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Review

The connexin43 carboxyl terminus and cardiac gap junction organization ☆

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ABSTRACT

The precise spatial order of gap junctions at intercalated disks in adult ventricular myocardium is thought vital for maintaining cardiac synchrony. Breakdown or remodeling of this order is a hallmark of arrhythmic disease of the heart. The principal component of gap junction channels between ventricular cardiomyocytes is connexin43 (Cx43). Protein–protein interactions and modifications of the carboxyl-terminus of Cx43 are key determinants of gap junction function, size, distribution and organization during normal development and in disease processes. Here, we review data on the role of proteins interacting with the Cx43 carboxyl-terminus in the regulation of cardiac gap junction organization, with particular emphasis on Zonula Occludens-1. The rapid progress in this area suggests that in coming years we are likely to develop a fuller understanding of the molecular mechanisms causing pathologic remodeling of gap junctions. With these advances come the promise of novel approach to the treatment of arrhythmia and the prevention of sudden cardiac death. This article is part of a Special Issue entitled: The Communicating junctions, composition, structure and characteristics.

Contents

1. Introduction

Gap junctions (GJ)s provide for cytoplasmic continuity between cells, permitting direct intercellular transmission of ions, second messengers, and other molecules less than 1 kDa [1–3]. Most mammalian cells exhibit abundant GJ contacts with neighboring cells, providing the basis for integrated networks within tissues. The propagation of electrical activation in the heart is one of the more widely known examples of tissue integration by an intercellular network of GJs. That GJs have additional roles in tissue homeostasis and dynamics is suggested by the near ubiquitous presence of GJs between excitable and non-excitable cells in vertebrates.

The channels that are responsible for electrical coupling of cardiomyocytes are composed of proteins encoded by the connexin gene family [4]. Connexins comprise four transmembrane spanning motifs, with both amino-(NT) and carboxyl-(CT) termini located on the cytoplasmic side of the plasma membrane. Connexin subunits

Abbreviations: Aa, amino acid; CT, Carboxyl-Terminal; CL, Cytoplasmic loop; Cx43, Connexin 43; Dlg, Disks Large; GJ, Gap Junction; LSCM, Laser Scanning Confocal Microscope; NT, Amino Terminal; MAGUK, Membrane-Associated Guanylate Kinase; ODDD, oculodentodigital dysplasia; PDZ, Postsynaptic density/Disks large/ZO-1; UKS, Unknown Significance; ZO-1, Zonula-Occludens-1

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form hexameric connexon oligomers, which traffic to the membrane, and dock with connexons from adjacent cells to form functional intercellular channels [5] reviewed in Ref. [6]. Aggregation of docked connexons results in the formation of the membrane organelle responsible for cell-to-cell communication — the GJ plaque (Fig. 1).

The CT sequence is the site of greatest amino acid (aa) variability between connexin isoforms [7] and thought to be the main regulatory domain of most connexins. The connexin CT has roles in GJ assembly and channel function, via numerous post-translational modifications and protein–protein interactions (Fig. 2). These assignments within the CT are also critical to governance of the trafficking, size, localization and turnover of GJs, as well as the level of intercellular coupling mediated by the plaque. Disruption in the distribution or extent of GJs is characteristic of various disease states, including slow healing wounds in the skin, epileptic seizures in the brain and arrhythmias in the heart. Mutations in Cx43 have been linked to the inherited human disease oculodentodigital dysplasia (ODDD), though interestingly relative to other domains of Cx43 there is a low incidence of mutation in the CT [8].

Connexin 43 (Cx43) is a GJ protein commonly found in excitable tissues in mammals, including humans [9–11]. The ventricular muscle of the heart is particularly enriched for Cx43, where it localizes preferentially to cardiomyocyte longitudinal termini — i.e. the intercalated disk. Mislocalization of GJs from polarized distributions at the intercalated disk and remodeling of Cx43 to the lateral surfaces of cardiomyocytes have been noted in nearly every cardiac disease state, and are correlated with the propensity of arrhythmia generation in the affected tissues [12–18].

There are numerous informative and detailed surveys of the literature on Cx43 remodeling and cardiac arrhythmic disease [9,19–21]. Reviews on the structure, molecular biology and function of the intercalated disk in its role of providing a platform for electromechanical coupling between cardiomyocytes are also available for interested readers [22–26].

Here, a perspective on regulation of GJ plaque size and localization in the plasma membrane (sarcolemma) of ventricular working myocardial cells will be provided. Emphasis in the review will be placed on the role of the Cx43 CT, and in particular, the Cx43 CT-interacting protein Zonula Occludens-1 (ZO-1). Where relevant, other cell types, connexins, junctional molecules and interacting proteins will be broached. The mechanisms determining normal GJ order in the healthy myocardium and pathological disorder in the diseased heart are a dual practical focus of this review. A working hypothesis is that the establishment of orderly arrangements of GJ organization in the sarcolemma is a key to stable propagation of electrical activation in myocardial tissues. Breakdown of this order can lead to cardiac conduction disturbance, arrhythmia and sudden death.

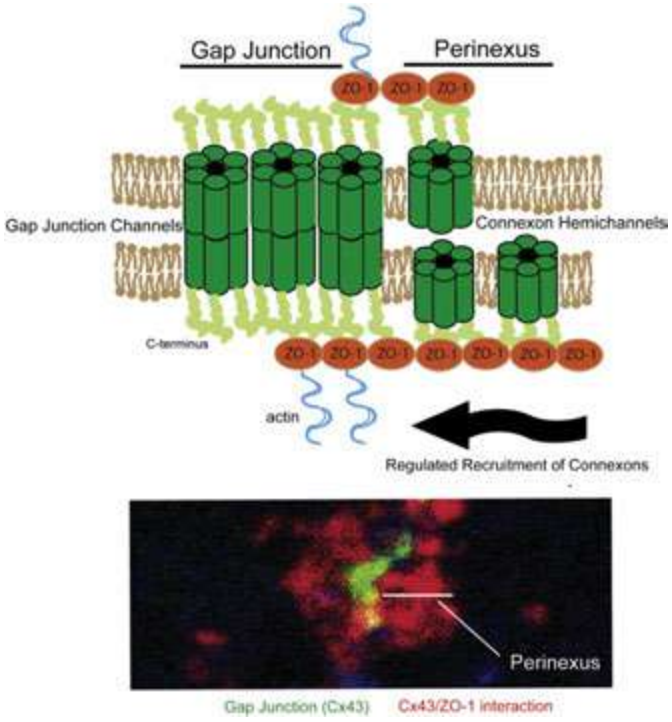

Fig. 1. The gap junction and perinexus. Top) Schematic of gap junction plaque with adjacent perinexus regulating hemichannel addition at the plaque edge. Bottom) Immunoconfocal view of rat epicardial cells labeled for Cx43 in green and Cx43/ZO-1 interaction in red as labeled in situ by the Duolink protein–protein interaction assay. Note that the extent of red signal in the perinexus exceeds that of the green immunolabeled gap junction. For more information see Ref. [95].

2. Historical background

The initial evidence that GJs in the mammalian heart demonstrated high levels of spatial order came from the electron microscope. Thin section electron microscopy localized large GJs at intercalated disks, particularly at lateral segments of apposed sarcolemma within disks [23,26–29]. Based on ultrastructural data, there were debates in the literature as to the size of the largest GJs at the intercalated disk [23,30], the resolution of which would await the advent of other approaches, including immuno-confocal microscopy. Small, interplicate GJs were also noted dispersed within the fascia adherens, the extended adhesive junctional complexes responsible for stabilizing mechanical interactions between cardiomyocytes [31,32].

