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Trafficking Highways to the Intercalated Disc: New Insights Unlocking the Specificity of Connexin 43 Localization

Shan-Shan Zhang[1] and **Robin M. Shaw**[1,2]

[1]Cedars-Sinai Heart Institute, Los Angeles, CA, USA

[2]University of California, Los Angeles, CA, USA

Abstract

With each heartbeat, billions of cardiomyocytes work in concert to propagate the electrical excitation needed to effectively circulate blood. Regulated expression and timely delivery of connexin proteins to form gap junctions at the specialized cell – cell contact region, known as the intercalated disc, is essential to ventricular cardiomyocyte coupling. We focus this review on several regulatory mechanisms that have been recently found to govern the lifecycle of connexin 43 (Cx43), the short-lived and most abundantly expressed connexin in cardiac ventricular muscle. The Cx43 lifecycle begins with gene expression, followed by oligomerization into hexameric channels, and then cytoskeletal-based transport toward the disc region. Once delivered, hemichannels interact with resident disc proteins and are organized to effect intercellular coupling. We highlight recent studies exploring regulation of Cx43 localization to the intercalated disc, with emphasis on alternatively translated Cx43 isoforms and cytoskeletal transport machinery that together regulate Cx43 gap junction coupling between cardiomyocytes.

Keywords

Connexin 43; internal translation; transcription; autoregulation; trafficking; cytoskeleton; intercalated disc; microtubules; actin

INTRODUCTION

In order to participate as functional units within an organized tissue system, individual cells utilize multi-functional adhesion proteins for structural support, cytoskeletal organization, subcellular protein localization, and precise coordination of external signals. The heart is an organ in which individual cardiomyocytes must adhere and communicate with neighboring cells along longitudinally oriented fibers in order to rapidly transmit electrical impulses and drive synchronized beat-to-beat muscle contraction. Cardiomyocytes achieve rapid coupling through gap junctions (GJs), which are associated with adhesion junction complexes at the intercalated disc (ID). GJs comprise connexin (Cx) channels formed by the pairing of

Address correspondence to Robin M. Shaw, MD, PhD, Cedars-Sinai Medical Center, 8700 Beverly Blvd, Davis, 1022, Los Angeles, CA 90048, USA. Tel: +310-967-3842. Robin.Shaw@cshs.org.

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abutting hexameric hemichannels (connexons) from adjacent cells (Unwin & Zampighi, 1980), and provide electrical, metabolic, and immunological connectivity between cells, generally facilitating the passage of molecules less than 1 kDa in size (Smyth & Shaw, 2012). Overall intercellular conductance is determined by individual GJ conductance, as well as by membrane GJ density. Intercellular conductance in the heart remains stable despite the reality that the Cx protein has a half-life of only 1–5 hours (Beardslee et al., 1998; Shaw et al., 2007). Cxs are constantly made, transported, and degraded in living cells, and these processes are rapidly and exquisitely regulated by intracellular as well as extracellular cues.

A total of 20 and 21 Cxs have been identified in the sequenced mouse and human genomes, respectively (Sohl & Willecke, 2003, 2004). Throughout the heart, GJs are made up of specific combinations of the following Cxs: Cx40, Cx43, Cx45, and Cx30.2, of which Cx43 is the most abundantly expressed in ventricular muscle (Zhang & Shaw, 2013; Boukens & Christoffels, 2012; Oyamada et al., 2013). In individual ventricular cardiomyocytes, Cx43 is localized in large aggregates at longitudinal ends of the cell, along with cadherins, desmosomes, and other ion channels (Delmar, 2004; Severs, 1989; Forbes & Sperelakis, 1985; Palatinus et al., 2012). This precisely arranged Cx43 cell surface localization facilitates rapid and directional action potential propagation. Critical to ventricular myocyte coupling is the tight regulation of the Cx43 lifecycle beginning with gene expression and followed by i) ER to Golgi assembly and transport, ii) forward trafficking of *de novo* connexons from the Golgi to the cell surface, iii) maintenance and organization within the GJ plaque region, and (iv) retrograde transport for degradation (D'hondt et al., 2013; Marquez-Rosado et al., 2012) (Smyth & Shaw, 2012; Zhang & Shaw, 2013; Hesketh et al., 2009; Thevenin et al., 2013; Johnstone et al., 2012). Post-translational modification occurring at the Cx43 C-terminus is key in regulating many of these steps including channel assembly, trafficking, channel gating, and degradation.

