *in vitro* transcription according to manufacturer instructions with some modifications. RNA Cap mixtures included ribonucleotides with a 4:1 ratio of ARCA M7G cap (New England Biolabs), or an A cap analog (New England Biolabs). *In vitro* transcribed RNA was purified by lithium chloride precipitation. Poly (A) tailing of transcripts was performed with Poly (A) polymerase (New England Biolabs) at 37°C for 30 min. Luciferase assays were performed with the Dual-Luciferase Reporter...
Assay System (Promega) according to manufacturer’s instructions. Briefly, NMuMG cells in 24 well plates were transfected with in vitro transcribed RNA constructs consisting of 200 ng of UTR-Fluc and 10 ng of control Rluc, or 200 ng of an Rluc-UTR-Fluc bicistronic transcript, using Lipofectamine 3000. Cells were lysed in 100 μl of passive lysis buffer 3.5 hours post transfection. Luminescence measurements were recorded using an automated GloMax-Multi+ luminometer using luciferase Assay Reagent II and Stop & Glo.

**Immunofluorescence**—Cells were fixed in 37°C 4% paraformaldehyde for 20 min and stored in PBS at 4°C until immunostaining was conducted as previously described (Smyth et al., 2012) with the following primary antibodies: mouse anti–N-cadherin (BD Biosciences; 1:200), mouse anti-pan cadherin (Novus Biologicals; 1:500), rabbit anti-Cx43 (Sigma; 1:3000), and mouse anti–α-tubulin (Sigma; 1:3000). Secondary antibodies used were goat anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 647 (Thermo Fisher Scientific; 1:500).

Heart tissue cryosections (10 μm) were prepared on glass slides and fixed in acetone for 1 minute followed by air drying. Sections were rehydrated for 10 minutes in PBS, followed by 1 h of blocking at room temperature with 5% NGS, 0.1% Triton X-100 in PBS. After blocking, sections were incubated overnight at 4°C with primary antibodies diluted in 5% NGS; rabbit anti-Cx43 (1:2500; Sigma) and mouse anti-N-cadherin (1:250; BD Biosciences). Washes included 10 quick rinses in PBS, two 10 min washes in 0.05% Tween 20 in PBS, two 5 min washes in PBS. Cells were then incubated for an additional hour at room temperature with goat secondary antibodies anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 647 (Thermo Fisher Scientific; 1:500) in 5% NGS, 0.1% Triton X-100 in PBS with DAPI. Slides were then washed as above, and coverslips were mounted using ProLong gold antifade reagent.

Image analysis was performed with FIJI ImageJ software (NIH) (Schneider et al., 2012). For cell lines, average fluorescence intensity profiles of Cx43 were measured from 5 μm lines bisecting, and perpendicular to, N-cadherin labeled cell–cell borders from confocal microscopy maximum intensity projections. For cryosections, three N-cadherin labeled intercalated discs were randomly selected for each image. At least four images were analyzed per heart from which averaged intercalated disc values were obtained. The Cx43 containing channel was then merged with the N-cadherin channel and Cx43 fluorescence intensity measured along a 10 μm wide line covering the N-cadherin signal.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All quantification was performed on experiments repeated at least three times. Data are presented as mean ± SEM. Statistical analysis was conducted with GraphPad Prism 8.0.2 (GraphPad Software, Inc. La Jolla, CA). Data were analyzed for significance using Student’s t test or one-way ANOVA with Tukey’s post hoc tests. A value of p < 0.05 was considered statistically significant.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
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REFERENCES


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Highlights

- In aged hearts and during hypoxia or TGF-β treatment, the $GJA1$ mRNA 5′ UTR is truncated
- Activation of P38 and ERK signal transduction pathways regulate $GJA1$ 5′ UTR length
- Truncated $GJA1$ 5′ UTRs are sufficient to suppress internal translation of GJA1–20k
- Reduced GJA1–20k expression is correlated with P38 activation in aged hearts
Figure 1. TGF-β Induces Translational and Transcriptional Changes to Gja1

(A) Western blot of cell lysates probed with Cx43 C-terminal antibody to detect full-length Cx43 and internally translated GJA1–20k in control (C) and following 48 h of TGF-β treatment (T). α-tubulin is the loading control. 

(B) Quantification of bands in (A).

(C) Schematic of primers in RACE procedure. Gene-specific reverse-transcription (RT) primer, primary RACE PCR (green arrows), and nested RACE PCR (orange arrows).

(D) Agarose gel electrophoresis of RACE PCR products in control (C), and following 48 h of TGF-β treatment (T).

(E) Location of RACE products in the Gja1 5’ UTR DNA.

(F) RACE products and CAGE reads mapped to mm10, UCSC genome browser. 
See Table S2 for RLM-RACE primers. Statistical analysis performed using the Student’s t test (n = 3), **p ≤ 0.01.
Figure 2. UTR Isoforms Regulate Alternative Translation
(A) qRT-PCR analysis of baseline levels of UTR isoforms.
(B) Schematic of UTR isoforms.
(C–E) qRT-PCR analysis of L (C), M (D), and S (E) UTR isoforms following treatment with vehicle (Veh; DMSO), TGF-β (T), SB 202190 (p), and SB431542 (SB).
(F) Western blot analysis of Cx43 following treatment as in graphs (C–E) with the addition of SP 600125 (G) and α-tubulin loading control.
(G) Quantification of bands from (F).
(H) Western blot of Gja1 translation products 24 h post-transfection into Gja1−/− cells with α-tubulin loading control.
(I) Quantification of bands from (H).
See Table S1 for primer sequences. Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparison test (n = 3). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
Figure 3. ERK Regulates Gja1 Transcript Isoforms and Alternative Translation
(A) Western blot of cell lysates probed with Cx43 C-terminal antibody to detect full-length Cx43 and internally translated GJA1–20k following treatment with DMSO (Veh) or ERK inhibitor (ERKi) SCH772984 with loading control α-tubulin.
(B) Quantification of bands from (A).
(C–E) qRT-PCR analysis of L (C), M (D), and S (E) UTR isoforms following treatment with Veh (DMSO) or ERK inhibitor. Statistical analysis performed using the Student’s t test (n = 3), *p ≤ 0.05, **p ≤ 0.01.
Figure 4. GJA1 Transcript Isoforms Are Conserved in Humans and Primary Mouse Tissue and Are Stress Responsive