Pioneering freeze fracture electron microscopy provided high spatial and temporal resolution views of GJ aggregates in beating myocardial tissues [33,34]. Aggregates of particles within GJs thought to represent individual connexons, were revealed to occur in closely packed arrays that were separated by particle-free aisles. Freeze fracture studies of embryonic, neonatal and perinatal ventricle also indicated the occurrence of developmental increases in the size of GJs [35,36], suggestive that the differentiation of GJ organization in the developing heart was in itself a progressive and regulated process.

The identification of connexin gene sequences in the late 1980s [37], including that encoding Cx43 [38] led to the advent of highly specific antibodies. These antibodies, coupled with powerful new light microscopic techniques, such as laser scanning confocal microscopy (LSCM), began to provide new views of the 3D order of GJs in the adult and developing heart [39–42].

Confirmation of the preferential localization of presumptive GJs at intercalated disks using immunohistochemistry and Cx43 specific antibodies was first provided by Beyer and co-workers in 1989 [39]. Shortly thereafter it was demonstrated that Cx43 GJ substructure showed further organization within the disk. Three-dimensional reconstructions of en-face views of the termini of rat ventricular cardiomyocytes from LSCM optical sections revealed circumferential distributions of large immunolabeled GJs [40,44]. This spatial configuration was subsequently confirmed to occur in the ventricles of mice, humans and other mammals [40–45]. Quantitative comparison of plaque size from immunofluorescent labeling and freeze fracture electron microscopy was among the approaches used to validate that myocardial GJs were being correctly identified and measured by the LSCM [41,46].

The distinct population of large GJs at the intercalated disk circumference did not occur in the embryonic ventricle, or indeed become fully manifest until quite late into postnatal development [40,42,43,47]. Several immunofluorescence studies indicated that distributions of Cx43 GJs in the neonate were uniformly organized around cardiomyocytes. But with subsequent maturational growth of the heart there was a loss of these lateral contacts between

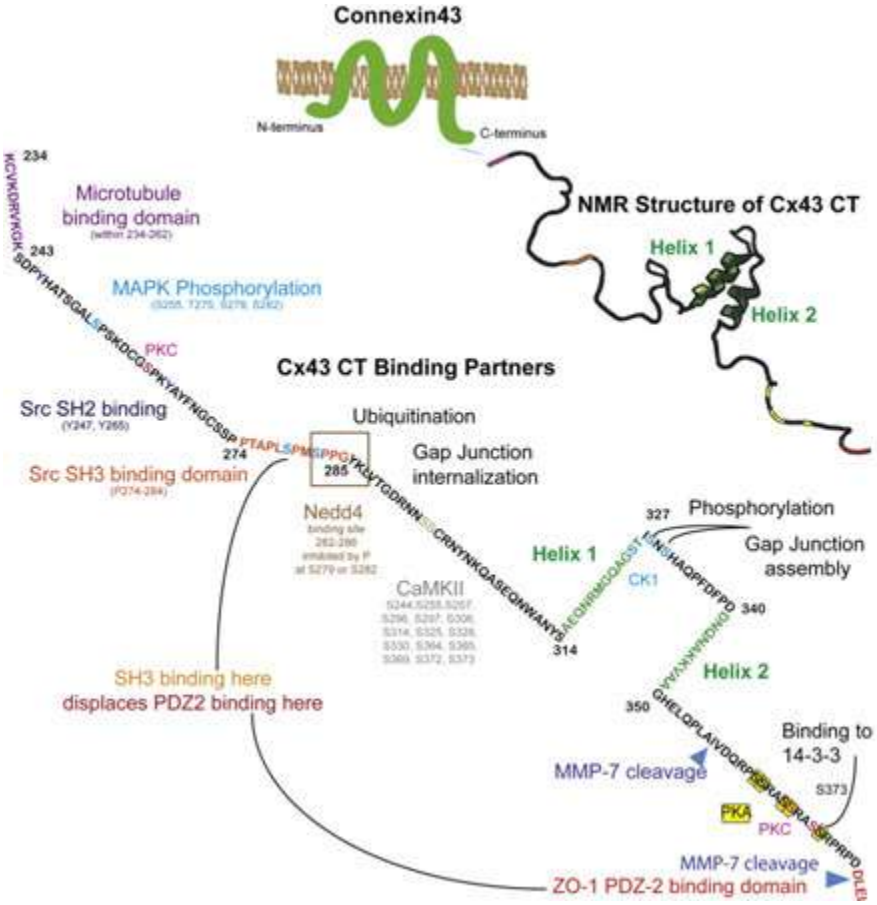

Fig. 2. The interacting proteins of the Cx43 carboxyl-terminus (CT). Amino acid sequence of human Cx43 from CT residues 234 to 382 labeled with sites of protein–protein interaction. NMR structure of Cx43 was acquired by Sorgen and others [65] and obtained from the World Wide Protein Data Bank (PDB ID: 1R5S).

cardiomyocytes and progressive increases in the relative abundance of Cx43 immunolabeling at intercalated disks.

The culmination of this developmental process resulted in 60% or greater of immunodetectable Cx43 at the disk in the ventricle of most mammals [32]. It is thought that this polarization of GJs to the ends of cardiomyocytes contributes to changes in conduction of the ventricle from isotropic to more directional propagation of electrical impulse along the longitudinal axis of myofibers [48,49]. This being said, mathematical modeling indicates that the postnatal increase in cardiomyocyte size is probably a larger factor in the emergence of directional propagation of cardiac action potentials than GJ distribution [50].

In 1991, studies of infarcted ventricular myocardium in humans [51] and dogs [52] provided the first indication that the pattern of Cx43 GJs is profoundly altered in a narrow zone of surviving muscle bordering the necrotic injury or scar — the infarct border zone. Since publication of these results, a number of groups have confirmed the close linkage between GJ remodeling in the infarct border zone and the propensity of ventricles to develop reentrant arrhythmias [15,31,53–59]. Typical of infarct border zone remodeling is a loss of GJs from the intercalated disk and redistribution of Cx43 to lateral domains of sarcolemma: an arrangement reminiscent of the developing ventricle.

With the parallels between disease and development being raised, an important distinction should be made. Cx43 shows homogenously lateralized distributions between cells throughout the myocardial tissues of the immature ventricle. By contrast, the lateralization of Cx43 at the border zone of healed coronary infarcts in an adult heart is an isolated and striking non-uniformity. Just a few cell layers away from the border zone, Cx43 GJs can be seen to assume normal distributions at intercalated disks. The remodeling of GJs in the infarct border zone to lateralized distributions occurs relatively rapidly, becoming notable within 24 h [60]. The extent to which Cx43 along myocytes sides exists as GJs or connexons, or indeed whether lateralized Cx43 has channel function remains unclear.

The GJ is not the only element of the intercalated disk to show progressive change over postnatal ventricular development. Adherens junctions and desmosomal mechanical junctions also change from lateral, side-by-side contacts between cardiomyocytes in the immature ventricle to preferential accumulation at disks in the adult [47,61]. The accumulation of GJs to the intercalated disk notably lags behind mechanical junctions, which assume mature localization at cardiomyocyte termini significantly earlier during postnatal development [61–63]. The important concept that intercalated disk assembly is a sequential process will be discussed further in relation to differing hypotheses regarding how GJs may be targeted to the intercalated disk.