Cx43 function is indispensable for normal development and maintenance of coordinated impulse propagation in the heart (Reaume et al., 1995; Huang et al., 1998; Ya et al., 1998; Guerrero et al., 1997; Thomas et al., 1998; Eloff et al., 2001; van Rijen et al., 2004; Morley et al., 1999; Eckardt et al., 2004; Danik et al., 2004; Gutstein et al., 2001; Shaw & Rudy, 1997). Mutations in *Gja1*, which encodes Cx43, are associated with atrial fibrillation, heterogeneous GJ loss, and sudden infant death (Thibodeau et al., 2010; Van Norstrand et al., 2012). Abnormal Cx43 post-translational modifications and trafficking defects can also result in lethal arrhythmias (Kalcheva et al., 2007; Peters et al., 1997; Remo et al., 2011; Beardslee et al., 2000; Ai & Pogwizd, 2005). In the setting of heart failure (HF), which is a growing epidemic in the United States (Go et al., 2013), disease progression is characterized by extensive tissue remodeling and altered expression of cardiac ion channels (Smith et al., 1991; Peters et al., 1993; Hong et al., 2012a, 2012b; Smyth et al., 2010). Cx43 is not only downregulated in HF patients; less Cx43 protein is localized to the ID to form GJ plaques. Common to these cardiomyopathies is aberrant Cx43 localization away from the ID.

In this review, we will discuss recent findings on the key checkpoints of the Cx43 lifecycle as summarized in Figure 1. We focus our discussion on transcriptional and translational control of Cx43 expression, ER to Golgi transport, cytoskeletal regulation of Cx43 forward

trafficking, and GJ organization at the dynamic ID. Given the fast Cx43 turnover rate (Beardslee et al., 1998; Jordan et al., 1999; Shaw et al., 2007), regulatory cues governing the Cx43 forward trafficking step play an important role in the regulation of intercellular coupling. Understanding this regulation is critical for future interventions that aim to rescue heart function by bolstering the delivery efficiency of Cx43 cargo. Together, the regulatory mechanisms presented will serve as a solid foundation upon which new clinical treatments can be developed.

MULTILAYERED REGULATION OF CX43 BIOGENESIS

Patterning of intercellular communication via transcriptional control

Changes in Cx43 expression level and region-specific patterning in the heart can alter cell–cell coupling and the special spread of excitation. Gene expression is an early primary step in the generation of functional GJ channels, and includes transcription, RNA splicing and processing, and translation. We begin our discussion on recent studies on the control of Cx43 expression.

Cx43 resides on mouse chromosome 10 and human chromosome 6, and has a relatively simple genomic structure of just two exons (Fishman et al., 1991; Sullivan et al., 1993). The first exon encodes most of the 5′-untranslated region (5′-UTR), while the entire coding sequence plus the 3′-UTR reside within the second exon. Two alternative promoters, along with four additional non-coding exons comprising alternative 5′-UTRs, have been reported (Pfeifer et al., 2004). While the canonical promoter is active throughout the heart, an alternative promoter is only active in the atria and septum, but not in the ventricle. An additional intronic promoter is utilized in the ventricles only. Based on promoter choice, a total of nine *Gja1* transcripts can be generated that only differ in their 5′-UTR to affect translational efficiencies of the corresponding mRNAs.

Several *T-box* and *Iroquois* homeobox transcription factors, which regulate cardiac development, have been shown to pattern areas of ion channel expression throughout the heart and parts of the conduction system (Boukens & Christoffels, 2012; Zhang et al., 2011; Gaborit et al., 2012; Bakker et al., 2008; Oyamada et al., 2013; Wiese et al., 2009). A central component of the cardiac conduction system is the sinoatrial node (SAN), which functions as the pacemaker to set the heartbeat. The atrioventricular node (AVN) then delays the traveling electrical impulse to ensure atrial ejection of blood before ventricular contraction, and to protect the ventricles from atrial arrhythmias. The ventricular conduction system, comprising the His bundle, bundle branches, and the His-Purkinje network, functions to rapidly direct the depolarizing impulse from the AVN to synchronously activate ventricular contraction.

