

Xin proteins and intercalated disc maturation, signaling and diseases

Qinchuan Wang¹, Jenny Li-Chun Lin¹, Kuo-Ho Wu², Da-Zhi Wang³, Rebecca S. Reiter¹, Haley W. Sinn¹, Cheng-I Lin², Jim Jung-Ching Lin¹

¹Department of Biology, University of Iowa, Iowa City, IA 52242 USA; ²Institute of Physiology, National Defense Medical Center, Taipei, Taiwan, ROC; ³Department of Cardiology, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115 USA

TABLE of CONTENTS

1. Abstract

2. Introduction

2.1. Advances in the anatomy of intercalated disc (ICD)

2.1.1. Area composita (mixed type of junctions) exist in mammalian ICDs

2.1.2. Defective adhering junctions generally lead to gap junction remodeling

2.1.3. Linkers involved in molecular crosstalk among different junctions of ICDs

2.1.3.1. Plakoglobin (γ -catenin)

2.1.3.2. Plakophilin 2 (PKP2)

2.1.3.3. p0071 (sometimes referred to as PKP4)

2.1.3.4. p120-catenin

2.1.3.5. ZO-1 (zonula occludens-1)

2.1.4. Transitional junction near ICD

2.2. The involvement of ICD in signaling

2.2.1. N-cadherin/ β -catenin signaling in the heart

2.2.2. Wnt/ β -catenin signaling in the heart

2.2.3. Interplay between Wnt/ β -catenin and adhering junction-mediated signaling

2.2.4. ICD influences ion channel surface expression

2.3. Discovery of Xin repeat-containing and ICD-associated protein family

3. Expression, domain structures and function of Xin

3.1. Xin is a striated muscle-restricted gene and a downstream target of Nkx2.5 and Mef2

3.2. Two phases of the Xin up-regulation during development correlate with chamber/valve formation and postnatal heart growth

3.3. Xin expression is significantly up-regulated in animal models of cardiac hypertrophy and hypertension

3.4. The origin of Xin coincides with the first appearance of true heart chamber, and mXin β is phylogenetically closer to ancestral Xin protein than mXin α

3.5. mXin β messages are preferentially localized to ICDs and enriched in cells of the left ventricle, interventricular septum and apex, particularly concentrated at the base of aorta and pulmonary artery of the heart

3.6. Domain structures of Xin proteins

3.7. ICDs are formed postnatally

3.7.1. Redistribution of junctional components during ICD formation

3.7.1.1. Adherens junctions

3.7.1.2. Desmosomes

3.7.1.3. Gap junctions

3.7.2. Molecular mechanism of ICD formation

3.7.3. Roles of mXin β in ICD formation

3.7.3.1. mXin β is required for ICD formation

3.7.3.2. Potential mechanism by which mXin β mediates ICD formation

3.8. mXin plays important roles in the surface expression of ion channels

4. Xin and cardiac diseases

5. Conclusions and Perspectives

6. Acknowledgement

7. References

1. ABSTRACT

Intercalated discs (ICDs) are cardiac-specific structures responsible for mechanical and electrical communication among adjacent cardiomyocytes and are

implicated in signal transduction. The striated muscle-specific Xin repeat-containing proteins localize to ICDs and play critical roles in ICD formation and cardiac function. Knocking down the *Xin* gene in chicken embryos collapses the wall of developing heart chambers and leads

to abnormal cardiac morphogenesis. In mammals, a pair of paralogous genes, *Xinalpha* and *Xinbeta* exist. Ablation of the mouse *Xinalpha* (*mXinalpha*) does not affect heart development. Instead, *mXinalpha*-deficient mice show adult late-onset cardiac hypertrophy and cardiomyopathy with conduction defects. The *mXinalpha*-deficient hearts up-regulate mouse *Xinbeta* (*mXinbeta*), suggesting a partial compensatory role of *mXinbeta*. Complete loss of *mXinbeta*, however, leads to failure of forming ICD, mis-localization of *mXinalpha*, and early postnatal lethality. In this review, we will briefly discuss recent advances in the anatomy and function of ICDs. We will then review what we know about Xin repeat-containing proteins and how this protein family promotes ICD maturation and stability for normal cardiac function.

2. INTRODUCTION

Intercalated discs (ICDs) are essential structures unique to cardiac muscle (1-4); they enable mechanical coupling and chemical communications among adjacent cardiomyocytes to achieve regulated contraction essential for cardiac function. Recent evidence also points to the involvement of ICD components in transducing signals important for cardiac remodeling in either the healthy or diseased state (5-10). The structure of the ICD and its function, deduced from electron microscopic studies, have been comprehensively summarized in a seminal review paper by Forbes and Sperelakis (1). In this classical description, the function of ICDs was assigned to three types of intercellular junctions: (i) gap junctions are responsible for electrical and chemical communications between cardiomyocytes; (ii) adherens junctions (*fasciae adhaerentes*) connect the myofibrils from neighboring cardiomyocytes, thus transmitting the contractile force; and (iii) desmosomes (*maculae adhaerentes*) anchor the desmin intermediate filament to provide mechanical strength to the ICDs. This classic view of the structure and function of ICDs has been supported by recent studies that employ genetic, biochemical, physiological and cell biological approaches in several animal models and cardiac diseases. In general, mutations or deficiencies in ICD components give rise to many types of cardiomyopathy, arrhythmias and other fatal heart diseases (for references see recent reviews (3, 7, 9-11)). Conversely, progression of cardiac disease to heart failure is generally associated with various degrees of ICD structural disruption.

Recent surveys from the human protein atlas web site (<http://www.proteinatlas.org>), ExPASY protein binding data (<http://ca.expasy.org/sprot/>) and published papers reveal nearly 200 proteins associated with ICDs (12), about 40% of them are altered in their expression and/or location in various cardiac diseases (12). The discovery of a subcellular domain (termed *transitional junction* (13), review in Bennett, this issue 2011) between the ICDs and the myofibrils, further increase the numbers of ICD-associated proteins. Thus, the inventory of ICD molecular components is far from complete and the molecular mechanisms by which these components support normal cardiac function remain to be elucidated. Adding to this list, in 1996, we identified a family of Xin repeat-

containing proteins in the heart, which co-localize with adherens junction proteins to the ICDs and play an important role in cardiac morphogenesis and function (14-18). In this review, we will briefly discuss recent advances in the anatomy of ICDs and in the functions (signaling) of adhering junctions (adherens junctions and desmosomes), and then focus on the recent advances in our understanding of how the Xin repeat-containing protein family promotes ICD maturation and stability for normal cardiac function.

2.1. Advances in the anatomy of ICD

Using immunogold electron microscopy and immunofluorescence microscopy, two new structures/domains, *area composita* and *transitional junction* (Figure 1), at the ICDs were recently identified (13, 19). Furthermore, characterizations of ICD components with molecular, cellular and genetic approaches revealed intricate connections among the intercellular junctions in the ICD.

2.1.1. *Area composita* (mixed type of junctions) exist in mammalian ICDs

The adhering junctions (adherens junctions and desmosomes) of the ICD are traditionally defined based on their morphological resemblance under transmission electron microscopy to corresponding junctions in epithelial cells. The adherens junction is characterized by a fuzzy electron dense plaque underlining the plasma membrane. Actin filaments extending from myofibril thin filaments apparently insert into the adherens junction, suggesting that these junctions are the anchorage sites of the termini of myofibrils. Adherens junctions of the ICD consist of N-cadherin as the transmembrane component, whose highly conserved cytoplasmic domain interacts with β -catenin, plakoglobin (γ -catenin), α -catenin (α E-catenin and α T-catenin), p120-catenin, vinculin and other actin-binding proteins to link to the actin filaments (Figure 2). Conversely, the desmosomes are characterized by straighter membranes, intermembrane bridges that form a line in the middle of the gap between two membranes, and the two-layered intracellular (cytoplasmic) plaques. Intermediate filaments insert into the cytoplasmic plaques. Desmosomes in the ICD consist of the desmosomal cadherins (desmoglein 2 and desmocollin 2) and intracellular linker proteins such as desmoplakin and plakophilin 2 (PKP2) (Figure 2). Such strict distinction between adherens junctions and desmosomes in the ICD has been challenged by recent work from Werner W. Franke and his colleagues with immunoelectron microscopy and immunofluorescence microscopy on mammalian hearts from different species (19, 20). These studies demonstrated in the adult mammalian ICDs, but not in the non-mammalian ICDs, that the molecular composition of adherens junctions and desmosomes are less exclusive than those in the epithelial junctions (21, 22). Adherens junctions of the ICD contain not only the typical adherens junctional components, but also desmosomal cadherins and cytoplasmic plaque proteins. Conversely, desmosomes in the ICDs contains not only typical desmosomal proteins, but also N-cadherin, β -catenin and α -catenin. Based on these observations, a new type of intercellular junction, *area composita*, was proposed. The formation of *area composita* by fusing

Intercalated disc and Xin repeat-containing protein

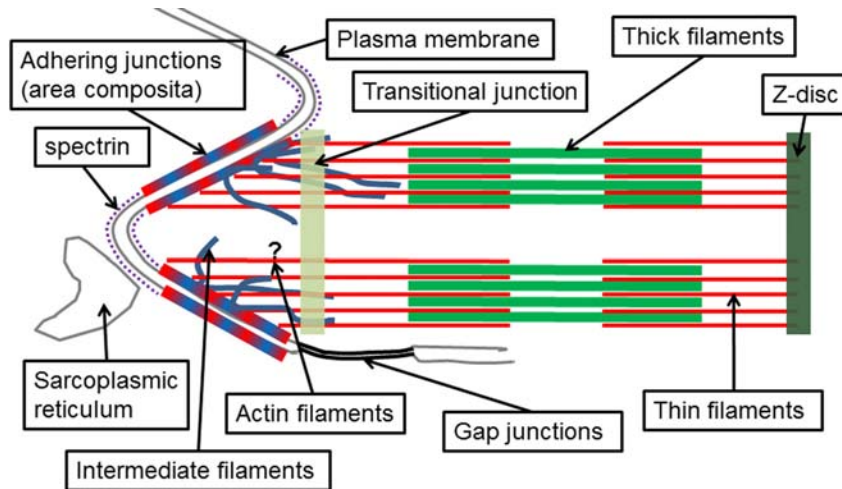


Figure 1. Diagram of an adult mammalian ICD. The adhering junctions (adherens junctions and desmosomes) intermix to form the *area composita*. Both actin filaments and intermediate filaments insert into the *area composita*. The transitional junctions are located at the level of the apices of the membrane folds and mediate the transition between the thin filaments and the actin filaments in the ICD. The question mark indicates that the organization of the actin filaments in the ICDs is not well understood. SR, sarcoplasmic reticulum.