3. Structure and function of the Cx43 carboxyl-terminus

The main regulatory locus of Cx43 is the CT. While the CT sequence distal to amino acid 258 appears dispensable for polarization to cardiomyocyte termini [64], the Cx43 CT is required for proper organization in the intercalated disk, as well as regulating the channel responses to various chemical stimuli. Sorgen and others reported that the CT of Cx43 is predominantly a random coil composed of 132 amino acids containing two helical domains and extending from the cytoplasmic face of the membrane-docked channel [65] and in Figs. 1 and 2. This group further demonstrated that binding partners of the Cx43 CT could significantly alter its secondary structure. Importantly, these structural alterations were observed at sites remote to the

region of binding. This suggested that the Cx43 CT was not simply a site of protein–protein interaction, but a platform through which connexin-binding partners could interact and signal using this sequence as an intermediary.

Proteins interacting with the Cx43 CT have been previously reviewed by Giepmans in 2004 [66] and Herve et al. in 2007 [2]. An updated survey of proteins interacting with the Cx43 CT is presented in Fig. 2 and Table 1. The Cx43 CT–protein interactions of particular note with respect to GJ channel assembly, regulation and trafficking are given particular attention. Fig. 2 shows the Cx43 CT sequence from amino acid 234 to 382, with the known sites of interaction of several Cx43 binding partners labeled. The consequence of CT-protein interaction is additionally noted.

Table 1 lists the Cx43 CT binding partners shown in Fig. 2, with additional information on site and functional consequences of interaction. The table is organized in order of interaction site from juxta-membrane to the distal terminus. Fig. 2 and Table 1 will serve as a reference for the discussion of the CT binding partners that follows. In the following sections we will address the role of these interactions in regulation of Cx43 GJ organization. Extra attention will be given to ZO-1, one of the most widely studied binding partners on the Cx43 CT.

In addition to participating in intermolecular interactions, the Cx43 CT has been reported to dimerize under acidic conditions in vitro [67]. This ability to dimerize suggests a potential for intramolecular interactions in which a Cx43 CT may be capable of interacting with itself or another domain of Cx43. The CT was proposed to regulate GJ channels via a "ball and chain" mechanism [68] and intramolecular interactions of this type appear to be involved in pH sensitive gating [69,70]. Mimetic peptides based on the CT have also been shown to interact with the cytoplasmic loop (CL) domain of Cx43 and effect hemichannel function in a Xenopus oocyte model [71]. Some of the ODDD mutations may inhibit the Cx43 CT–CL intramolecular interaction [8]. Presently, it is unclear whether Cx43 CT intramolecular interactions contribute to GJ size control or cellular distribution.

4. The role of ZO-1 interaction with Cx43 in regulation of GJ organization

A key step in understanding control of GJ organization in the heart came with the identification of the interaction of Cx43 with ZO-1 [72,73]. While there are numerous Cx43 CT binding partners, many of which may be involved in regulating GJ size and cellular distribution, few other interacting proteins have been studied as extensively as ZO-1 [11,74–79].

The second Postsynaptic density/Disks large/ZO-1 (PDZ) domain of ZO-1 interacts directly with the distal extremity of the Cx43 CT [72,73]. Originally discovered in association with tight junctions [80], ZO-1 is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins that function in protein targeting, signal transduction, and determination of cell polarity [81,82]. MAGUKs, such as ZO-1, synaptic protein PSD95 and the *Drosophila* tumor suppressor disks large (dlg) are characterized by amino-terminal protein–protein binding motifs, including one or more PDZ domains.

Initial immunoprecipitation and yeast 2-hybrid studies showed that a 4 amino acid PDZ-binding ligand at the extreme CT of Cx43 was necessary for interaction with the second of three PDZ domains on ZO-1 [72,73]. Ongoing work has indicated that the ZO-1-binding domain comprises up to the CT-most 19 amino acids of Cx43 and this sequence likely interacts with a ZO-1 dimer [83,84]. Structural studies have demonstrated roles for ZO-1 binding in regulating Cx43 CT structural confirmation and signaling [65]. It is also now known that numerous connexins interact with ZO-1 via a CT PDZ-binding sequence similar to that of Cx43 [83,85–89]. A second MAGUK, ZO-2, has been reported to interact with Cx43 in a cell-cycle dependent manner [90].

Table 1
Proteins interacting with the Cx43 carboxyl-terminus. Note: While some studies have clearly identified certain serines and tyrosines as targets of specific kinases in vivo, it is apparent that multiple kinases can phosphorylate the same serine residue under different conditions. In vivo it is difficult to determine if a specific kinase is mediating the phosphorylation event directly or via activation of another kinase. UKS = unknown significance.

Interacting protein	Amino acids	Consequence	Reference
β-tubulin	234–262	Trafficking and TGFβ signaling	[140,141,144]
CaMKII	S244	Phosphorylation, UKS	[175]
Src SH2	Y247	Phosphorylation (disrupts communication)	[96,176]
MAPK/CaMKII	S255	Phosphorylation, UKS	[175,177,178]
CIP85	P253–P256 (*rat Cx43)	Induce GJ turnover	[179]
CaMKII	S257	Phosphorylation, UKS	[175]
PKC	S262	Phosphorylation, (reduces DNA synthesis)	[180,170]
Src SH2	Y265	Phosphorylation (disrupts communication)	[181,182]
V-Src SH3	274–287	SRC-Cx43 putative binding	[183]
MAPK	Y274 S279 S282	Phosphorylation (disrupts communication)	[178]
Nedd4	282–286	ubiquitination	[184]
CaMKII	S296, S297, S306, S314,	Phosphorylation, UKS	[175]
CK1, CaMKII	S325 S328, S330	Phosphorylation Enhanced GJ assembly	[175,185,186]
MMP-7	A357-I358	Potentially generate free carboxy-terminal peptides?	[173]
PKA, CaMKII	S364	Enhances channel assembly	[165,175]
PKA CaMKII	S365	Phosphorylation gatekeeper: must be dephosphorylated for PKC to phosphorylate S368	[175,187]
PKC PKA	S368	Phosphorylation (disrupts communication); Stabilizes GJs at Intercalated disks; Decreased assembly during S-phase	[163,169,188,189]
PKA, CaMKII	S369	Increased Channel formation/activity	[189]
PKC	S372	Phosphorylation, UKS	[190,191]
PKA, 14-3-3	S373	Phosphorylation required for 14-3-3 binding	[192]
MMP-7	D379-L380	Potentially cleave PDZ binding domain?	[173]
ZO-1-PDZ2	379–382	Reduced GJ size	[72,100]
Proteins with uncharacterized binding sites			
NOV/CCN3	Between 257 and 374	Reduced cell proliferation	[193]
Caveolin-1/-2/-3	Binds whole C-terminus but not D244 mutant	Indirect regulation of channel activity	[194,195]
Drebrin	Unknown	Cytoskeletal anchor	[196]

4.1. ZO-1 interaction with Cx43 and GJ internalization

When the interaction between Cx43 and ZO-1 was first identified in the late 1990s, it was proposed that its function was to stabilize GJs in the plasma membrane. However, subsequent findings by a number of groups, suggested that the view of ZO-1 as a passive scaffold at the GJ was inadequate. Notably, ZO-1 has an actin-binding domain at its CT, enabling linkage of GJs to cytoskeletal dynamics [91]. Consistent with this ability to mediate linkage to actin, ongoing reports indicate that the interaction between Cx43 and ZO-1 encompasses active functions, including in GJ remodeling [74,92–103].