Of the T-box transcription factor family, *Tbx3* and *Tbx18* can directly bind to the canonical promoter of Cx43 to pattern its region-specific expression. *Tbx3* is required in developmental patterning and controls the pacemaker gene program *in vivo*, including repression of Cx43 transcription (Hoogaars et al., 2007a, 2007b). *Tbx3* is also sufficient to downregulate Cx43, along with Cx40 and Nav1.4, in the reprogramming of mature cardiomyocytes to form SAN-like pacemaker cells (Bakker et al., 2012). *Tbx18* acts

upstream of *Tbx3* and is required for specification of the SAN (Wiese et al., 2009). *Tbx18* directly represses Cx43 transcription and cell–cell coupling in postnatal cardiomyocytes when overexpressed, and is sufficient for the reprogramming of mature cardiomyocytes to form SAN-like cells in a guinea-pig model (Kapoor et al., 2011, 2013). Common to both peacemaker cell conversion approaches is the need to suppress GJ coupling and downregulate resident channels of the ID that normally function together to drive depolarization and rapid directional impulse propagation across aligned ventricular cardiomyocytes.

Homeobox factors, which are characterized by a 60 amino acid DNA-binding domain known as the homeodomain, function within a transcriptional complex with other co-factors to switch gene cascades on or off in various tissues (Oyamada et al., 2013; Scott et al., 1989). Within this gene family, *Nkx2.5* and *Iroquois* can function as either activators or repressors of gene transcription, depending on the cellular context. *Nxk2.5* has highly evolutionarily conserved roles in heart development and function, and acts as a transcriptional repressor of *Cx43* (Kasahara et al., 2001, 2003; Dupays et al., 2005; Tanaka et al., 1999; Benson et al., 1999; Lyons et al., 1995; Schott et al., 1998; Goldmuntz et al., 2001). The *Iroquois homeobox gene 3* (*Irx3*) contains a homeodomain belonging to the three amino acid loop extension (TALE) superclass (Gomez-Skarmeta & Modolell, 2002), and has been shown to regulate Cx expression in the ventricular conduction system to tightly control electrical excitation spread for ventricular activation (Zhang et al., 2011).

The ventricular conduction system is extensively coupled via GJs containing specific combinations and region-specific patterns of mostly Cx40 and Cx43 (Oyamada et al., 2013; Boukens & Christoffels, 2012; van Veen et al., 2001). *Irx3* antithetically regulates Cx40 and Cx43 whereby *Irx3* indirectly activates Cx40 expression but directly represses Cx43 transcription. *Irx3* loss-of-function resulted in reduced Cx40 throughout the developing ventricular conduction system, and ectopic Cx43 expression in the proximal bundle branches. These changes were accompanied by abnormal cell–cell coupling, disruption of rapid spread of ventricular excitation, as well as right bundle branch block. Taken together, *Nkx2.5* and *Irx3* function in the tight regulation of Cx expression, and represent a transcriptional regulatory pathway of cell–cell coupling that can contribute to arrhythmogenesis when disrupted.

Found in translation: the secret life of *Gja1* mRNA

We continue our Cx43 biogenesis discussion on how such a rich Cx43 protein repertoire is generated from a two-exon encoded gene (Figure 1), focusing specifically on the recent finding that multiple Cx43 isoforms are made via alternative translation (Smyth & Shaw, 2013). Given its relatively simple gene structure, yet unique functions at various cellular locations, Smyth and Shaw asked whether additional mechanisms exist to explain the vast functional diversity.

An initial clue came from the finding that cap-independent translation occurs at internal ribosome entry sites (IRES) in eukaryotic systems (Candeias et al., 2006; Ingolia et al., 2011). In addition to the previously known IRES in the *Gja1* 5′ UTR (Schiavi et al., 1999), we identified that the *Gja1* mRNA is polycistronic, encoding at least four smaller Cx43

isoforms via alternative start site (AUG codon) selection within the coding mRNA. This alternative translation step is negatively regulated by PI3K/AKT/mTOR signaling, which promotes cap-dependent translation. While transcriptional control determines how much full-length Cx43 is made, this novel regulatory step during translation fine-tunes the precise type of Cx43 isoform that is generated in response to signaling and cellular need. As discussed below, smaller Cx43 isoforms facilitate anterograde transport of full-length Cx43 to autoregulate the degree of GJ coupling at cell–cell borders.