(A) Agarose gel electrophoresis of RACE PCR products in control (C) and following 48 h of TGF-β treatment (T).

(B) RACE products and CAGE reads mapped to hg18 in UCSC genome browser.

(C) Agarose gel electrophoresis of RACE PCR products from primary astrocytes (astros).

(D) Agarose gel electrophoresis of RACE PCR products from primary neonatal ventricular cardiomyocytes (NMVCMS). Arrows indicate excised bands.

Cell Rep. Author manuscript; available in PMC 2019 November 15.
(E) Astrocytes and NMVCM RACE products and CAGE reads mapped to mm10, UCSC genome browser. Vertical dotted lines indicate position of L (blue), M (red), and S (black) UTRs.

(F) Top panel: RACE PCR products in normoxia (N) and following 48 h of hypoxia (H). Bottom: western blot of iPSC-CM cell lysates probed with Cx43 C-terminal antibody to detect full-length Cx43 and internally translated GJA1–20k following 24 h of normoxic or hypoxic conditions (1% oxygen).

(G) Fixed cell confocal immunofluorescence (×100) of cells labeled with an antibody directed against the Cx43 C terminus (green) with cell borders detected using N-cadherin (red) in cells exposed to 48 h of normoxic or hypoxic conditions. Scale bars: 20 μm. Enlarged region of cell border shown below.

(H) Quantification of average fluorescence intensity profiles of 5-μm lines bisecting, and perpendicular to, cell-cell borders from confocal microscopy maximum intensity projections. n = 13.

(I) Western blot of NMuMG cell lysates probed with Cx43 C-terminal antibody to detect full-length Cx43 and internally translated GJA1–20k (20k) following 48 h of normoxic or hypoxic conditions (1% oxygen).

(J) Fixed cell confocal immunofluorescence (×60) of cells labeled with an antibody directed against the Cx43 C terminus (green) with cell borders detected using pan-cadherin (red) in cells exposed to 48 h of normoxic or hypoxic conditions. 4′,6-Diamidino-2-phenylindole (DAPI; blue). Enlarged region of cell border shown below.

(K) Quantification of NMuMG immunofluorescence as in (H). n = 12. black line = normoxia, blue line = hypoxia.
Figure 5. *Gja1* 5′ UTRs Regulate Translation Efficiency

(A) Schematic of RNA constructs.

(B–D) Firefly luciferase (Fluc) reporter gene expression normalized to renilla luciferase (Rluc) for L (B), M (C), and S (D) 5′ UTR sequence when capped (m7G), uncapped or A-capped (A), and located internally in a bicistronic construct (dual).

(E–G) Fluc reporter gene expression normalized to Rluc for L (E), M (F), and S (G) m7G-capped 5′ UTR sequences following transfection into cells exposed to TGF-β for 48 h. Statistical analysis was performed using the Student’s t test (n = 3), ***p ≤ 0.001, ****p ≤ 0.0001.
Figure 6. Aging Hearts Have Increased p38, Altered UTRs, and Reduced Internal Translation

(A) Immunofluorescence (×60) of mouse heart cryosections. Young (Y) = 3.8 months and old (O) = 29 months labeled with an antibody directed against the Cx43 C terminus (green), with intercalated discs labeled with N-cadherin (red) and DAPI DNA stain (blue). Scale bar: 10 μm.

(B) Quantification of average Cx43 fluorescence intensity within N-cadherin-labeled intercalated discs (n = 5 animals, 23 averaged intercalated disc intensities from young and n = 6 animals, 29 averaged intercalated disc intensities from old).

(C) Western blot of mouse heart lysates probed with antibodies directed against phosphorylated p38 (p38\textsuperscript{T180/Y182}), total p38, and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loading control (n = 6 per group).
(D) Quantification of bands from (C).

(E–G) qRT-PCR analysis of L (E), M (F), and S (G) UTR isoforms from mouse heart RNA (n = 6 per group).

(H) Western blot of mouse heart lysates probed with a Cx43 C-terminal antibody to detect full-length Cx43 and internally translated isoform GJA1–20k, and a GAPDH loading control (n = 6 per group).

(I) Quantification of GJA1–20k relative to Cx43 from western blot in (H).

(J) Correlation between GJA1–20k relative to Cx43 and phosphorylated p38\(^{T180/Y182}\); blue dotted lines represent 95% confidence interval. Statistical analysis performed using the Student's t test, *p ≤ 0.05, **p ≤ 0.001.
Figure 7.
Model of Transcription Start Site Selection in the *Gja1* 5′ UTR and Transcript Isoform Effect on Alternative Translation and Gap Junction Formation
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<td>Run et al., 2013</td>
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Software and Algorithms

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