In the early 2000s, we published results showing that Cx43/ZO-1 interactions may be involved in the internalization of GJs [77,92]. The approach was to demonstrate that ZO-1 and Cx43 association showed an acute and conspicuous increase following collagenase dispersal of cardiomyocytes from intact ventricles. Severs and others demonstrated in an earlier study that cardiomyocyte dissociation prompted mass endocytosis of GJs, forming a stable population of vesicular annular junctions within the cytoplasm [104]. We hypothesized in the 2002 study that a function of ZO-1 may have been to link Cx43 to actin-based motors, and that this contributed to the rapid internalization of GJs when cardiomyocytes were disaggregated from intact ventricular muscle by collagenase.

Integral to this discussion is the parallel story of Cx43/Src interaction. Src was identified as a potent inhibitor of Cx43 GJ communication in 1990 [105,106]. Tyrosine phosphorylation was found to be a hallmark of Src-mediated Cx43 inhibition, and it was demonstrated that Src phosphorylates the Cx43 CT on residues Y247 and Y265 [107]. Saliently, Toyofuku and co-workers later determined that phosphorylation mediated by Src played a role in GJ communication deficits in the cardiomyopathic hamster heart [108]. The same group subsequently demonstrated that Src binding and phosphorylation are mutually exclusive to ZO-1 interaction with Cx43 [97]. In a final twist, recent data has indicated that Src decreased interaction between Cx43 and ZO-1 by competitively binding to ZO-1 in the infarct border zone of a canine coronary ligation model [109], with an associated loss of Cx43 from the intercalated disk.

The above findings on Cx43, Src and ZO-1 indicated the occurrence of complex interplay between the three proteins. Studies by Pointis, Segretain and co-workers have provided further insight into this set of interactions [74]. The aforementioned laboratories undertook their work on association between Cx43 and ZO-1 during GJ internalization in Sertoli and other cell lines [74]. In 2008, this group made the observation that during a chemically induced GJ endocytosis, ZO-1 dissociated from one side of the GJ, but remained associated with the other side of the plaque [98]. This loss of ZO-1 from a cytoplasmic face of the GJ appeared to occur in response to competition for binding to the Cx43 CT by Src. Asymmetric maintenance of ZO-1 on the other plaque face culminated in focal concentration of the actin-binding protein within the interior of endocytozed GJ vesicles. Interestingly, the co-localization pattern reported by this group, wherein ZO-1 signals appeared to be contained with larger Cx43 particles, was similar to that located at annular junctions in enzymatically-dispersed cardiomyocytes [92]. More recent data from the French group shows that internalization of annular junctions is dependent on the protein dynamin2, which they demonstrate to interact with Cx43 during plaque endocytosis [110].

Evidence that ZO-1 interaction with Cx43 is a mechanistically necessary participant in GJ endocytosis came from papers examining the response of Cx43 to inflammatory mediators. In 2007, van Zeijl et al. reported that ZO-1 was required for the down-regulation of GJ intercellular communication observed in response to endothelin-1 and thrombin-like agonists in Cx43-expressing Rat-1 cells [111]. In a subsequent paper from the Falk group, using endothelial and HeLa cells, it was demonstrated that thrombin and endothelin-1 inhibited intercellular coupling via inducing rapid and acute internalization of GJs [103]. Importantly, these authors showed that in the absence of competent ZO-1 interaction with Cx43, internalization of GJs and annular junction formation in response to treatment with the inflammatory agonists did not occur.

Taken together the evidence from these studies suggested that ZO-1 interaction with Cx43 is necessary for certain types of GJ internalization processes. However, it is widely recognized that certain GJ endocytotic events may not require ZO-1 [112,113]. In one instance, classical clathrin-based mechanisms efficiently operate to mediate internalization of GJs from the plasma membrane [112]. Furthermore, HeLa cells expressing Cx43 rendered incompetent to interact with ZO-1 by a CT GFP tag form large junctions without a significant change in Cx43 half-life, suggesting that in this context loss of the PDZ2 interaction does not increase GJ turnover [100,101]. In ongoing work, our group finds that ZO-1 binding to Cx43 CT can also direct actin-based force in the plane of the plasma membrane, mediating the fusion and pulling apart of GJ plaques [114].

Significant questions remain regarding the molecular motors that remodel Cx43 GJs. For example, in internalization events involving ZO-1, it remains unknown how actin forces are imparted on the GJ. Does it involve pulling or pushing of double-membrane GJs from the plasma membranes of adjoined cells? What are the other linkage and force generating constituents of these motors? Does the molecular machinery that operates constitutively during GJ turnover differ from that which operates during acute events such as in response to ischemia, mechanical destabilization of cell–cell contacts or inflammatory cytokines?

4.2. ZO-1 interaction with Cx43 and regulation of GJ size: in vitro studies

Insight into a second role for ZO-1 at the GJ in the control of plaque size was initiated from studies of Cx43–ZO-1 co-localization. In a 2002 paper we noted that the level of ZO-1 association with Cx43 in intact ventricular myocardium was modest, with some GJ plaques at intercalated disks demonstrating no detectable ZO-1 [92]. Immuno-confocal microscopy of cultured neonatal rat ventricular cardiomyocytes provided greater clarity of the association between the two proteins than could be achieved in tissue sections of cardiac muscle [115]. Confocal optical sectioning of monolayers of cardiomyocytes revealed that ZO-1 preferentially localized at the edge of GJ plaques.

Having observed this peripheral accumulation of ZO-1 at the GJ edge, the question arose as to what would happen if this association was inhibited. We determined that treatment of neonatal rat cardiomyocytes with a peptide mimetic of the Cx43 ZO-1-binding domain decreased co-localization of ZO-1 at the edge of GJs and increased plaque size [101]. This result was interpreted as implying that ZO-1 interaction with Cx43 at the GJ edge may be acting to constrain the extent of GJ channel aggregation.

Consistent with a role in inhibiting plaque size growth, it was also noted that abnormally large GJs formed between HeLa cells heterologously expressing a Cx43-GFP construct incompetent to interact with ZO-1 [79] These large junctions were reduced to more normal sizes when the cells were transduced by adenovirus expressing wildtype Cx43 [100,101]. Subsequent work by Andrew Hunter in the lab established that the Cx43-GFP GJs also had deficits of ZO-1 at the plaque edge, and that the reduction in plaque size caused by co-expression of wildtype Cx43 was accompanied by recruitment of ZO-1 to the edge of GJs [101].

It was also determined that the GJ size increase prompted by the Cx43 CT peptide, did so via inhibiting the interaction of the second PDZ domain of ZO-1 with Cx43 [101]. The reduction in interaction mediated by the PDZ2-binding peptide in turn resulted in a shift within the total pool of Cx43 protein in cells from detergent-soluble to GJ-associated detergent-insoluble pools. Taken together, the results indicated that ZO-1 controlled the rate at which Cx43 transitioned from non-junctional to junctional pools of the protein [101].