TRAFFICKING HIGHWAYS TO THE ID

After biogenesis, the Cx43 lifecycle continues with ER to Golgi transport, vesicular trafficking from the trans-Golgi network (TGN) to the cell surface, organization, and maintenance within the GJ plaque, and retrograde transport for degradation (D'hondt et al., 2013; Smyth & Shaw, 2012; Thevenin et al., 2013). Changes in regulation of the balance of these steps can quickly affect the amount of Cx43 available for coupling at cell–cell junctions. In this section, we focus on recent developments on targeted delivery of Cx43 to the ID, and discuss how Cx43 GJs are organized once they reach their destination. Cx43 GJ internalization, which depends on post-translational modifications of the Cx43 C-terminal tail, is also a highly regulated process that controls surface GJ availability. Interested readers, please refer to these studies (Su et al., 2010; Fong et al., 2013; Cochrane et al., 2013; Thevenin et al., 2013; Fong et al., 2012; Johnstone et al., 2012; Falk et al., 2009) for developments on this front.

Keeping up with the connexons—novel isoforms function as chaperones

Prior to forward transport along the cytoskeleton, Cx43 traverses through the ER and Golgi apparatus to oligomerize and acquire the appropriate posttranslational modifications (Musil & Goodenough, 1993; Smith et al., 2012). Unlike other channels such as the L-type calcium channel that utilize channel-specific auxiliary subunits for transport and function (Zhang & Shaw, 2013; Gerhardstein et al., 2000; Gao et al., 2001; Hulme et al., 2005; Hulme et al., 2006; Domes et al., 2011; Fu et al., 2011), no such subunits have been identified for Cx43. Smyth and Shaw discovered alternative internal translational events as the mechanism generating N-terminally truncated Cx43 isoforms (Smith & Shaw, 2013). To understand the functional significance of the smaller isoforms, mutations of four internal AUG start sites were made to reveal Cx43 accumulation in the ER, and a marked decrease of Cx43 at the cell surface. This change in localization was rescued by overexpressing a 20-kilo-dalton isoform (GJA1-20K), which is the most abundantly expressed smaller isoform. When full-length Cx43 and GJA1-20K were differentially tagged with fluorescent reporters and imaged, they were found to be associated in reticulated ER structures, but only full-length Cx43 was found on the cell surface. Together, these findings indicate that the smaller isoforms can function as chaperones for Cx43 transport through the ER/Golgi, prior to vesicular trafficking along the cytoskeleton.

Cx43 isoform generation, affecting GJ-based intercellular conductance, was also found to be under mTOR signaling regulation. Inhibition of PI3K/AKT/mTOR, which normally promotes cap-dependent translation and thus full-length Cx43 levels (Folkes et al., 2008;

Feldman et al., 2009), increased GJA1-20K chaperone expression and yielded larger GJ plaques at neonatal ventricular cardiomyocyte cell–cell borders. Many GJ-independent roles have been attributed to the Cx43 C-terminal tail (Palatinus et al., 2012; Francis et al., 2011; Rhee et al., 2009; Xu et al., 2006). Involvement of mTOR indicates that mTOR inhibitors, which are common therapeutic immunosuppressants used in organ transplantation, may possibly be used to restore cell–cell coupling in failing hearts as well.

Our findings also offer a potential mechanism to explain how Cx43 plays vastly different roles independent of cell–cell communication, such as trafficking of Nav1.5 (Rhett et al., 2012), mitochondrial function (Rodriguez-Sinovas et al., 2006), and cell cycle regulation (Olbina & Eckhart, 2003). It is possible that some of these functions could be carried out by alternatively translated isoforms acting at different intracellular organelles. We speculate that alternative translation could also contribute to some of the cardiac phenotypes of Cx43 C-terminal truncation mouse models that either retain, or completely lack, a wild type copy of full-length Cx43 (Maass et al., 2007; Maass et al., 2009).

Leaving the TGN: directed targeting along microtubules

Once the correct Cx type, isoform, and amounts are made, what regulatory cues direct final delivery of the precious cargo to form Cx43 GJs? In the secretory transport pathway, vesicles emerge from the Golgi apparatus and are transported to the cell–cell junction along the filamentous microtubule and actin networks (Ross et al., 2008). This dynamic process is dependent upon coordinated action of microtubule-based motors, kinesin, cytoplasmic dynein/dynactin, as well as actin-based myosin motors. Cx43 is a short-lived protein with a half-life of only 1–5 h (Beardslee et al., 1998; Jordan et al., 1999; Shaw et al., 2007), suggesting its intracellular movements are tightly regulated. How cytoskeletal transport machinery contributes to Cx43 hemichannel trafficking specificity in cardiomyocytes is an active area of investigation.