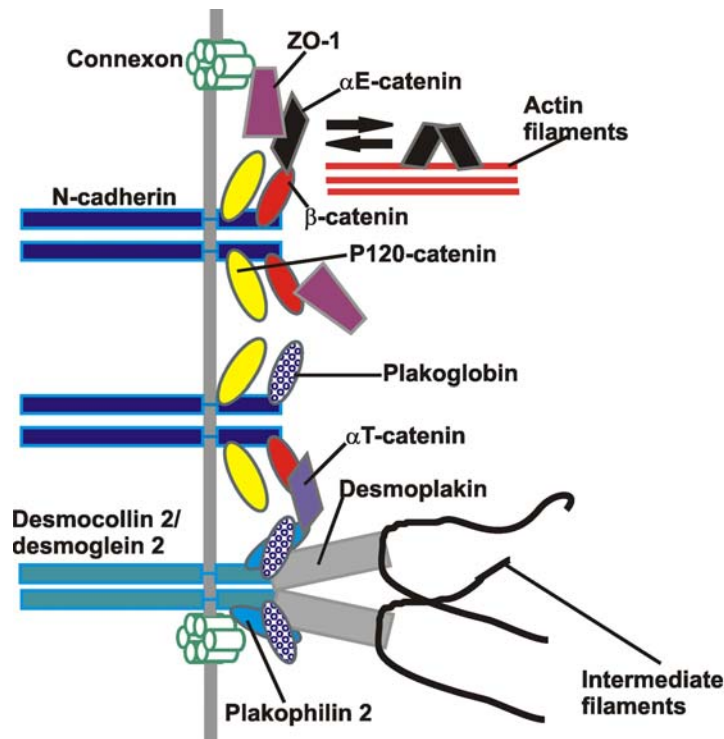


Figure 2. Major molecular components of the ICDs. Proteins that may shuttle between different intercellular junctions are shown. ZO-1 interacts with Cx43, α E-catenin and β -catenin and thus it may provide a link between the gap junctions and the adherens junctions. Plakoglobin is known to interact with both N-cadherin and desmosomal cadherins (desmocollin 2 & desmoglein 2) and thus it may play an important role in the association between adherens junctions and the desmosomes. α T-catenin may be a direct link between the adherens junctions and desmosomes through its interaction with the β -catenin and plakophilin 2. p120-catenin is a critical component of the adherens junctions and it has been shown to associate with the desmosomes. However, the mechanism of p120-catenin's association with the desmosomes is not clear; thus such association is not shown. Plakophilin 2 links the desmosomes and the gap junctions together. P0071 is not shown because its interaction partner in the ICDs has not been defined.

adherens junctions and desmosomes appears to be a late process both in ontogenesis and in evolution (21); the area composita is only found in cardiomyocytes of maturing and adult mammalian hearts (23). The significance of area composita in mammalian hearts remains to be determined; however, it may strengthen mechanical coupling among neighboring cardiomyocytes and may enhance crosstalk among different types of junctions. On the other hand, the absence of the area composita found in the ICDs of non-mammalian hearts may advantageously assist in the regeneration of damaged hearts. This possibility is supported by the recent finding that the mouse heart retains impressive regenerative capacity at birth but not at one week of age (24). The timing of the loss of regenerative capacity coincides with the maturation of area composita.

2.1.2. Defective adhering junctions generally lead to gap junction remodeling

Despite the apparent mixing of the molecular components in different types of junctions of mammalian ICDs, the morphology of adherens junctions and desmosomes are nevertheless discernable and the associated filament systems are clearly defined. The targeted deletion/disruption of mouse genes encoding ICD-associated and actin-interacting proteins such as Ena/VASP, mXin α , non-muscle myosin IIB, α E-catenin or vinculin, seems to affect the morphologically defined adherens junctions specifically and spare the desmosomes (17, 25-28). In addition to alterations in the expression levels of adherens junctional components, most of these hearts from the above mutant animals exhibit reduced levels of connexin 43 (Cx43) expression and altered localization of Cx43 (gap junction remodeling).

Gap junction remodeling is also commonly observed in human patient and animal model hearts with mutations in desmosomal protein components. Desmoplakin is one of the major components of desmosomes and is capable of interacting with many other desmosomal components, including PKP2 and plakoglobin, as well as with desmin intermediate filaments (29). The global or cardiac restricted deletion of *desmoplakin* in mice results in embryonic lethality, and mutant embryos display a severe deficiency of desmosomes (5, 30); unfortunately, effects on the structures of adherens junctions and gap junctions of ICD cannot be examined in these mutant lines. However, cardiac-restricted *desmoplakin* heterozygous mice recapitulate the phenotype of human arrhythmogenic right ventricular cardiomyopathy (ARVC), a major cause of sudden cardiac death, ventricular tachycardia and heart failure (5). Gap junction remodeling has also been observed in human patients with ARVC (31, 32) due to *desmoplakin* mutations (33, 34), *plakoglobin* deletion (Naxos disease) (35) or *PKP2* mutations (36). Similarly, cardiac-restricted overexpression of a desmoplakin missense mutation (disrupting the binding of desmin) results in changing the expression and localization of Cx43 as well as widening the gaps of ICDs (34). It is also known that gap junction remodeling can occur in human patients with ischemic cardiomyopathy, dilated cardiomyopathy and heart failure (10, 37-44).

In summary, the defective linkage between adhering junctions (adherens junctions and desmosomes) and the cytoskeleton (actin and intermediate filaments) affects formation and maintenance of gap junctions (for a more extensive discussion see (7)). As will be described below, gap junction remodeling was detected in mouse hearts completely lacking one of the Xin repeat-containing and adherens junction-associated proteins, mXin α . Whether mXin α can directly or indirectly interact or associate with gap junctional components remains to be determined. Following is a brief review of what is known about junctional proteins that can co-exist in more than one junction of the ICD.

2.1.3. Linkers involved in molecular crosstalk among different junctions of ICDs

Junctional proteins shuttled and/or linked among different junctions of the ICD could potentially play important roles in the formation of area composita and/or in molecular crosstalk among the ICD junctions. Recent studies have revealed that many junctional proteins can co-exist in different junctions, potentially providing linkers to strengthen the mechanical coupling and to enhance molecular crosstalk (Figure 2). In addition, many protein components of one type of junction can associate with protein components of another junction (Figure 2). Understanding these associations and interactions may unveil underlying mechanisms for pathogenesis of many cardiac diseases, such as cardiomyopathy, arrhythmias and heart failure.

2.1.3.1 Plakoglobin (γ -catenin)

Plakoglobin is the first known junctional component present in both adherens junctions and desmosomes of the ICD (45). *Plakoglobin*-null mice die between E12 and E16 due to severe heart defects (46, 47). In these mutant mice, typical desmosomes are no longer detectable in the heart but are still present in epithelial organs, and the desmosomal cadherin, desmoglein 2, becomes diffusely distributed. The extended adherens junctions of mutant ICDs contain desmoplakin, most of which co-localizes with β -catenin, thus prematurely forming a “mixed type” of adhering junction (47). Furthermore, similar phenotypes have been observed in mice with targeted deletion of *PKP2*, another armadillo protein plaque constituent of desmosomes (48). These results suggest that both plakoglobin and PKP2 are not only essential for the formation of cardiac desmosomes, but also critically involved in the segregation of the two sets of molecules into desmosomes and adherens junctions. Studies with *desmoplakin* heterozygous knockout mice and cardiomyocytes to understand the pathogenesis of ARVC have suggested that desmoplakin deficiency leads to mislocalization of plakoglobin from the ICD to the nucleus. Plakoglobin has structural and functional similarity to β -catenin, and is able to compete with β -catenin to suppress the Wnt/ β -catenin signaling pathway through T cell factor/lymphoid-enhancer factor (Tcf/Lef) (49-51). Suppression of Wnt/ β -catenin signaling could promote adipogenic and fibrogenic gene expression in cardiomyocytes, leading to adipocytic replacement of cardiomyocytes, the hallmark of ARVC (5). These results

suggest that plakoglobin can function as a signaling protein in addition to a linking protein between cadherins and the cytoskeleton. Recent studies with cardiac-restricted overexpression and deletion of plakoglobin further support this signaling (crosstalk) function of plakoglobin (52, 53).