In ongoing studies the question of how and where ZO-1 may perform this function has been addressed [95]. The approach taken was to use in situ protein–protein interaction assays to further define the relationship between Cx43 and ZO-1 in a HeLa cell model [95]. Using the Duo-link assay to fluorescently amplify Cx43 ZO-1 association, a previously unknown zone of interaction proximal to the GJ edge was identified. This sector, which we termed the *Perinexus*, appears to be the primary site at which control of the transition of non-junctional connexons into the junctional plaque occurs.

Based on the data reported, it was proposed that the mechanism of ZO-1-mediated control of GJ size is via governing the connexon hemichannel to GJ channel transition from the perinexus [95]. The perinexal scaffold is envisaged as a sieve-like network containing ZO-1 that extends from the GJ edge. This network is part of a mechanism that regulates, but does not prevent, the flow of connexons from surrounding membrane available to dock with connexons from the apposed cell (Fig. 1). In this manner, it was hypothesized that ZO-1 regulates a balance between connexon-mediated membrane permeance and GJ intercellular communication. Indeed, it was demonstrated that the level of Cx43/ZO-1 interaction concomitantly and inversely affected hemichannel-mediated dye uptake and GJ-mediated cell-to-cell dye transfer [95].

The model of connexon flow from the perinexus is consistent with the widely held view that GJs are assembled from the outside edge of the plaque inwards [116,117]. This was most notably illustrated in the elegant study of Gaietta et al., in which pulse-chase labeling of tetracysteine-tagged Cx43 with biarsenical compounds showed that Cx43 accreted to the edge of GJs [117]. The new insight provided by the Duolink assay is that the recruitment process draws from a pool of non-junctional connexons located proximal to the GJ edge in the perinexus [95].

The study also provided unexpected new data on the association between actin, ZO-1 and Cx43 at GJs. Co-immunoprecipitation demonstrated that ZO-1 interacted with both detergent soluble and insoluble Cx43-containing connexons, but only those connexons docked in the detergent insoluble fraction were also complexed with actin [95]. These data correlated well with immunofluorescenent data showing preferential overlap of actin signal with Cx43/ZO-1 interaction within the GJ as compared to the perinexus. At present, how this partitioning of Cx43 interaction with the actin cytoskeleton is regulated is unclear. Nonetheless, the linkage of actin to connexons in the GJs, but less avid association with connexons in the perinexus, may provide one explanation as to how ZO-1 appears to have dual functions: One in the transmission of force to the GJ via tethering to the actin cytoskeleton and the other in controlling the flow of connexons at the plaque perimeter.

One key question for ongoing work is the specifics of how perinexal ZO-1 acts to regulate connexon recruitment. Another outstanding issue is whether other connexins that interact with ZO-1, such as Cx40 [89] and Cx45 [86,118], exhibit mechanisms of GJ size control similar to those of Cx43. There are also interesting related questions on whether heteromeric and heterotypic partnering of connexins affect control of GJ aggregation. In an example from the literature, coexpression of Cx43 with Cx45 in HeLa cells has been reported to decrease GJ size [119]. Characterization of CT interactions in cell models expressing and/or co-expressing different connexin isoforms may provide further insight into the role of perinexus in regulating GJ organization.

4.3. ZO-1 interaction with Cx43 and regulation of GJ size: in vivo studies

There is growing evidence that ZO-1 contributes to regulation of GJ size in vivo. Delmar and co-workers determined that expression of a CT-truncated Cx43 incompetent to interact directly with ZO-1 resulted in formation of abnormally large GJs in lateral sarcolemma adjacent to the intercalated disk [64]. In patients with certain types of cardiomyopathy, increased ZO-1 interaction with Cx43 was reported to prompt attendant decreases in the abundance and size of GJs [11].

Recent studies from our lab have also elucidated how patterns of ZO-1 interaction with Cx43 may generate the characteristically large GJs ringing the intercalated disk. GJs at the periphery of the disks in the rat ventricle were found to exhibit greater ZO-1 colocalization at their inward, as compared to their outward facing edges [120]. Disruption of this asymmetric co-localization by expression of a ZO-1 truncation mutant resulted in loss of size distinction between GJs at the peripheral edge of the intercalated disk versus those in the disk interior.

Together with in vitro studies [95,101], it was proposed that the larger size of GJs at the intercalated disk periphery resulted from preferential accretion of connexons at the outward facing edge (i.e., ZO-1-lacking) of GJ plaques. Therefore, when connexon recruitment is not regulated by the presence of a perinexal ZO-1 network, such as in the case of GJs at the intercalated disk periphery [120] or between Cx43-GFP expressing HeLa cells [100], plaques tend to grow larger.

4.4. ZO-1 interaction with Cx43: GJ size and Cx43 level in cardiac disease

While spatial remodeling of GJs is clearly associated with arrhythmic disease, the functional significance of changes in GJ size to pathologic disturbance of conduction in the heart is less clear. Maass and co-workers demonstrated that an increase in GJ size from CT truncation of Cx43 was correlated with a propensity of acutely injured hearts to develop inducible arrhythmias [121]. Whether this effect is due to changes in GJ size or other phenomena associated with CT truncation – such as changes in junctional conductance – remain unclear. Conversely, Nick Severs group showed a decrease in GJ size in ischemic and dilated cardiomyopathies [11]. Interestingly, in some forms of severe epilepsy in humans, decreases in Cx43 GJ size have been reported to accompany an increase in overall GJ area and expression [122]. As is the case with cardiac arrhythmia, epilepsy is characterized by the propagation of chaotic electrical activity.

In 2007, Yamada and co-workers found that ZO-1 and Cx43 levels were reduced concomitant with a reduction in co-localization between these two proteins at intercalated disks in humans at end-stage heart failure [123]. Interestingly, although Cx43 levels were diminished, the percentage of disks positive for Cx43 was not different from that of controls. In a short report, Kostin described a similar diminution in ZO-1 in heart failure patients, but noted that Cx43 was decreased in intercalated disks in which ZO-1 was also reduced [124]. Related analyses were performed in 2007 on a rat model of right ventricular hypertrophy. This study found increased GJ internalization and some lateralization of Cx43, but in apparent contrast with the aforementioned papers, no change in total Cx43 levels or the co-distribution of Cx43/ZO-1 in diseased hearts [125].

As touched upon in the first paragraph of this section, Severs' group in London performed an in-depth study on ventricular tissues sampled from human patients in end-stage heart failure [11]. In line with earlier results, these workers found that the abundance of Cx43 was significantly reduced in patients compared to controls subjects. Moreover, ZO-1 levels were increased at the intercalated disks in these individuals, and ZO-1 levels showed a negative correlation with Cx43 levels at disks. Furthermore, co-localization and co-immunoprecipitation assays indicated that interaction between Cx43 and ZO-1 was increased in these heart failure patients.

Recent data from the laboratories of Sorgen and Duffy on the border zone of infarcted myocardium in a canine model of coronary occlusion found that reductions in Cx43 level were associated with decreases in Cx43/ZO-1 binding [109]. A novel interaction between activated Src and ZO-1 showed a complementary increase in infarct border zone tissues. Finally, in a report published in 2010, a rat model of pressure-overload hypertrophy over an 8-week period was described in which Cx43 levels initially increased, then declined back to control levels by 8 weeks [126].

A similar variation over time was observed for Cx43 phosphorylated at serine 368 on its primary sequence. ZO-1 co-immunoprecipitation with Cx43 remained unchanged 2 weeks after pressure overload, but declined slightly at 8 weeks.