A critical aspect of Cx43 forward transport in the heart is microtubule-based localization to the ID of ventricular cardiomyocytes (Shaw et al., 2007; Smyth et al., 2010). The ID mediates mechanical as well as electrical coupling between cardiomyocytes, and is enriched with the Ca^{2+}-dependent adhesion molecule N-cadherin (Delmar, 2004; Severs, 1989; Palatinus & Gourdie, 2007; Forbes & Sperelakis, 1985). A number of cardiomyopathies are characterized by disrupted mechanical junctions associated with altered Cx43 localization and dissociation from N-cadherin and desmosomal proteins (Hesketh et al., 2010; Akar et al., 2004; Kostin et al., 2004; Sepp et al., 1996; Fidler et al., 2009; Oxford et al., 2007). Thus, the ability of cardiomyocytes to form adhesion contacts is critical for Cx43 GJ localization to the ID. How the microtubule-based trafficking machinery contributes to polarized localization of Cx43 GJs in ventricular cardiomyocytes is discussed below.

Data exist for multiple, but not mutually exclusive, models of Cx43 localization to the ID (Gaietta et al., 2002; Lauf et al., 2002; Shaw et al., 2007; Smyth & Shaw, 2012; Zhang & Shaw, 2013; Johnson et al., 2002). It has been established that microtubules participate in the delivery of Cx43 to the plasma membrane. Initial landmark studies provided evidence that *de novo* Cx43 appears at the perimeter of the GJs before diffusing into the plaque center (Jordan et al., 1999; Lauf et al., 2002). Based on these findings, it was proposed that Cx43

hemi-channels are inserted into the general plasma membrane and quickly diffuse to the edge of GJ plaque, before slowly diffusing into the plaque center. Subsequently, it was observed that Cx43 can be inserted directly at the plaque and that membrane fluidity exists within the GJ space (Falk et al., 2009; Shaw et al., 2007; Majoul et al., 2013). Plaques can also be internalized from regions away from the plaque center, and that full GJ plaque internalization can occur in a single step (Falk et al., 2009; Piehl et al., 2007). Recent studies identified that collections of Cx43 hemichannels, which exist in a “perinexus” space around the GJ plaque, interact with scaffolding proteins as well as other ion channels (Rhett et al., 2011; Rhett et al., 2012). These studies provide evidence that the GJ plaque and the surrounding membrane domain are highly dynamic, exhibiting complex behavior of targeted insertion and internalization events to organize communication between cells.

Given the low density of Cx43 hemichannels in membrane regions well away from the GJ plaque, and the technical difficulty of distinguishing Cx43 already inserted into the membrane from submembranous collections in the cytoplasm, the lateral diffusion coefficient of membrane-bound Cx43 has been difficult to quantify. It is possible that hemichannels could diffuse within the plasma membrane before arriving at the GJ plaque. Our directed targeting paradigm is based on the observation that *de novo* Cx43 hemichannels are targeted directly to adhesion junctions with specificity directed by the Cx43 hemichannel, microtubule plus-end tracking proteins EB1 and p150(Glued), and the adherens junction structure including β-catenin. In the directed targeting model, dynamic microtubules act as highways that terminate and anchor at adherens junction complexes to allow direct delivery of Cx43 cargo (Shaw et al., 2007; Smyth et al., 2010, 2012). Based on these studies, instead of long-range lateral diffusion, local hemichannel movement within the plaque and the perinexus is likely to occur to organize GJs and modulate coupling conductance.

In line with the short Cx43 half-life and constant need to maintain cellular coupling in the heart, the dynamic model of directed forward trafficking offers an efficient way to control GJ coupling in response to changes in the cardiomyocyte microenvironment. A similar cytoskeleton-based directed-targeting paradigm is conserved for Cav1.2 delivery to the cardiac T-tubule, which is a specialized membrane subdomain facilitating calcium influx deep within the cardiomyocyte to drive excitation–contraction coupling (Hong et al., 2012b). In particular, the BIN1 scaffolding protein was shown to anchor microtubules laden with Cav1.2 channels to T-tubules, similar to the role of N-cadherin in anchoring EB1/p150(Glued)-tipped microtubules at the ID. Further work supporting this model revealed that Cx43 and Cav1.2 were decreased at their respective membrane subdomains, along with displacement of their associated trafficking machinery, in the setting of heart disease (Hong et al., 2012a, 2012b; Smyth et al., 2010). We speculate that this trafficking paradigm can be generalizable to other cardiac ion channels, and be explored in context of other cytoskeletal elements and anchor proteins.