2.1.3.2. Plakophilin 2 (PKP2)

Mutations in both plakoglobin and PKP2 have been identified in ARVC patients (35, 54-58); about 70% of familial ARVC is caused by a *PKP2* mutation. Using small interference RNA (siRNA) techniques, it has been shown that inhibition of PKP2 expression in primary cultures of neonatal rat ventricular myocytes leads to progressive loss of area composita-like structures and undetectable desmoplakin in the residual ICD-like structures (58). In addition, knocking down PKP2 by siRNA causes gap junction remodeling (a reduction in Cx43 expression, a decrease in dye coupling between cells, and a significant redistribution of Cx43), further suggesting intra-ICD crosstalk (59). Similar to that observed in *PKP2*-null cardiomyocytes (48), knocking down PKP2 also results in the loss of desmoplakin in residual junctions; instead, there is an accumulation of many cytoplasmic vesicles/aggregates containing desmoplakin, PKP2 and desmoglein 2 (58). Thus, PKP2 acts not only as an “organizer” protein in the formation, stabilization and function of the area composita, but also functions in the molecular crosstalk between desmosomes and gap junctions (8, 11). The exact mechanism mediating junctional organization and molecular crosstalk likely involves the multiple functional domains of PKP2, the only plakophilin isoform expressed in the heart (60). It is known that PKP2 can bind to a large number of desmosomal proteins, including desmoplakin, plakoglobin, desmoglein and desmocollin (61). Through these interactions, PKP2 may zip up desmosomal cadherins and tighten the desmosomal plaque. In addition, PKP2 is capable of interacting with α T-catenin but not α E-catenin; α T-catenin is a component of the adherens junctions which co-localizes with α E-catenin at the ICD (62). Through its interaction with α T-catenin, PKP2 could link components of adherens junctions and desmosomes to form and/or stabilize mixed type adhering junctions (area composita). Moreover, it has been shown by pull down and co-immunoprecipitation assays from rat heart lysates that PKP2 and Cx43 coexist in the same macromolecular complex; the head domain of PKP2 appears to be sufficient for this association (59). Through this head domain, PKP2 is also able to associate with β -catenin and up-regulate the signaling activity of Wnt/ β -catenin/Tcf in an overexpression system (61). Furthermore, the *Cx43* gene is known to be a functional target of Wnt/ β -catenin signaling and its C-terminus can associate with β -catenin, which can account, in part, for the crosstalk (63, 64).

2.1.3.3. p0071 (sometimes referred to as PKP4)

Another junctional protein having dual localization in desmosomes and adherens junctions on epithelial and endothelial cells is p0071 (65, 66). Although dual localization of p0071 has not been demonstrated in cardiomyocytes, the *p0071* message is detected in mouse hearts (67), and its protein product is localized to ICDs

(20). p0071 (PKP4) belongs to a member of the p120-catenin subfamily of armadillo related proteins; p120-catenin is the prototype of this subfamily that comprises p0071, ARVC protein, NPRAP/ δ -catenin and the more distantly related plakophilins 1-3 (68). Structurally different from p120-catenin, p0071 contains a PDZ domain-binding motif at its C-terminus. The head domain of p0071 interacts with desmocollin and desmoplakin, whereas the armadillo repeat domain binds to classical cadherins (65, 66). In addition, both head and armadillo repeat domains interact with plakoglobin (65). Moreover, p0071 and p120-catenin can bind to the same region of the cytoplasmic tail of VE-cadherin, and thus, p0071 can compete p120-catenin off from intercellular junctions (66). Functionally similar to p120-catenin, p0071 can organize small Rho-GTPase signaling, in particular, spatially increasing RhoA activity via its interaction with Ect2, a guanine nucleotide exchange factor (GEF) for Rho, and subsequently regulating cell adhesion, cytokinesis and motility (69). However, the exact roles of p0071 in ICD maturation, stability and function remain to be determined.

2.1.3.4. p120-catenin

p120-catenin is an armadillo-repeat protein that directly binds to the juxtamembrane region of classical cadherins and regulates cadherin-based adhesion, cell shape determination and migration. Recent evidence suggests that like plakoglobin, p120-catenin is another component common to adherens junctions and desmosomes, at least in epithelial cells. In addition to binding E-cadherin, p120-catenin can associate with desmoglein 1 and desmoglein 3 when desmosomes are assembled in high Ca^{2+} medium but not in low Ca^{2+} medium (70). These observations of conditional dual localization suggest that p120-catenin may play an important role both in the regulation of desmosome assembly and disassembly, as well as in junctional crosstalk. The region required for the association of p120-catenin with desmosomes has been identified to aa#758-773 of desmoglein 3, which is different from the plakoglobin-binding site (71). However, results from *in vitro* pull-down assays and yeast two hybrid assays suggest that p120-catenin cannot directly interact with desmoglein 3 (71, 72). Similar to p0071, p120-catenin can induce Rac1 and Cdc42 activation via its interaction with Vav2 (a GEF for Rac1 and Cdc42) and subsequently regulate cell adhesion, shape and motility (73, 74). However, this regulatory role of p120-catenin has not been demonstrated in cardiomyocytes. We have previously reported that the loss of the Xin repeat-containing and ICD-associated protein, mXin β , in the developing heart impairs N-cadherin clustering during the formation of mature ICDs, alters the expression and localization of p120-catenin, and reduces Rac1 activity (18). How mXin proteins influence p120-catenin and Rac1 remains to be determined. Finally, p120-catenin has been shown to link the adherens junctions to the minus end of microtubules through PLEKHA7 and Nezha, which is required for the establishment and maintenance of the zonula adherens/adherens junctions (75).

2.1.3.5. ZO-1 (zonula occludens-1)

ZO-1 is a member of the membrane-associated guanylate kinase family of proteins and originally

discovered in association with the tight junction (76). In the heart, ZO-1 is expressed in endothelial cells, interstitial cells and cardiomyocytes and localized to ICDs of cardiomyocytes (37, 77, 78). The N-terminal half of ZO-1 contains 3 PDZ domains, a SH3 domain and a catalytically inactive guanylate kinase domain (78, 79). Through its second PDZ (PDZ-2) domain, ZO-1 binds to the extreme C-terminus of Cx43 (78, 80, 81). The N-terminal fragment of ZO-1 can also bind directly to α -catenin, whereas the C-terminal specifically co-sediments with actin filaments *in vitro* and localizes to microfilament bundles in non-muscle cells (79). Therefore, ZO-1 appears to be able to link between gap junctions and adherens junctions. These associations between adherens junctional proteins and Cx43 are shown to be required for the development/formation of gap junctions in non-muscle cells (82, 83) and in cardiomyocytes (84). In addition, as described above, depletion of the Cx43-associated desmosomal protein, PKP2, by siRNA treatment of cultured cardiomyocytes leads to gap junction remodeling and a decrease in dye coupling between cells (58, 59). Therefore, molecular crosstalk/associations between adhering junction (adherens junction and desmosome) components and gap junction proteins (α -catenin, ZO-1, Cx43 and PKP2) at the ICD likely account for the underlying mechanisms for gap junction remodeling observed in many human cardiac diseases and heart failure.

In the heart, Cx43-associated ZO-1 may also play a key role in regulating size, number and distribution of gap junctions (85-87). ZO-1 was found to preferentially localize to the periphery of gap junction plaques, presumably either to inhibit further recruitment of connexons or to favor their removal from gap junctions on reaching a certain size (37, 77, 86). Within the ICDs *in vivo*, only low level co-localization between ZO-1 and Cx43 is found, as compared with the relatively high level co-localization between ZO-1 and N-cadherin (77). However, during remodeling of cardiac gap junctions, such as in the enzymatically isolated cardiomyocytes (77) or in the human failing heart (37), co-localization and interaction between ZO-1 and Cx43 strikingly increase. This increased interaction of Cx43 with ZO-1 could constrain the growth of gap junctions and contribute to reduction in the Cx43 levels observed in the human failing heart (10, 37, 40, 41). Further support of this hypothesis comes from studies of both *in vitro* cell systems (86) and *in vivo* hearts of mice expressing C-terminally truncated Cx43 (K258stop/KO) (88). Specific disruption of the interaction between ZO-1 and Cx43 leads to increased size, decreased number, and altered localization of gap junction plaques. Thus, both ZO-1 and the C-terminal domain of Cx43 are involved in regulating the organization of Cx43 plaques. As will be described below, *mXin* α -null mouse hearts display gap junction remodeling (17). Like ZO-1, *mXin* α has been shown to be a β -catenin-binding and actin-binding protein located at ICDs (89). It would be of interest to investigate whether *mXin* α and ZO-1 may cooperatively be involved in the assembly and maintenance of gap junction plaques in the heart.

2.1.3. Transitional junction near ICD

A recent study has also advanced our understanding of how sarcomeres are connected to the ICD.

It has long been noticed that in addition to the intercellular junction-covered membrane, the ICD membrane also contains regions free of gap junctions, adherens junctions and desmosomes (1). Bennett and coworkers observed that these regions are mainly located at the apex of the membrane interdigitations and are associated with spectrins (13). It has been shown that this junction-free region of the ICD membrane is at the level where the Z-disc of the last sarcomere would have been located, if the last sarcomere formed a Z-disc close to the ICD. Consistently, some of typical Z-disc proteins such as α -actinin, titin, ZASP are identified in this region. In addition, although the thin filaments extend from the sarcomeres into the ICD seamlessly, the ICD actin seems to be β -actin instead of the sarcomeric α -actin (90). This isoform switch appears at the Z-disc-like region, where non-muscle myosin IIB (26) and NRAP (91, 92) are also found; this specialized Z-disc-like structure was thus defined as the transitional junction (13). The finding not only explains how the last sarcomere retains regular organization even though its thin filaments insert into the highly convoluted ICD, but also suggests that new sarcomeres can be added onto the end of the myofibril in the convoluted region of ICDs without disturbing the overall organization of the myofibrils. Indeed, addition of new sarcomeres in the ICD was recently observed in cardiomyocytes whose myofibrils are elongating under volume overload (93). The ICD has been shown to change the organization of its interdigitation to accommodate the addition of forming sarcomeres without disrupting the overall organization of the myofibrils, supporting an important role for the ICD in myofibril formation.

In this special issue, the article "From myofibril to membrane: the transitional junction at the intercalated disc" by Pauline Bennett reviews new and known information on the structure and function of the transitional junction. Xufei Ma and Robert Adelstein review the role of non-muscle myosin II in cardiac development and function. Particularly relevant to this issue is that similar to *mXin* α -null mice (17), *myosin IIB* conditional knockout mouse hearts develop cardiomyopathy and fibrosis (26). In addition, the loss of myosin IIB in the heart results in a significant reduction in *mXin* α protein. How the actin-binding protein, *mXin* α , collaborates with non-muscle myosin IIB in such cardiac defects remains to be determined.