In sum, the literature on relationships between Cx43/ZO-1 interaction, GJ size, and Cx43 level in diseased myocardium is not amenable to simple interpretation. Further reports of Cx43 remodeling in the diseased ventricle include those by Goldfine et al. and Benes et al. [127,128]. The different conclusions reached by different groups may be grounded in part in the varying models that have been used. These include two rodent models of hypertrophy, a canine ischemia model, and samples from human patients at various stages of heart failure with differing disease etiologies. Lining up assays of specific microscopy and biochemically based end-points with specific cardiac diseases and disease stages may also be key considerations in uncovering mechanism. Further work in this important area is evidently warranted.

5. GJ targeting to the intercalated disk

How GJs come to be targeted to and retained in the intercalated disk is presently unknown. This being said, two main hypotheses have been proposed to explain how GJs assume polarized distributions at cardiomyocyte termini. The first hypothesis posits that polarization results from intercalated disks having lower rates of loss or turnover of GJs than other parts of the sarcolemma [61,63,129]. The second hypothesis proposes that order is generated by direct trafficking of Cx43 to the intercalated disk via cytoskeletal pathways — most notably via Cx43 CT interaction with microtubules. The two propositions are not mutually exclusive and indeed, when eventually understood, the actual mechanism may embody aspects of both.

Axiomatic to the first hypothesis, is that Ca^{2+}-dependent adhesion junctions (adherens junctions and desmosomes) provide mechanically stable domains of sarcolemma, within which GJs are less rapidly turned over. The intercalated disk contains high concentrations of adherens junctions and desmosomes. As such, in this model GJs would tend to be preserved in or near disks in the active tissues of the beating heart. Conversely, GJs in lateral domains of sarcolemma would be mechanically destabilized at higher rates, being more susceptible to shearing stress at lateral interfaces between contracting muscle cells.

The GJ turnover model was first proposed based on descriptive studies of postnatal maturation of the rat and dog heart [61]. During cardiac maturational growth it was observed that the distribution of GJs displayed a transient dissociation from those regions of sarcolemma exhibiting the greatest concentrations of cell–cell adhesion junctions. This lack of GJ-mechanical junction association resulted from GJs accruing from lateral sarcolemma to the intercalated disk more slowly than the bulk of adherens junctions and desmosomal contacts between cells (Fig. 3). In rats, the majority of both classes of adhesion junctions (i.e., >60%) become polarized to the intercalated disk between 10 and 20 days following birth [61]. By contrast, the rodent is full grown at 90 postnatal days before the majority of Cx43 is lost from myocyte sides and immunolabeling in the disk matches that of adherens or desmosomal junctions.

Peters et al., found that humans display a similarly extended time course of intercalated disk formation, with lateralized GJs still apparent in children up to 6 years of age [43]. The multi-year time course for establishing polarized distributions of GJs is orders of magnitude slower than the GJ trafficking (<5 min) kinetics of Cx43. Additionally, these data suggest that over extended periods of time (e.g., such as that occurring over mammalian postnatal development), small deviations in turnover of plaques between disk and lateral sarcolemma could generate large differences in GJ abundance between these domains of membrane.

Electron microscopy of ventricle from normal adult mammals has indicated that internalized or annular GJs are extremely rare [23,125]. However, a conspicuous increase in the frequency of annular GJs has been described from ultrastructural studies of immature human [43], rabbit [130], and dog [131] ventricle. Importantly, Mühlfeld and Richter found that annular GJ numbers increased sharply between birth, 5 and 14 postnatal days in the rat [132]. The timing of this postnatal uptick in internalization would be consistent with GJ stability being dependent on proximity to mechanical junctions, as it coincides with the developmental phase in this species within which the sarcolemmal distribution of adhesion junctions shows greatest dissociation from that of GJs [61].

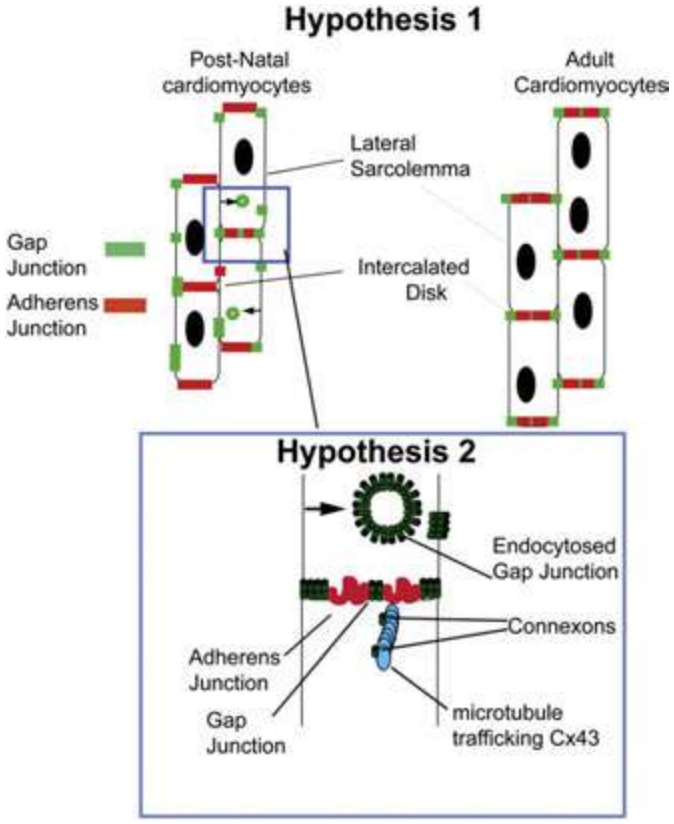

Fig. 3. Two hypotheses for gap junction recruitment to intercalated disks. 1) Following birth, mechanical junctions in the ventricle, including adherens junctions (red), undergo rapid polarization to intercalated disks, while the emergence of polarized distributions of Cx43 GJs (green) from lateral sarcolemma progresses on a slower time course. In Angst et al. [61] it was proposed that this pattern emerges as GJs tend to be internalized (open green circles) from lateral domains of sarcolemma during postnatal maturation, whereas those GJs co-located with adherens junctions at disks are more liable to be retained in the membrane. 2) Microtubules traffic connexon hemichannels directly to the Adherens junctions at the intercalated disk for direct assimilation into GJ plaques [142].

Work in vitro and in vivo provides further support for the dependence of GJ spatial distribution on that of adhesion junctions between ventricular myocardial cells. In a study using aligned cultures of neonatal rat ventricular cardiomyocytes, Matsuda et al. recapitulated the maturational dissociation of GJs and adherens junctions, showing that polarization of Cx43 to cell termini was preceded by and required the localization of N-cadherin at intercalated disks [133]. Consistent with biomechanics having a determinative role in the differentiation of the intercalated disk, levels of expression and initial localization of N-cadherin to cardiomyocyte termini have been reported to be dependent on the configuration of active mechanical stress and strain [134–138].

Direct evidence of the requirement for stable cell–cell adhesion junctions in GJ stability has been provided in experiments in which a dominant negative N-cadherin was expressed in cultured cardiomyocytes. This mutant N-cadherin prompted loss of Cx43 from the sarcolemma and disruption of GJ contacts between cardiomyocyte pairs [139]. In line with these in vitro studies, Luo and Radice showed that cardiac-specific knockout of the N-cadherin gene in transgenic mice

resulted in reductions of Cx43 GJs [140]. Our group has recently reported that expression of a dominant negative disruptor of adherens junctions in the postnatal rat ventricle leads to increases in lateralized GJs and complementary decreases in Cx43 at intercalated disks [120] — results consistent with adhesion junctions being necessary for establishing and maintaining normal GJ organization.