Actin’ on cargo trafficking specificity

In addition to microtubule-based trafficking, the actin cytoskeleton is implicated in hemichannel delivery to the cell–cell junction. In cardiomyocytes, α actin comprises the thin

filaments of the sarcomere (Boateng and Goldspink, 2008), while β and γ actin form filamentous actin (F-actin) not associated with generating contractile force (Hayakawa et al., 1996). In line with an organizational role, formation and maintenance of membrane subdomains such as the ID, caveolae, and T-tubules depend upon F-actin (Noorman et al., 2009; Itoh et al., 2005). However, actin is also highly dynamic, known to regulate intracellular vesicular transport through motor protein-based trafficking and vesicular fusion with the cell membrane (Jaiswal et al., 2009; Rogers & Gelfand, 2000), as well as mediate long-range vesicular trafficking in plants and mouse oocytes (Schuh, 2011; Akkerman et al., 2011). Moreover, dye transfer studies identified the dependence of GJ formation and maintenance on actin (Theiss and Meller, 2002; Qu et al., 2009; Thomas et al., 2001).

Building on these roles, we set out to test whether F-actin directly participates in the regulation of Cx43 trafficking to form GJs between cardiomyocytes (Smyth et al., 2012). We found that Cx43 co-immunoprecipitates with β actin, and colocalizes with non-sarcomeric actin structures along the vesicular transport pathway at the perinuclear region, as well as ID regions. Using live-cell imaging, we determined the time course and directionality of Cx43 cargo movement relative to actin. Cx43 cargo slowed down when associated with actin, with the majority traveling at speeds slower than that of microtubule-based transport (<0.25 μm/s) (Fort et al., 2011; Shaw et al., 2007), but consistent with myosin-based transport on actin (Howard, 1997). This finding is consistent with previous studies of actin–myosin based transport of melanosomes, slowing of endocytic vesicles at actin-rich regions in the cell cortex (Aschenbrenner et al., 2004; Ross et al., 2008), and Cx32 pausing at actin structures en route to the hepatocyte cell surface (Fort et al., 2011).

By monitoring timed release of Cx43 accumulated in the ER (Misumi et al., 1986), we identified F-actin integrity as necessary for *de novo* Cx43 delivery to cell–cell borders in neonatal ventricular cardiomyocytes. Cx43 plaque formation at N-cadherin-containing IDs was examined in Langedorff-perfused mouse hearts subjected to acute no-flow ischemia, actin disruption by latrunculin A, or both treatments. Cx43 localization at the cell–cell junction was comparably reduced with either treatment, but the effect was not additive with both treatments, suggesting a common pathway. In control hearts, Cx43 and β actin interact biochemically and this was disrupted when actin polymerization was inhibited, or when the heart became ischemic. Together, these studies reveal that F-actin is associated with a slow-moving Cx43 cargo population, and that this interaction is an essential component of the Cx43 forward trafficking machinery.

Based on the growing evidence supporting directed delivery, the microtubules that load Cx43 at the Golgi apparatus may not end up delivering the cargo to the cellular junction. This flexibility in transport provides cardiomyocytes with a means to respond quickly to the microenvironment such that Cx43 deposited along F-actin can be readily recruited by dynamic EB1-tipped microtubules. Our studies revealed noncontractile F-actin as a cytoplasmic Cx43 reservoir, serving as an alternate trafficking route and a means to mass acute Cx43 delivery in response to cellular stress.

Future work is needed to test how the F-actin network determines cargo directionality toward the cellular junction, and identify additional cytoskeletal players involved in this

dynamic process. Interestingly, secretory vesicles in plant cells are transported by an actin–myosin system involving two types of F-actin. Vesicles exhibit faster and directional motility along long F-actin tracks, but move slowly with little directionality in areas containing a fine F-actin meshwork (Akkerman et al., 2011). Could separate types of cytosolic actin structures exist in the cardiomyocyte cytoplasm to affect Cx43 trafficking? How is Cx43 trafficking specificity controlled by actin? Does interplay exist between the actin and microtubule machineries in directing Cx43 delivery to the cell–cell junction? These are intriguing questions that remain to be answered.