2.2. The involvement of ICD in signaling

Recent evidence from studies with transgenic overexpressing and knockout animals clearly points to the involvement of ICD components in transducing signals important for cardiac remodeling in both physiological and pathological states (see references in (5-8, 10, 52, 53, 94)). Here, we only briefly discuss signaling relevant to β -catenin in the heart, because the β -catenin-binding domain is present in Xin repeat-containing proteins (89). β -catenin is a multifunctional protein and plays a central role in regulating both canonical Wnt (Wnt/ β -catenin) signaling and cadherin-mediated (cadherin/ β -catenin) signaling in many cell types and tissues (95-97). The interplay between these two signaling pathways has been shown to be crucial in the process of epithelial-mesenchymal transition (EMT), which occurs not only in normal embryonic development such

as cardiac morphogenesis, but also in tumor formation and metastasis (98). Both Wnt/ β -catenin and N-cadherin-mediated signaling pathways likely operate in postnatal and adult hearts, and a faulty component of these pathways could result in cardiac hypertrophy and cardiomyopathy (5, 6, 52, 53, 99, 100). In the presence of canonical Wnt signaling, cytoplasmic β -catenin is stabilized and enters the nucleus, where it interacts with TCFs, such as Lef1, to regulate gene expression. In the absence of Wnt signaling, cytoplasmic β -catenin is targeted for destruction by the APC, axin, and GSK3 β complex that phosphorylates β -catenin and directs it to a destruction pathway (95). β -catenin is known to bind N-cadherin at ICDs to regulate N-cadherin-mediated adhesion. Therefore, canonical Wnt and N-cadherin-mediated signaling pathways potentially compete for the same pool of β -catenin.

2.2.1. N-cadherin/ β -catenin signaling in the heart

It has been shown that either too much or too little N-cadherin in the heart leads to dilated cardiomyopathy, suggesting that delicate signaling through N-cadherin is required for normal adult heart function. Transgenic mice over-expressing N-cadherin in the heart develop cardiomyopathy, whereas ectopic expression of E-cadherin in the heart leads to a much more severe cardiomyopathy (101). Ectopic expression of E-cadherin in the heart would interfere with N-cadherin-mediated signaling and result in a more severe cardiomyopathy. Conditional deletion of N-cadherin in the adult heart leads to a complete dissolution of ICD structure and a significant decrease in the gap junction protein, Cx43 (102). Consequently, N-cadherin-deficient mice exhibit dilated cardiomyopathy, impaired cardiac function, ventricular arrhythmias and sudden death (103, 104). These results suggest that N-cadherin-mediated adhesion and signaling pathway are essential for the structural integrity and function of the heart.

The most characterized cellular signals involving cadherin/catenin complexes are those generated locally upon cadherin-cadherin engagement during cell-cell contact formation. In non-cardiomyocytes, small GTPases (Rho, Rac and Cdc42) have been shown to transduce such local signals to control cell adhesion, survival, shape change and motility (105-107). Following the engagement, the juxtamembrane domain of cadherin interacts with p120-catenin, which can activate Rac1 and Cdc42, by binding to Vav2, a GEF for these GTPases (73). In addition to initiating cellular signals during contact formation, cadherin/catenin complexes in established junctions are also involved in mediating signal transduction. The adherens junctions are recognized as a sensor for mechanical forces and transduce signals that influence the actin cytoskeleton, which relies on the actin cytoskeleton and the proteins linking adherens junctions to the actin cytoskeleton (108, 109). Such mechanical signal transduction likely involves small GTPases (110). Interestingly, we have shown that mXin α is capable of interacting not only with β -catenin but also with p120-catenin (89). Furthermore, mXin β -null hearts show a significant decrease in active Rac1, a failure to form mature ICD and a misaligned myocardium (18). These results together suggest an involvement of Xin repeat-containing proteins in the N-cadherin-mediated signaling pathway.

2.2.2. Wnt/ β -catenin signaling in the heart

The role of Wnt/ β -catenin signaling in cardiac development has been intensively studied in a variety of organisms, although controversy remains. During early cardiac development, Wnt/ β -catenin signaling appears to have developmental stage-specific biphasic effects on cardiogenesis (111, 112). Activation of Wnt/ β -catenin signaling before gastrulation promotes mesoderm formation and cardiogenesis, whereas signaling during and after gastrulation inhibits cardiomyocyte differentiation by opposing bone morphogenetic protein (BMP) signaling (113, 114). However, the hypothesis that Wnt actively inhibits cardiogenesis is still too simple. Recent studies using both gain and loss of Wnt/ β -catenin function have shown that the Wnt/ β -catenin pathway acts cooperatively with fibroblastic growth factor (FGF) and BMP signaling to promote expansion of the second heart field progenitors (115-117), which contribute to outflow tract and right ventricle (118, 119). In postnatal and adult hearts, the importance of Wnt/ β -catenin signaling for cardiac remodeling under physiological and pathological conditions has also been intensively addressed. Activation of β -catenin in cultured rat neonatal cardiomyocytes was found to be not only sufficient but also necessary to induce cardiomyocyte hypertrophy (120, 121). *In vivo* studies using inducible cardiac-specific knockout or transgenic mice to modulate the expression levels of Wnt/ β -catenin signaling components or their mutants have further confirmed that stabilization of β -catenin or activation of Wnt signaling is required for both physiological and pathological cardiac hypertrophy (99, 122-124). However, conflicting results have also been reported (125). The precise reason for such discrepancy is unknown but may reflect the pleiotropic effects of Wnt signaling depending on the experimental conditions. Recently, studies with conditional transgenic mice expressing either no β -catenin or stabilized β -catenin generated by using a ventricle-specific driver (MLC2v-Cre) have revealed that mice lacking β -catenin in the adult ventricles do not have an overt phenotype (100), due to an up-regulation of plagglobin, as suggested previously (126). In contrast, mice expressing stabilized β -catenin develop cardiac hypertrophy and dilated cardiomyopathy at 2 months of age, and do not survive beyond 5 months (100). Furthermore, stabilized β -catenin was only found at the ICDs but never detected in the nucleus (100). These results suggest that β -catenin's role in the nucleus may be of little significance in the healthy adult heart, and that similar to N-cadherin, too much β -catenin at the ICD may critically affect the N-cadherin/ β -catenin signaling and subsequently lead to dilated cardiomyopathy. It should be noted that increased β -catenin levels were also detected in the hypertrophic hearts from human cardiomyopathy patients and from spontaneously δ -sarcoglycan-deficient hamsters (127). The accumulation of β -catenin at the ICD, but not in the nucleus, is accompanied by an increase in Wnt5a (a noncanonical Wnt) expression, a decrease in GSK3 β expression and differential expression of APC isoforms. The existence of multiple Wnt signaling pathways in the heart has added another level of complexity to Wnt signaling related to cardiac remodeling.

2.2.3. Interplay between Wnt/ β -catenin signaling and adhering junction-mediated signaling

Down-regulation of Wnt/ β -catenin signaling by nuclear plakoglobin as detected in ARVC hearts might be part of the molecular mechanism for the pathogenesis of ARVC (5, 53). Adult mice heterozygous for the deletion of *desmoplakin* in the heart recapitulate the phenotype of ARVC (5). Apparently, desmoplakin deficiency leads to an impaired desmosome assembly, which could free plakoglobin from the desmosomes and increase its nuclear localization in cardiomyocytes. Plakoglobin is known to be able to compete with β -catenin at multiple cellular levels with a net negative effect on the Wnt/ β -catenin signaling pathway (50, 51). Thus, increasing plakoglobin nuclear localization in *desmoplakin* heterozygous mice should suppress Wnt/ β -catenin signaling, which in turn would promote adipogenesis, fibrogenesis and apoptosis (128-130), the characteristic hallmarks of human ARVC. This mechanism has been further supported by two recent studies with transgenic mice over-expressing *plakoglobin* in cardiomyocytes as well as mice with a conditional knockout of *plakoglobin* in cardiomyocytes. Over-expressed plakoglobin translocated to the nucleus and suppressed Wnt/ β -catenin signaling. The association of plakoglobin, instead of β -catenin, with Tcf712 increases the expression of Wnt5b and BMP7, which promotes adipogenesis, and decreases the expression of connective tissue growth factor, which is an inhibitor of adipogenesis (53). The adipocytes in mouse and human ARVC hearts were identified to originate from the second heart field progenitors, accounting for a predominant involvement of the right ventricle in human ARVC (53). On the other hand, Wnt/ β -catenin signaling was activated in the hearts of mice with inducible cardiac-restricted *plakoglobin* deletion (52). Upon deletion of plakoglobin, expression levels of Wnt/ β -catenin target genes, such as c-Myc and c-Fos, were increased significantly. Stabilization of β -catenin following the loss of plakoglobin may be due to activation of Akt and inhibition of GSK3 (52), which could affect the β -catenin phosphorylation state/stability and promote cardiac hypertrophy. Interestingly, stabilized β -catenin in *plakoglobin*-null hearts became associated with Tcf4, a transcription factor primarily binding to plakoglobin. These results are consistent with the idea that β -catenin directly competes with plakoglobin for Tcf4 binding. The mutant mice exhibit progressive loss of cardiomyocytes, extensive inflammation, fibrosis, altered desmosome structure and cardiac dysfunction similar to ARVC patients. However, in contrast to the *desmoplakin* heterozygous hearts, neither adipocyte replacement nor lipid droplet accumulation was observed in the conditional *plakoglobin* knockout hearts, suggesting that plakoglobin itself might be required for the full spectrum of ARVC phenotype.