In the second hypothesis, targeting of Cx43 GJs to the intercalated disk is proposed to involve trafficking via microtubules. Falk, Giepmans and colleagues demonstrated that the CT of Cx43 could directly interact with β-tubulin and based on this interaction, it was proposed that microtubules trafficked connexins directly to the GJ [141]. Subsequent work provided evidence that Cx43 targeting to the plasma membrane involved delivery of connexons to N-cadherin complexes at the plus ends of microtubules [142]. In this respect it is notable that N-cadherin, β-catenin and Cx43 have been reported to co-immunoprecipitate as part of a multi-protein complex [143].

Intercalated disks are enriched with N-cadherin — the Ca^{2+} dependent cell adhesion molecule found in cardiac adherens junctions. It therefore follows that Cx43 should accumulate preferentially at sites of N-cadherin enrichment where microtubules tether, such as the intercalated disk. Further support for this trafficking-based model has come from more recent work from Shaw's group in which it was shown that both Cx43 and the microtubule binding protein EB1 were decreased at the intercalated disk of patients with ischemic cardiomyopathy [144]. This observation was associated with a decreased localization of microtubules at adherens junctions and decreased Cx43 trafficking to the disk.

Experiments utilizing the expression of a truncated Cx43 raise some considerations for interpreting the role of microtubules in trafficking of connexins to GJs. It has been reported that amino acids 234 to 262 of the Cx43 CT contain the necessary and sufficient sequences to mediate interaction with β-tubulin [141,145]. As outlined previously in this review, a CT-truncation of Cx43 has been expressed in transgenic mice [64]. This CT mutant was truncated from amino acid 258 and presumably able to interact with microtubules normally, as it retained a consensus microtubule-binding domain. The Cx43 mutant protein accumulated at cardiomyocyte termini and formed functional GJs in this transgenic. However, the structure of intercalated disks of mice expressing the truncated Cx43 was disturbed, with GJs being located in domains of sarcolemma lateral to, but not within the body of the disk proper. Moreover, the GJs that formed in these domains were unusually large and showed abnormal associations with ZO-1, N-cadherin and desmosomal proteins.

Taken together, the data suggest that microtubule trafficking may be necessary for general localization of Cx43 at adherens junction-rich regions of sarcolemma. However, these cytoskeletal elements may not be sufficient to maintain Cx43 distribution at the intercalated disk long-term. Additional microtubule independent CT-interactions, or at least Cx43 CT-interactions distal to the presumptive microtubule-binding domain may be required for normal GJ-adherens junction configuration within the disk.

Moreover, consonant with the first hypothesis, numerous lines of evidence support the concept of a hierarchical dependence of GJ pattern and stability on the formation and distribution of adherens junctions. The role of this relationship in establishing the cellular pattern GJ connections may be particularly important in early postnatal life when adherens junctions and desmosomes undergo their rapid polarization to cardiomyocyte termini in response to changes in mechanical activity following birth. This hierarchy is probably also evolutionarily conserved, or at least been converged upon by other species, as cadherin junctions have been reported necessary for membrane retention of innexins, the genetically unrelated GJ proteins of invertebrates [146].

Proper localization of cardiac GJs in the physiologic state appears to be ultimately determined by Ca^{2+}-dependent adhesion junctions, independent of whether GJ stability or targeting by microtubules individually or jointly determine the pattern. Subsequent regulation of GJs via protein–protein interaction and post-translational modification seems likely to be downstream of this requirement. A hypothetical model for how GJs assume localization at the intercalated disk that distils results from various studies is provided in Fig. 3.

Questions remain as to if, how and when the different processes hypothesized in the model might act. Given that targeting GJs to the intercalated disk occurs over the entire course of postnatal development, it is conceivable that separate mechanisms may contribute at differing phases or time scales. There are also specific outstanding matters of interest. For example, the molecular mechanism responsible for the spike in GJ internalization during early postnatal life remains to be characterized. Cardiac-specific expression in mouse of a Cx43 mutant protein missing the consensus CT microtubule-binding domain could provide useful insight into the role of trafficking in the differentiation of the intercalated disk.

6. GJ lateralization in cardiac disease

GJ order in the healthy myocardium and pathological disorder in the diseased ventricle appear to show similarities. As has already been broached, in disease GJs often assume lateral distributions that are independent of intercalated disks. The lateralization of Cx43 observed in many pathological scenarios is reminiscent of arrangements observed during maturational growth of the ventricle. This remodeling of GJ contacts in disease is thought responsible for heterogeneities in electrotonic coupling that generate tissue substrates for re-entrant arrhythmias.

In a further notable parallel to postnatal maturation, a number of studies of experimental and clinical cardiomyopathies have shown that Cx43 distribution dissociates from that of N-cadherin [147–151] or desmosomal proteins [152,153]. In this pathological recapitulation of developmental patterns in the adult, cell–cell adhesion junctions appear to remain prominently localized at the intercalated disk, whereas Cx43 distributes to lateral sarcolemma (Fig. 4). Also similar to the situation observed during postnatal growth, endocytozed GJs have been reported to demonstrate an increase in frequency in some of these pathologies [149,150,153].

Mutations of desmosomal proteins have been identified in a number of rare arrhythmic cardiomyopathies including arrhythmic right ventricular cardiomyopathy, Carvajal syndrome and Naxos disease [154–157]. Disruption of mechanical junctions in these syndromes has been noted to be associated with a loss and lateralization of Cx43 in ventricular muscle [158,159]. The fact that the normal order of the intercalated disk can also be disrupted by mutations in components of desmosomal junctions [160,161], reinforces the notion of a hierarchical dependence of GJ stability on the normal function of mechanical contacts between cardiomyocytes.

The kinases and phosphatases responsible for regulating Cx43 CT phosphorylation have important roles in determining GJ organization in the sarcolemma in health and disease [97,98,162–166]. These Cx43-interacting proteins, and the phosphorylation events that they mediate, have potential in the development of anti-arrhythmia therapies [167,168]. Phosphorylation of serine 372 and 373 destabilizes Cx43 CT binding to the ZO-1 PDZ2 domain [169], with presumptive downstream consequences for GJ size regulation. In another example with clinical implications, Fishman and colleagues identified a mechanistic link between casein kinase-phosphorylation of the Cx43 CT, and blunting of pathological GJ remodeling, in a pressure overload-induced model of cardiac hypertrophy [168].