Organization and maintenance within the dynamic ID

Once delivered to the ID, GJ coupling conductance is dependent upon the stability and organization of resident proteins. The original view, first articulated in the late 1980s, held that the ID contains adhesion molecules (desmosomes and adherens junctions), and GJ channels that exist independently from each other (Unwin & Zampighi, 1980; Forbes & Sperelakis, 1985; Severs, 1989). Recent data found that the ID is a highly complex and dynamic membrane space, which is continuously organized by scaffolding proteins linking the cytoskeleton to resident transmembrane proteins.

During development, polarization of mechanical junctions at end-to-end contacts was observed before that of Cx43, which is initially widely distributed throughout the sarcolemma, including the lateral sides of the cell (Muhlfeld & Richter, 2006; Maass et al., 2007; Fromaget et al., 1992; Gourdie et al., 1991, 1992; Peters et al., 1994). Preferential Cx43 localization to IDs over developmental time was proposed to be a consequence of slower Cx43 internalization rates at IDs in comparison with other membrane areas, which are sparsely decorated with adhesion junctions and are thus less stable (Hirschy et al., 2006; Angst et al., 1997; Palatinus et al., 2012). Moreover, a close link between Cx43 localization and mechanical adhesion was demonstrated in experimental and clinical cardiomyopathy studies, which revealed Cx43 dissociation from N-cadherin and desmosomes, as well as membrane lateralization (Akar et al., 2004; Smyth et al., 2010; Akar et al., 2007; Hesketh et al., 2010; Kostin et al., 2004; Nygren et al., 2007; Sepp et al., 1996; Uzzaman et al., 2000; Li et al., 2008). These studies demonstrate a hierarchical dependence of Cx43 on mechanical junctions and that cardiomyocytes form polarized physical adhesions with neighboring cells before organizing communication via Cx43 GJs.

The GJ plaque, and the surrounding perinexus space, are increasingly appreciated as a dynamic structure in which resident proteins form composite structures that are constantly delivered, organized, and turned over (Majoul et al., 2013; Agullo-Pascual et al., 2013; Palatinus et al., 2012; Rhett et al., 2013). The perinexus is envisaged as a sieve-like network containing Cx43 hemichannels interacting with the tight junction protein, zonula occludens protein 1 (ZO-1), via the postsynaptic density/disc large/ZO-1 (PDZ) domain in the Cx43 C-terminal tail (Giepmans et al., 2001; Toyofuku et al., 1998). Interactions between Cx43 and ZO-1 were initially implicated in Cx43 GJ endocytosis under specific settings (Barker et al., 2001, 2002, 2008; van Zeijl et al., 2007; Piehl et al., 2007; Duffy et al., 2004). Moreover, given the ability of ZO-1 to link GJs, adherens junctions and the actin cytoskeleton, it was proposed that the ZO-1/Cx43 interaction held an additional role in GJ aggregation.

Supporting evidence revealed preferential localization of ZO-1 at the edge of the Cx43 GJ plaque, and an increase in GJ size with the loss of ZO-1/Cx43 interactions (Palatinus et al., 2011; Hunter et al., 2005; Rhett et al., 2011; Maass et al., 2007). The ZO-1/Cx43 interaction is now understood to have multiple roles including restricting the accrual of new connexons into the GJ plaque.

A mouse model expressing a Cx43 C-terminal truncation mutant at amino acid 258, lacking the PDZ domain to interact with ZO-1, exhibited no disruption of GJ formation at the ID (Maass et al., 2007). However, GJ plaques appeared bigger and accumulated at the periphery of the ID, in line with a role for ZO-1 in sequestering Cx43 in the perinexus space. Adding to the complexity of the dynamic ID disc, deletion of the last five amino acids (encoding one of two PDZ domains) of the Cx43 C-terminus in another mouse model did not disrupt ZO-1 binding, localization at the ID, or GJ channel function. Instead, these last five residues were required for maintaining proper functional properties of the sodium and potassium channels (Lubkemeier et al., 2013).

The concept that the ID is a hub for cell adhesion, electrical coupling, as well as cellular excitability, is rapidly emerging. The pore-forming alpha subunit of the sodium channel encoded by Nav1.5, along with the scaffolding proteins plakophilin-2 (Pkp2) and Ankryin-G (AnkG), have been detected at the ID space to interacting with Cx43 (Kucera et al., 2002; Lowe et al., 2008; Petitprez et al., 2011; Malhotra et al., 2004; Rhett et al., 2013; Sato et al., 2011). Further support comes from a super-resolution florescence microcopy study that revealed colocalization of Cx43, Pkp2, and AnkG inside the GJ plaque (Agullo-Pascual et al., 2013). This study added another layer of complexity to the ID space, by implicating AnkG–actin interactions as a means to further restrict the flow of Cx43 hemichannels within the perinexus.