Thus, based on mouse models with various manipulations of the components of adhering junctions (adherens junctions and desmosomes), it seems that the molecular mechanism of ARVC consists of both nuclear and desmosomal signaling pathways. In one mechanism, elevated plakoglobin in the nuclei alters Wnt/ β -catenin signaling, which appears to be critical for the manifestation

of ARVC phenotypes such as apoptosis, fibrosis and adipogenesis. On the other hand, signals generated by desmosome likely play a role in the pathology of ARVC, because down-regulation of Wnt/ β -catenin signaling by simple conditional deletion of *β -catenin* in the postnatal heart does not lead to ARVC. The specific involvement of signals from the desmosomes in ARVC is further supported by the lack of ARVC phenotype in mice with conditional deletion of adherens junction components, such as N-cadherin (102), α E-catenin (27), mXin α (17) and β -catenin (99, 100, 126). These mice exhibit dilated cardiomyopathy with neither myocyte loss nor inflammation, which is different from the conditional *plakoglobin* knockout mice (52) and other animal models of ARVC (34, 53, 131). These differences suggest that different signaling may transduce through adherens junctions versus desmosomes.

2.2.4. ICD influences ion channel surface expression

As a functional unit, the ICD also plays important roles in organizing and/or regulating surface ion channels and receptors. Previous studies have shown that the pore-forming α -subunit, Nav1.5, of the voltage-gated sodium channel is preferentially localized to the ICD (132-136). This population of sodium channel complexes is composed of Nav1.5, tyrosine-phosphorylated β 1 subunit, and ankyrin G in close association with both N-cadherin and Cx43 (137, 138). A recent study has shown that Nav1.5 can be pulled down by the head domain of PKP2 from heart lysates, suggesting that PKP2 participates in the same molecular complex at ICDs. Knockdown of *PKP2* expression in cultured cardiomyocytes by siRNA leads to a decrease in peak current density, changes in the current kinetics (inactivation and recovery from inactivation), and a slower velocity of action potential propagation (139). In this special issue, Dr. Cheng-I Lin's group presents evidence that ICD-associated mXin α protein influences surface expression of transient outward potassium current (I_{TO}) through its ability to interact with Kv channel interacting protein 2 (KChIP2) (140), an auxiliary subunit of I_{TO} , and filamin, an actin-crosslinking protein. Taken together, these results further suggest a link among all 4 components of the ICD: desmosomes, adherens junctions, gap junctions and the voltage-gated channel complex. It seems relevant to consider the ICD as an overall functional unit when seeking to understand the pathogenesis of cardiac disease.

2.3. Discovery of the Xin repeat-containing and ICD-associated protein family

Prior to the availability of genome-wide microarray and functional genomics, we used differential mRNA display screening in conjunction with whole-mount *in situ* hybridization to clone novel genes that are temporally and spatially expressed during cardiac morphogenesis (14). Cardiac cushion formation and valvuloseptal morphogenesis are essential for a four-chambered heart. These processes involve inductive interaction between myocardium and endocardium as well as EMT, which occur temporally in chicken embryos between Hamburger and Hamilton (HH) stage 15 and 21 and spatially at the future atrioventricular canal (AVC) and future outflow tract of the linear heart tube (141-143). Therefore, we performed differential display cloning on the

total RNAs prepared from the AVC region of stage 15 and 21 chicken hearts. Whole-mount *in situ* hybridization was used as a secondary screening method to confirm the temporal and spatial expression patterns of isolated genes. From this screen a novel gene, *21C*, among others was identified. Subsequently, we used antisense oligonucleotide treatment of culture chicken embryos to show that this *21C*, later called chicken Xin (*cXin*), plays a very important function in cardiac morphogenesis and looping (15). Furthermore, we cloned mouse homologs of *cXin* and identified the gene as a downstream target of *Mef2C* and *Nkx2.5* (15). Because of its critical role for normal heart development, but not because of its strong cardiac expression as wrongly cited in Otten *et al.* (144), we then called this gene *Xin* (a Chinese word meaning heart). *Xin* encodes a modular protein that contains a N-terminal 16 amino acid repeating unit (called Xin repeat) with a consensus sequence of GDV(K/Q/R/S)XX(R/K/T)WLFET(Q/R/K/T)PLD (15, 145, 146). Since the initial discovery of *cXin*, two homologous genes, each containing a Xin repeat region, have been identified in mammals: *mXin α* and *mXin β* (also called *Myomaxin*) in mouse (15, 17, 18, 147, 148) as well as *hXin α* (also called *cardiomyopathy associated 1*, *CMYA1*, or *Xin actin-binding repeat containing 1*, *XIRP1*) and *hXin β* (also called *CMYA3* or *XIRP2*) in humans (145, 146, 149).

3. EXPRESSION, DOMAIN STRUCTURES AND FUNCTION OF XIN

3.1. *Xin* is a striated muscle-restricted gene and a downstream target of *Nkx2.5* and *Mef2*

Multiple tissue Northern blot analyses revealed that *cXin* (9.0kb), *mXin α* (5.8kb) and *mXin β* (12kb) messages were detected only in heart and skeletal muscle (15, 145, 147). Occasionally, low level *Xin* expression could be detected in the pulmonary vein of lung tissue, which may represent associated cardiomyocytes in this tissue. Whole-mount *in situ* hybridization revealed that expression patterns of *cXin* and *mXin α* in the developing heart and somites (15) are very similar to that of *Nkx2.5* and *Mef2C* (150, 151). In an anterior medial mesoendoderm explant system, the induction of *cXin* expression by BMP-2 followed activation of *Nkx2.5* and *Mef2C*, but preceded expression of myosin heavy chain (15). Similar effects on cardiac looping morphogenesis observed in embryos after *Nkx2.5* (152) or *Mef2C* (153) deletion or *cXin* antisense treatment (15) further suggest that *cXin* participates in a *BMP-Nkx2.5-Mef2C* pathway to regulate chicken cardiac morphogenesis. Either *Nkx2.5* or *Mef2C* alone is able to trans-activate the expression of a *luciferase* reporter gene driven by the *mXin α* promoter in non-muscle cells. These results together with drastically down-regulated *mXin α* messages in *Nkx2.5*-null and *Mef2C*-null mouse embryos (145) suggest that *mXin α* also participates in a *Nkx2.5-Mef2C* pathway to control cardiac differentiation and morphogenesis in the mouse. Moreover, *mXin β* has been shown to be a direct target of *Mef2A* (147) and to function downstream of angiotensin II (AngII) signaling to modulate pathological cardiac remodeling (154). Therefore, the Xin repeat-containing protein family

plays an important role in cardiac development and function through the *Nkx2.5-Mef2* pathway.

3.2. Two phases of *Xin* up-regulation during development correlate with chamber/valve formation and postnatal heart growth

During embryogenesis, the *cXin* transcript is first detected at HH stage 8 in the paired lateral plate mesoderm that forms the primordial of the heart (15). At stage 9, *cXin* expression increases substantially in the heart forming fields, which migrate anteriorly and ventrally toward the midline of the embryo. At stage 10, *cXin* is expressed exclusively in the linear heart tube. Cardiac specific expression continues until stage 15, when somite expression can be detected. Skeletal and cardiac muscle-restricted expression of *cXin* continues throughout development and into adulthood. The relative expression level of *cXin/glyceraldehyde 3 phosphate dehydrogenase (GAPDH)* determined from Northern blot analysis of developing hearts reveals that two major phases of *cXin* up-regulation exist at stage (st.) 16-25 and post-hatch day (D)12-14 (Figure 3). These two peaks of *cXin* up-regulation appears to coincide with the timing for chamber/valve formation and postnatal heart growth, respectively (141), suggesting a role for *cXin* in normal cardiac morphogenesis. Supporting this role, knocking down *cXin* by antisense oligonucleotide collapses the heart chamber wall, leading to abnormal cardiac morphology (15).

The *mXin* protein was first detected in the developing linear heart tube of E8.0 mice by whole-mount immunofluorescence microscopy with the antibody recognizing both *mXin α* and *mXin β* (148). At this stage, one to seven somites are present but contain no *mXin* protein. At E10 (equivalent to HH st. 17 in chick), *mXin* is expressed throughout the myocardium but not the endocardium of the truncus arteriosus, common atrial chamber and ventricle; *mXin* expression is also detected within the myotome of the first two rostral most somites (148). As development progresses to E13.5 (equivalent to HH st. 30 in chick), staining detects strong *mXin* expression throughout the myocardium of the ventricle and atria. Co-localization of *mXin* with both β -catenin and N-cadherin is observed in the cell periphery but not the nucleus of early mouse embryonic hearts (148). Later in embryogenesis co-localization becomes punctate structures around the cardiomyocytes. During the first 3 weeks of postnatal life, these co-localized structures gradually move into the termini of elongated cardiomyocytes to form mature ICDs. Similar to the second phase of *cXin* up-regulation, peaking at D14, expression of *mXin α* and *mXin β* in postnatal mouse hearts increase significantly from newborn to postnatal day 13.5 (P13.5) (18). During this developmental period, N-cadherin expression in the mouse heart also exponentially increases, reaching a plateau at two weeks of age and maintaining this level throughout adulthood. The timing of the *Xin* up-regulation coincides with the maturation of ICD (3, 148), T-tubule and sarcoplasmic reticulum (155), as well as diastolic function (156), suggesting a vital role for *Xin* in cardiac development and function. Supporting this role, the *mXin β* -null mouse hearts fail to form mature ICDs, exhibit diastolic dysfunction and premature death (18).