Specific patterns of Cx43 phosphorylation appear to be associated with the conferring of resistance to pathological lateralization of GJs. Ek-Vitorin et al. reported that in response to transient ischemia, upregulation of PKC-epsilon mediated phosphorylation of Cx43 CT at serine 368 resulted in retention of subpopulations of GJs at intercalated disks, even as total Cx43 underwent remodeling to lateral cardiomyocyte surfaces [170]. Similarly, Kardami and co-workers demonstrated that ischemic-

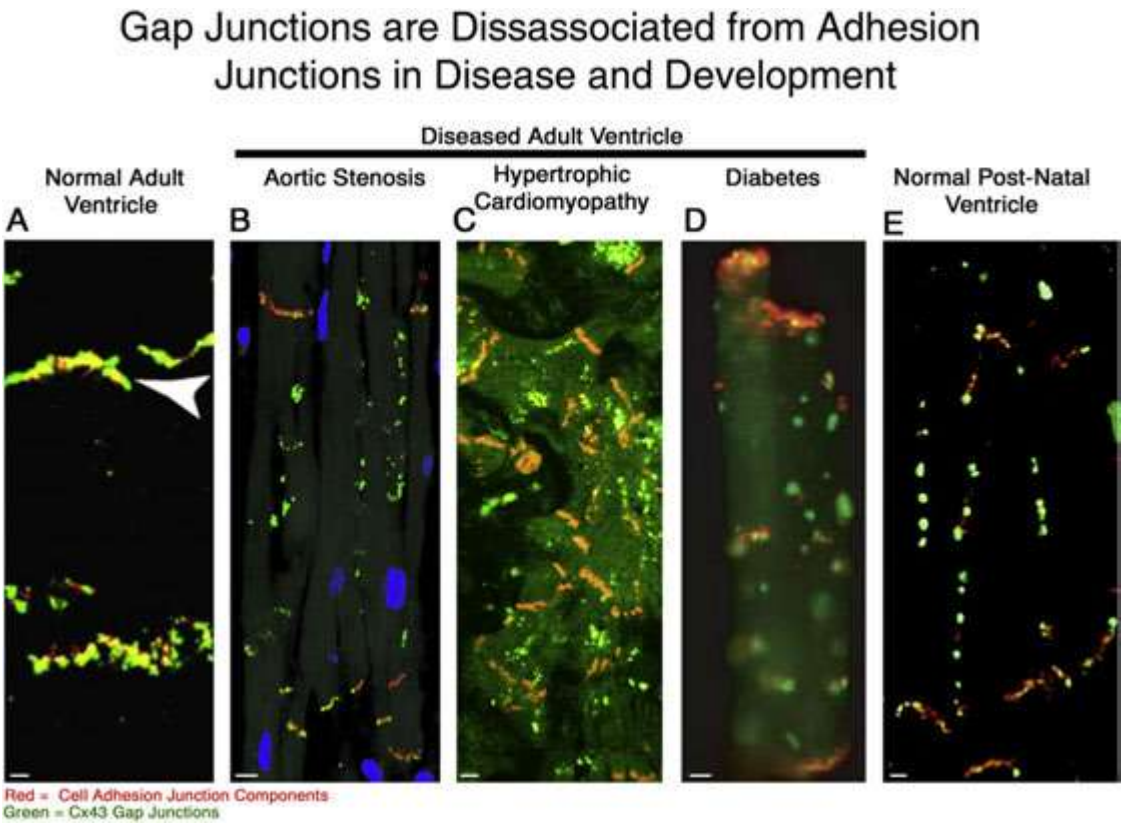

Fig. 4. Gap junctions and adhesion junctions in cardiac development and disease. Immunolabeling of Cx43 and cell–cell adhesion junctions in ventricular myocardium from various reports are shown from normal (undiseased) adult, diseased adult, and normal postnatal ventricle demonstrate how arrangements of intercellular junctions seen in the still developing postnatal heart are recapitulated in adult disease. While these figures have not been modified, the color tables have been adjusted such that Cx43 is consistently indicated by green signal and Ca^{2+}-dependent adhesion junctions are in red A. Normal Adult Human Left Ventricle from Smyth JW, Hong TT, Gao D, Vogan JM, Jensen BC, Fong TS, Simpson PC, Stainier DY, Chi NC, Shaw RM. Limited forward trafficking of connexin 43 reduces cell–cell coupling in stressed human and mouse myocardium. J Clin Invest, 120 (2010) 266–79 used by permision of the American Society for Clinical Investigation. B. S. Kostin, S. Dammer, S. Hein, W.P. Klovekorn, E.P. Bauer, J. Schaper, Connexin 43 expression and distribution in compensated and decompensated cardiac hypertrophy in patients with aortic stenosis, Cardiovasc Res, 62 (2004) 426–436 used by permision of Oxford University Press. C. Reproduced from Sepp R, Severs Nj, Gourdie Rg, Altered Patterns Of Cardiac Intercellular Junction Distribution In Hypertrophic Cardiomyopathy, Heart, 76 (1996) 412–417 with permission from BMJ publishing group. D. Reproduced from A. Nygren, M.L. Olson, K.Y. Chen, T. Emmett, G. Kargacin, Y. Shimoni, Propagation of the cardiac impulse in the diabetic rat heart: reduced conduction reserve, J Physiol, 580 (2007) 543–560. With permission from John, Wiley and Sons. E. Postnatal rat demonstrates uniform Cx43 distribution at the lateral membranes and the disk — taken from [61]. Note that in all cases of disease Cx43 is detected in lateral domains of sarcolemma. Scale bars A = 2.5 μm, B–E = 10 μm.

preconditioning increased PKC-epsilon-mediated Cx43 phosphorylation of serines at positions 262 and 368, and inhibited Cx43 lateralization following an ischemic insult [171]. Studies that examined cell-cycle regulation of Cx43 have shown that during S-phase GJ assembly is decreased and is correlated with increased phosphorylation of serine 368 [164]. Phosphorylation may also have effects on intramolecular interaction between the Cx43 CT and CL [71] and the targeting of Cx43 to the intercalated disk [64,168].

The Cx43 CT-interacting kinase PKC-ε also seems to have promise as a therapeutic target. Dhein and colleagues determined that a short peptide, AAP10, inhibited Cx43 dephosphorylation at the PKC-ε consensus serine 368 and remodeling of GJs away from intercalated disks following induction of ischemia in a rabbit model [172]. Our laboratory has demonstrated that a peptide mimetic of the Cx43 CT can activate PKC-ε phosphorylation in vitro. Treatment of cardiac injuries with this peptide in a mouse model resulted in increases in the levels of Cx43 at intercalated disks and consistent with experiments in vitro, prompted serine 368 phosphorylation of Cx43 [173].

Interestingly, Matrix metalloproteinase-7 (MMP-7) has been shown capable of cleaving peptides from the extreme CT of Cx43 [174] (Fig. 2). It is unclear at this point if this can occur physiologically, as MMP-7 is canonically extracellular whereas the Cx43 CT in cytoplasmic. Nonetheless, this interesting result points to the possibility that endogenous Cx43 may itself be a source of bioactive cleavage products. Given the data on the actions of synthetic peptides based on the Cx43 CT [95,101,173,175], any endogenously generated peptides of related sequence would have the potential to regulate GJ size and configuration in the sarcolemma via modulation of PKC-ε, ZO-1 and other interacting proteins.

7. Concluding remarks

The Cx43 CT, through its protein–protein interactions and post-translational modifications, plays a direct role in regulating Cx43 GJ size, localization and sub-organization at the intercalated disk in the developing and adult ventricle. The proper arrangement of Cx43 GJs at the disk is critical to the maintenance of a stable cardiac rhythm and further understanding of Cx43 CT interactions could provide new paths for therapies that prevent arrhythmia and sudden cardiac death. Exciting future work would usefully focus on further elucidating the processes that result in perturbations of Cx43 localization and function in disease. By identifying the network of Cx43-binding partners, and understanding their dynamic patterns of interaction, a fuller understanding of the molecular determinants of pathological GJ remodeling will emerge.

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