Taken together, these studies offer distinct, but not mutually exclusive, mechanisms for polarized Cx43 GJ localization in cardiomyocytes. Dynamic cytoskeletal-based Cx43 trafficking directly to adherens junctions accounts for rapid Cx43 delivery in response to cellular need. Other models focus on how specific protein–protein interactions afforded by the Cx43 C-terminal tail can affect GJ organization and stabilization over time. These studies highlight the complexity of the dynamic ID and surrounding space in which GJs intermingle with other ion channels important for membrane depolarization. Future work is required to elucidate how these trafficking and organizational mechanisms, which could function in concert, determine intercellular conductance in context of specific cardiac demands and remodeling processes.

CONCLUSIONS

To achieve precise and timely Cx43 expression, multiple regulatory steps have been identified to include initial transcription in the nucleus, translational control, ER to Golgi transport, directed trafficking along the cytoskeletal network, and finally delivery and organization within the ID. Together, the studies presented expand on previous knowledge of the ID as a collection of separate protein entities either involved in communication or adhesion. The GJ and the surrounding perinexus space are appreciated as a highly dynamic

structure within which resident channels and adhesion proteins intermingle, while interacting with scaffolding proteins and cytoskeletal elements to effect intercellular coupling and excitation. We bring to light that in addition to previously known mechanisms influencing how much Cx43 is expressed in the cardiomyocyte, alternative translation of *Gja1* mRNA generates truncated Cx43 isoforms that can act as autoregulatory chaperones during ER to Golgi transport. The need to understand cytoskeletal control of the specificity and directionality of Cx43 trafficking toward the GJ plaque is also highlighted.

Cx43 is expressed in most excitable as well as nonexcitable tissues, and is indispensable for their development and function. Various pathologic conditions, including cancer and heart disease, are characterized by altered Cx43 expression and cellular distribution. Together the studies presented illustrate the importance of Cx43 in maintaining cellular communication and tissue function, and emphasize the need to identify specific mechanisms regulating the Cx43 lifecycle. The field is rapidly evolving, driven by the need to understand how perturbations of these highly regulated processes contribute to disease progression in a variety of tissues, and introducing new therapeutic classes such as mTOR inhibitors to regulate cell–cell coupling. This knowledge will be key for clinical interventions aimed at affecting intercellular communication in patients with heart failure, cancer, and other common maladies.

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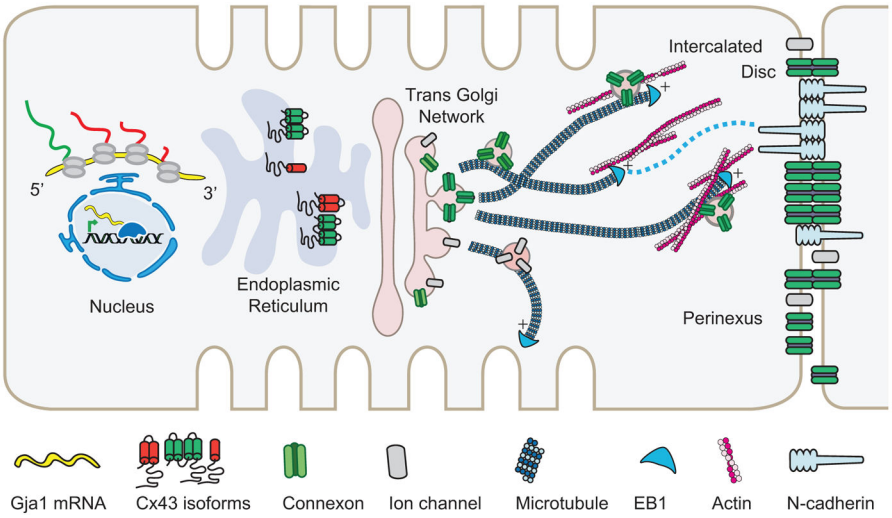

Figure 1.

The Cx43 lifecycle. Key steps of the lifecycle are highlighted: transcriptional control of Cx43 expression pattern and level, mTOR-regulated alternative internal translation of *Gja1* mRNA, the role of smaller N-terminally truncated Cx43 isoforms in ER-to-Golgi transport, and directed targeting to the ID via the microtubule and actin cytoskeleton.