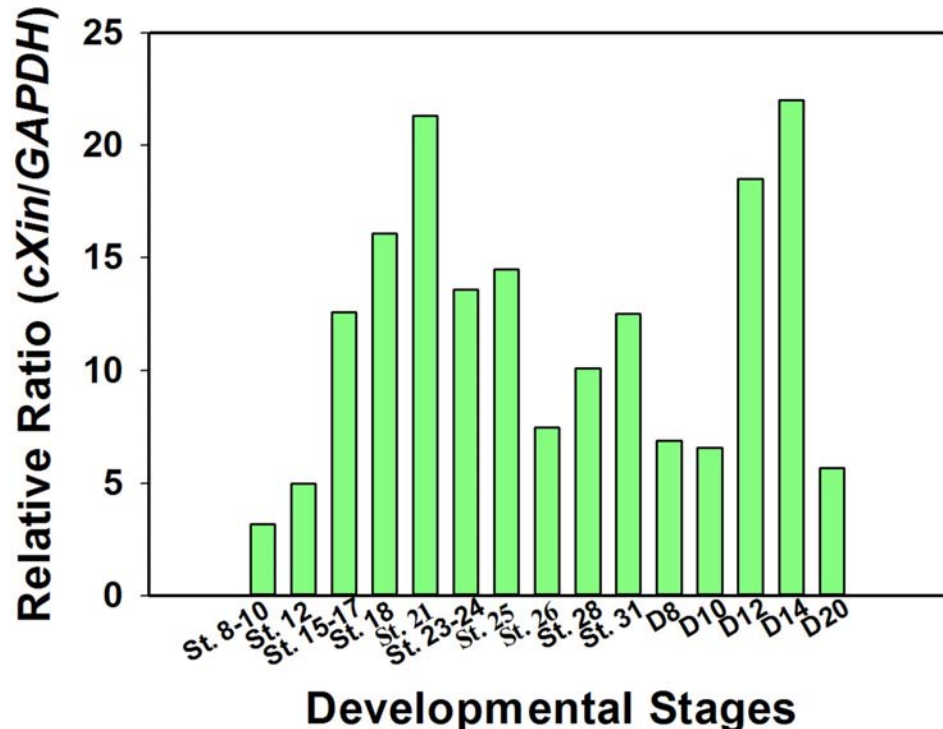


Figure 3. *cXin* expression profile during cardiac development. Developing hearts were dissected from chicken embryos at various Hamburger-Hamilton Stages (St.) and from chicks at various post-hatch day (D) and used for total RNA isolation and Northern blot analysis. After scanning the blots, the expression levels of *cXin* message were normalized with the expression levels of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) at respective time point, and the ratios were plotted against developmental stage. Two *Xin* up-regulation peaks correlate with valve/chamber formation (St. 26–25) and postnatal heart growth (D10–20).

3.3. *Xin* expression is significantly up-regulated in animal models of cardiac hypertrophy and hypertension

In response to abnormal stresses, such as hypertension, pressure overload, endocrine disorders and myocardial infarction, adult cardiomyocytes undergo pathological hypertrophy. Hypertrophy can be a compensatory mechanism that helps to preserve pump function in pathological conditions. Frequently, this hypertrophy progresses to dilated cardiomyopathy. Using pressure overload-induced hypertrophy by thoracic aortic banding (157–159), we detected up-regulation of both *mXinα* and *mXinβ* messages in the banded hearts, as compared with sham-operated controls (Figure 4A). In addition, immunofluorescence microscopy showed that banded hypertrophic hearts had thickening ICDs containing much more *mXin* and N-cadherin proteins (Figure 4B), suggesting that *mXin* may play an important role in modulating hypertrophy/stress responses in the adult heart. The up-regulation of *mXinβ* has also been observed in hypertensive hearts induced only by Ang II infusion (within 6 hours) but not by salt, suggesting that its up-regulation is due to Ang II-induced myocardial damages and not to blood pressure elevation per se (160). Thus, up-regulation of *mXinβ* appears to be one of the earliest molecular events triggered by Ang II. It is likely that *mXinα* up-regulation would also be observed in this Ang II-induced

hypertension/myocardial damage model, because both *mXinα* and *mXinβ* are transcriptional targets of MEF2 (15, 145, 147). It has recently been shown that *mXinβ* hypomorphic mice with 80% reduction in *mXinβ* message results in cardiac hypertrophy (154). Hearts from these hypomorphic mice display less myocardial damage when exposed to Ang II (154). These results suggest that *mXinβ* functioning downstream of Ang II signaling can modulate cardiac function in health and disease.

3.4. The origin of *Xin* coincides with the first appearance of the true heart chamber, and *mXinβ* is phylogenetically closer to the ancestral *Xin* protein than *mXinα*

A phylogenetic analysis has been performed with 40 vertebrate *Xins* to elucidate the evolutionary relationship between *Xin* proteins and to identify the origin of *Xin* (16). Multiple sequence alignment (161, 162) of the *Xin* repeats from vertebrates was analyzed in maximum likelihood and Bayesian analyses (163–165). The constructed evolutionary tree replicates the phylogeny of taxa with mammals, other land vertebrates and teleost phylum-level groups. Clearly, whole genome duplication that occurred early in evolution to produce both *Xinα* and *Xinβ* (16). The additional gene duplication of *Xinβ* was detected in teleosts. BLAST searches only detect *Xin* in chordates but not in other organisms such as

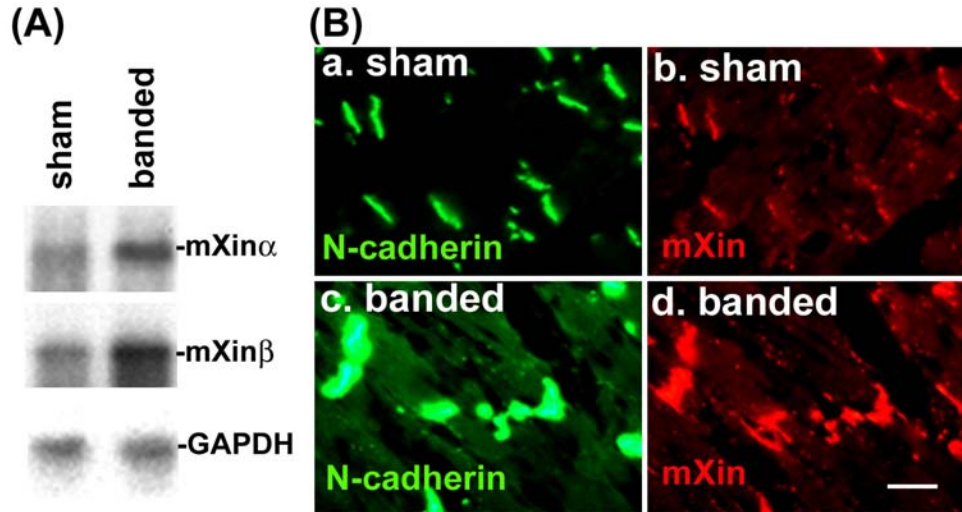


Figure 4. Up-regulation of both messages and proteins of mXin α and mXin β in stressed hearts. (A) Northern blot analysis of total RNAs prepared from sham-operated and thoracic aortic banded hearts. *GAPDH* blot was used as a control for RNA loading. (B) Double-label immunofluorescence microscopy of heart sections from sham-operated and thoracic aortic banded mice with anti-N-cadherin antibody (in green) and anti-Xin antibody (in red). N-cadherin and Xin co-localize to ICD of both sham-operated and banded hearts. The thickening ICD in banded hearts supports the up-regulation of both N-cadherin and mXin proteins.

Saccharomyces cerevisiae, *Candida albicans*, *Arabidopsis thaliana*, *Dictyostelium discoideum*, *Caenorhabditis elegans*, *Anopheles gambiae* (mosquito) and *Drosophila melanogaster*. Further analyses failed to identify Xin repeat-containing proteins in the Urochordate tunicate (*Ciona savignyi*) or the Cephalochordate amphioxus (*Branchiostoma floridae*). A Xin protein, defined as a protein containing Xin repeating units, was first identified in the Craniate lamprey (evolved about 550 million years ago). Importantly, both the Urochordate tunicate and the Cephalochordate amphioxus have hearts with only a single layer of contracting mesoderm or contracting vessel coupled with an incomplete endothelial cell layer, whereas the Craniate lamprey has a true chambered heart with complete endothelial and myocardial layers. Thus, the origin of Xin proteins coincides with a critical evolutionary modification of the heart, namely, the origin of true chambers. This finding is consistent with the chamber genesis role for Xin repeat-containing proteins in vertebrates.

In the avian lineage, there is a loss of Xin β , however, its sole Xin protein still retains 27 Xin repeats (compared to 28 repeats in the ancestral lamprey Xin) in order to carry out the essential functions of Xin (16). Therefore, chicken embryos treated by antisense oligonucleotide targeting *cXin* showed defects in cardiac looping and morphogenesis (15). In the mammalian lineage, Xin α contains a reduced number of Xin repeating units with 17 repeats in opossum and 15 in placental mammals. This strongly suggests that there was selective pressure within the mammalian lineage that resulted in the reduction of Xin repeats. We previously found that *mXinα*-null mice are viable but develop cardiac defects in adults (17), and *mXinβ*-null mice die postnatally with chamber defects (18). Thus, it is likely that mammalian Xin α s

evolved quickly to form many unique traits for neofunction in the adult heart, whereas all mammalian Xin β s are highly conserved with the ancestral lamprey Xin and retain its function in embryonic development (16). Evolutionary studies have also identified a putative DNA-binding domain conserved in the N-terminus of all Xins, in addition to a highly conserved β -catenin binding domain within the Xin repeat region (16). In the C-terminus, Xin α s and Xin β s are divergent relative to each other but each isoform from mammals shows a high degree of within isoform sequence identity (16). These results suggest different but conserved functions for mammalian Xin α and Xin β .

3.5. *mXinβ* messages are preferentially localized to ICDs and enriched in cells of the left ventricle, interventricular septum and apex, particularly near the base of the aorta and pulmonary artery of the heart

We have previously shown by *in situ* hybridization that *mXinβ* mRNAs are specifically expressed at discrete regions in cardiomyocytes that highly resemble ICDs (17). On the other hand, only a less extent of *mXinα* mRNAs are localized to the ICD-like structures, whereas *cardiac troponin T* messages are never found at ICDs (Figure 5). The localization of *mXinβ* mRNAs to the ICD, where the protein is utilized, may provide an efficient way for mXin protein localization, given the fact that mXin is also capable of binding to actin filaments. Although the molecular mechanism for this ICD localization of *mXinβ* RNAs remains to be determined, it may suggest an initiating role for mXin β protein in the formation of ICDs (more discussion below).

To further identify which region of the heart mXin β is preferentially expressed, serial sections of the entire hearts were prepared for *in situ* hybridization. At high magnification, positive hybridization signals were

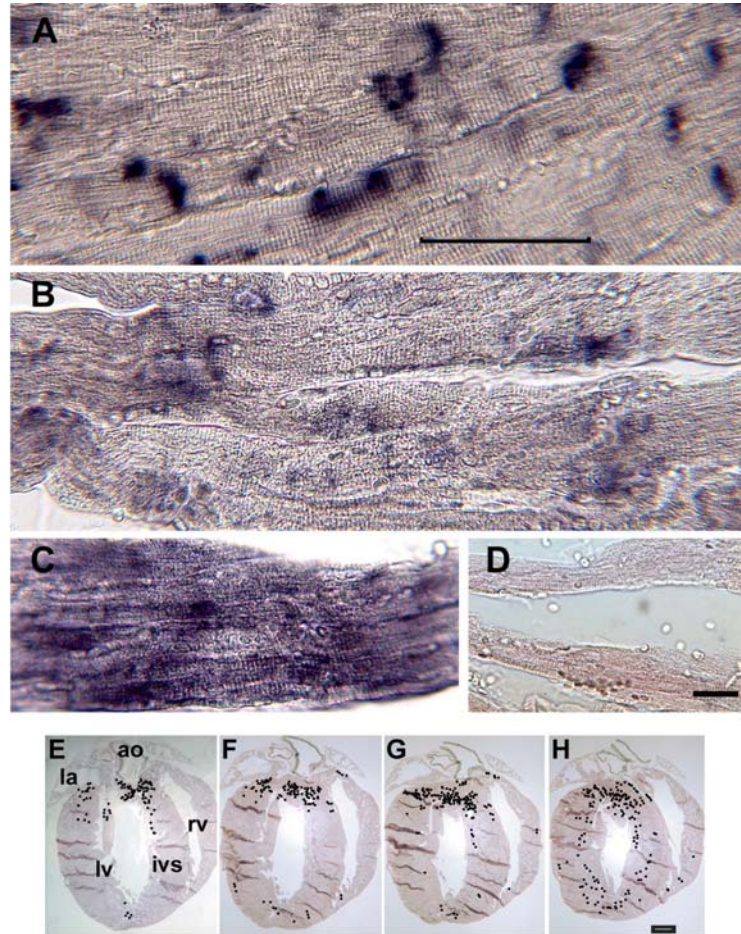


Figure 5. *mXinβ* RNA was specifically expressed at the ICD-like region. In situ hybridization of heart sections was performed with digoxigenin-labeled *mXinβ* antisense riboprobe (A), *mXinα* antisense riboprobe (B), *cardiac troponin T* antisense riboprobe (C), or *mXinβ* control sense riboprobe (D). bar in A = 50μm for A~C, bar in D = 25μm. (E~H) In situ hybridization was performed with *mXinβ* antisense riboprobe on serial 15μ-sections of an entire heart (total 264 sections/heart). The locations of *mXinβ* RNAs were carefully and correspondently mapped by black dots on the low-magnification images of serial sections after examining each section at high magnification. (E~H) represents sections from 22 to 25. Bar in H = 0.5mm for E~H. la, left atrium; ao, aorta; lv, left ventricle; rv, right ventricle; ivs, interventricular septum.

again found at discrete ICD-like regions; however, *mXinβ* expression is not uniformly distributed throughout the heart. The location of *mXinβ* mRNAs were carefully and correspondently mapped by “black” dots on the low-magnification images of serial sections while examining each section at high-magnification, examples are shown in Figure 5E-H. Overall, *mXinβ* messages were enriched in the left ventricle (lv), interventricular septum (ivs) and apex, particularly concentrated at the base of aorta (ao) and pulmonary artery, where cardiomyocytes may be subjected to relatively high stress. Relatively low levels of *mXinβ* expression were found in the right ventricle (rv), right atrium (ra) and left atrium (la). The significance of this expression pattern remains to be determined.

3.6. Domain structures of Xin proteins

The *Xin* gene encodes a striated muscle-specific protein containing a region with 15~28 Xin repeats. The Xin repeat defines a new class of actin binding domains

with a minimum of 3 repeats required to bind actin filaments (146). The *mXinα* is further shown to not only bind but also bundle actin filaments (89). The pink box shown in Figure 6 represents the Xin repeat region found in all Xin proteins from chick and mouse. Within the Xin repeat region, there is a highly conserved β-catenin-binding domain (β-catBD, indicated by a light green box), which has been mapped previously on *mXinα* (17). Similar to *hXinα* (149), *mXinα* undergoes an unusual intraexonic splicing of exon 2 to generate two variants differing in its C-terminus (17). The larger variant is termed *mXinα-a*, which contains a region homologous to the filamin c-binding motif (red box in Figure 6) identified in *hXinα* (149); however, this filamin c-binding motif is not found in *cXin* and *mXinβ* (16). Another feature of all Xin repeat-containing proteins is the existence of multiple proline-rich (PR) regions. The highly conserved PR1 or Mena/VASP-binding domain (E/V BD in Figure 6) at the N-terminus has

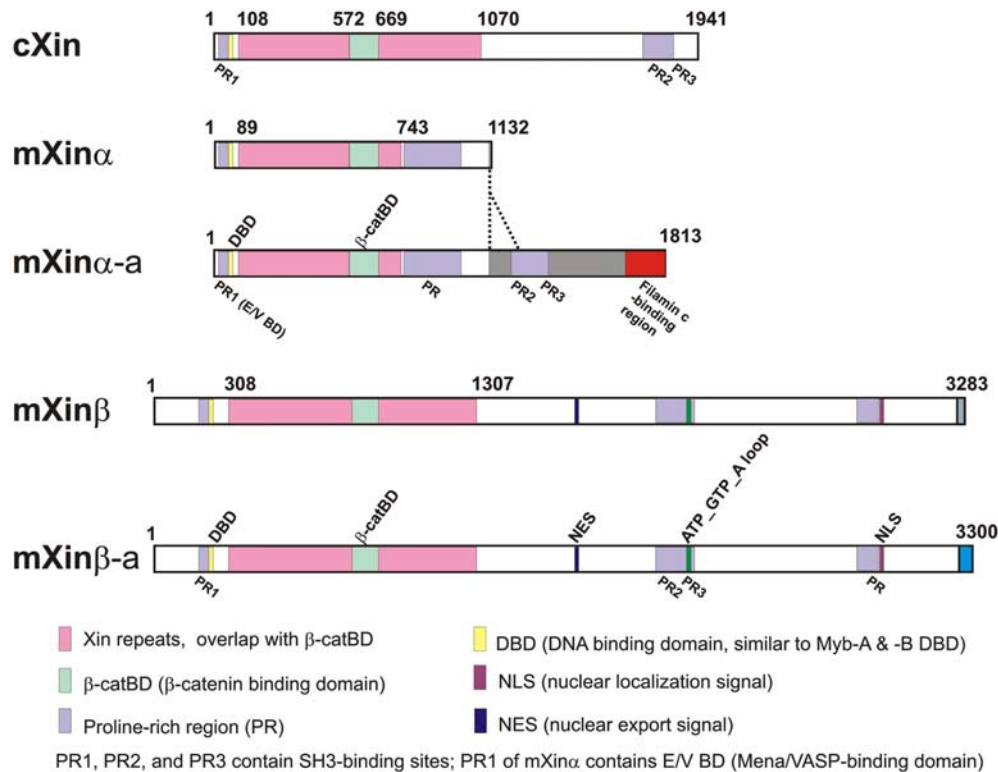


Figure 6. Domain structures of Xin from chick and mouse. Amino acid residue numbers are labeled above on each Xin protein diagrams. Through alternative splicing, both *mXin α* and *mXin β* genes encode two protein variants, which differ only in the very C-terminal sequences. The actin-binding motifs are contained in the Xin repeat region (indicated by pink box), within which a conserved β -catenin binding domain (β -catBD, indicated by light green box) is located. Other binding domains defined on large variant of human Xin α (hXin α -a) include filamin c-binding region (indicated by red box in mXin α -a) and Mena/VASP-binding domain (PR1, E/V BD, indicated by purple box in all Xin proteins). The functions of other proline-rich regions (PR, PR2, PR3) and other consensus sequences such as DNA-binding domain (DBD), nuclear export

been shown to bind to Mena/VASP proteins (16, 149). Interestingly, the sequences downstream of the E/V BD are highly conserved among all Xin proteins (indicated by yellow box in Figure 6) and homologous to the Myb DNA-binding domain, despite that the function of this putative DNA binding domain (DBD) is unknown. The roles of the other PR regions including PR2, PR3 and PR distributed at the C-terminus of the protein remain unclear.

In *mXin β* , alternative splicing of the primary transcript leads to an inclusion or exclusion of exon 8 and results in two protein variants differing in its very C-terminus (18). The large variant is called mXin β -a. The significance of this difference remains to be determined. Both mXin β and mXin β -a also possess consensus sequences for nuclear export signal (NES), nuclear localization signal (NLS) and ATP/GTP-binding domain (ATP_GTP_A loop) (18); however, the functions of these domains are still unknown.

3.7. ICDs are formed postnatally

Adult ventricular cardiomyocytes are rod-shaped, highly bi-polarized cells. A structural and functional segregation exists between parts of the plasma membrane that are either parallel or perpendicular to the long axis of

the cell. The membrane that is parallel to the long axis (lateral membrane) associates with the extracellular matrix through costameres. On the other hand, the membrane that is perpendicular to the long axis (terminal membrane) forms the highly specialized ICDs that mediate cell-cell adhesion and communication (3). Early observations with transmission electron microscopy had hinted that formation of ICDs is a late event of cardiac development. With the electron microscopy, Legato found that in neonatal dog hearts, cardiomyocytes were tightly packed together and extensively contacted each other, contrasting with the limited cell-cell contacts at the ICDs in adult hearts (166). Forbes and Sperelakis noticed that the ICDs of the P2 mouse hearts were more primitive than adult ICDs, in that the former had less inter-digitation and less dense cytoplasmic plaques (1). However, electron microscopy could only reveal ICD components that are already incorporated into specialized intercellular junctions; thus the extent of developmental changes during ICD formation had largely been overlooked until specific antibodies against ICD components were utilized to study the development of ICDs.

Although all three types of intercellular junctions localized to the adult ICDs could be identified by electron

Intercalated disc and Xin repeat-containing protein

microscopy in mouse cardiomyocytes at embryonic day 10 (E10) (1), recent studies with specific antibodies demonstrated that ICDs are formed through a series of events that happen mainly postnatally. In mice and rats, typical adult ICDs are not completely formed until P90, while in humans, maturation of ICDs continues until age 7 (21, 167-169). The formation and maturation of ICDs, in essence, is a process of specialization of the subdomains of the cardiomyocytes' membrane. During the formation of ICDs, cardiomyocytes redistribute the adhering cell-cell junctions (adherens junctions and desmosomes) and gap junctions to the terminal ends, and through poorly understood processes, further recruit an extensive panoply of junctional, channel, signaling and auxiliary proteins to the ICDs.

3.7.1. Redistribution of Junctional components during ICD formation

3.7.1.1. Adherens Junctions

The leading role of adherens junctions in the hierarchy of establishment and maintenance of different intercellular junctions has been demonstrated in various *in vitro* and *in vivo* systems, including cardiomyocytes (102, 170-172). Thus, the developmental changes of adherens junctions are particularly important when studying ICD formation. Indeed, the components of adherens junctions are extensively redistributed during embryonic and postnatal development in the heart. Consistent with the tight packing and extensive contacting of cardiomyocytes in embryonic hearts (166), immunofluorescence staining localizes N-cadherin to the cardiomyocyte surface almost homogeneously at E10.5 (148). Later during embryonic development, N-cadherin staining becomes heterogeneous and the cardiomyocyte surface is characterized by bright spots interspaced by weakly and diffusely stained areas (148). These bright spots likely represent N-cadherin clusters (and their intracellular partners) involved in strong homophilic interaction with opposing cells. On the other hand, the adherens junctional components not incorporated in such bright spots may represent N-cadherin molecules that are yet to be clustered and engaged in strong cell-cell adhesion. Consistent with this idea, adherens junctions identifiable in transmission EM micrographs only occupy a small fraction of the cell-cell contacting interface (166), despite the extensive staining of N-cadherin on the surface of cardiomyocytes during these embryonic stages.

The extensive coexistence of brightly stained spots and more diffusely distributed N-cadherin signals can be found on virtually the entire surface of cardiomyocytes until P3.5 (21, 148, 167, 168). The trend for N-cadherin to become heterogeneously distributed continues after P3.5, leading to the loss of N-cadherin staining at some lateral regions in the P5.5 mouse heart. However, at P5.5, very few ICD-like features can be found and the N-cadherin spots are still extensively scattered on the entire surface of cardiomyocytes, suggesting that ICD formation requires further steps.

At P7.5, a few contacts between the longitudinal termini of cardiomyocytes are strongly stained for N-cadherin, which also adopt a transverse orientation relative to the long axis, resembling the ICDs in adult hearts.

Compared to P3.5, the number of N-cadherin spots and the intensity of the N-cadherin signal in them decrease at the lateral surface of the cardiomyocytes. The diffusely stained N-cadherin at the lateral membrane of the cardiomyocytes also occupies smaller areas. By P15.5, a majority of the ventricular cardiomyocytes form ICD-like structures, suggesting ICDs are established rapidly between P7.5 and P15.5. Coinciding with the intense staining of N-cadherin at the cell termini, the diffuse staining at the lateral surface disappears and the number and intensity of the lateral spots greatly decreases.

The switch of N-cadherin distribution from the lateral membrane to maturing ICDs is also supported by quantification of the N-cadherin signal at these loci by confocal imaging. The proportion of the integrated density of the N-cadherin signal (pixel number \times intensity) at the maturing ICDs increases slowly during the first week of postnatal development and then rapidly in the second week and reaches a plateau after one month of ages (our unpublished observation).

3.7.1.2. Desmosomes

The dependence of desmosomes on the adherens junctions for their formation and maintenance has been well documented. Consistent with this, the time course of incorporation of desmosomal components into the ICDs seems to follow that of adherens junctions (167). However, the establishment of area composita and thus the almost perfect co-localization between adherens junctions and desmosomes by immunostaining seems to be a late event during postnatal development. Indeed, the amalgamation of the transmembrane proteins N-cadherin and desmoglein-2 is an ongoing process even at 3 weeks postnatal in mice, even though both proteins are highly localized to the cell termini at earlier stages (21).

3.7.1.3. Gap junctions

The developmental redistribution of gap junctions is one of the first phenomena noticed by researchers demonstrating the late maturation of ICDs (169). The incorporation of gap junctions into the cell termini seems to happen much later than the adherens junctions and desmosomes, and this phenomenon is shared by different mammalian species examined so far. In the mouse, at 3 weeks postnatal development, prominent Cx43 staining is seen as large puncta located at both the ICD and the lateral surface (167). Quantitative analysis of the distribution of adherens junctions and gap junctions confirmed delayed incorporation of gap junctions to the cardiomyocyte termini (167, 169). Interestingly, through double immunostaining, we have noticed that at three weeks of age the lateral gap junction puncta are associated with weakly labeled N-cadherin spots (unpublished observation). This observation suggests that the delayed incorporation of gap junctions may in fact be a manifestation of the N-cadherin mediated cell-cell contacts that persist on the lateral surface of cardiomyocytes at this time.

3.7.2. Molecular mechanisms of ICD formation

As reviewed above, the components of adherens junctions seem to undergo two phases of redistribution in

order for ICDs to form. During embryonic and early postnatal development, adherens junction components form discrete spots from a diffusely localized pool. Next, the contacts at the cell termini expand to form mature ICDs while the lateral spots reduce both in number and signal intensity. Little is known about the molecular mechanisms controlling these processes. However, since cadherin mediated cell-cell interaction is fundamental in multicellular organisms, many principles learned from other models of adherens junction establishment, maintenance and regulation likely hold true for cardiomyocytes.

The process of forming brightly stained spots and the reduction of diffusely-stained N-cadherin on the lateral surface of cardiomyocytes might be mediated by cadherin clustering. Cadherin clustering is a critical aspect in adherens junction establishment and maintenance; however, its mechanism is still under debate. One model suggests that the cadherin extracellular domain interacts weakly with neighboring cadherin molecules and the intracellular domain is required for lateral clustering (173, 174). Yet studies with tailless E-cadherin mutants indicate that the extracellular domain of cadherin can form strong dimers; clustering and incorporation into cell-cell junctions might be an intrinsic property of cadherin's extracellular domain (175, 176) and the clustering of cadherin at the initial stage of junction formation is mediated by a diffusion-mediated trapping mechanism (177). Since extensive evidence supports a critical role of the cytoplasmic tail of cadherin in clustering, it is likely that for intact cadherin molecules, their intrinsic clustering tendency is tightly regulated by intracellular mechanisms. Supporting this idea, it was shown that in 4-cell stage embryos, E-cadherin and other adherens junctional components are diffusely distributed on the entire surface of each blastomere. Junctional assembly is initiated at the 8-cell stage when compaction starts and protein kinase C plays a critical role through regulating β -catenin and other factors (178). Interestingly, the formation of N-cadherin clusters in the embryonic heart is akin to E-cadherin clusters in the compacting embryo. Therefore, it might be worthwhile investigating if similar mechanisms exist in the heart for the formation of N-cadherin clusters.

In epithelial cells, it has been shown that E-cadherin clusters are highly dynamic structure. New E-cadherin molecules are continuously added to the clusters and ATP-dependent mechanisms maintain the size of clusters by actively removing E-cadherin molecules from the cluster (175). Depletion of ATP in cells leads to very large cadherin clusters, suggesting cadherin cluster size is a result of dynamic equilibrium (175). The active removal of cadherins from clusters requires both endocytosis and other unidentified mechanisms (175, 179). If similar mechanisms exist for N-cadherin clusters in the heart, they could have important implications for the formation of ICDs. In principle, tipping the balance between N-cadherin clustering and dissociation could account for both the reduction of N-cadherin spots at the lateral surface of cardiomyocytes and the expansion of adherens junctions at the cell termini. Indeed, the role of cadherin endocytosis

and recycling has been shown to be critical for epithelial junction formation and remodeling (180, 181). It has been well recognized that p120-catenin is the central player regulating internalization of cadherins (182). Thus, molecular partners of p120-catenin are also likely to be important regulators of cadherin stability.

The actin cytoskeleton plays an important role in the establishment and maintenance of adherens junctions. Therefore, regulating the interaction between cadherin-catenin complexes with the actin cytoskeleton may also be an important aspect in adherens junction remodeling during ICD formation. Although it has been shown that a direct and stable link between the cadherin- β -catenin complex and actin filaments mediated solely by α -catenin is unlikely (183, 184), emerging evidence indicates that molecules other than or in addition to α -catenin can stably couple the cadherin complex to the underlining actin filaments. Cavey and colleagues demonstrated in *Drosophila* embryonic epithelial cells that spot adherens junctions (SAJs) mediated by DE-cadherin is tightly coupled to very stable, actin depolymerizing drug-resistant actin patches (need to add ref 183 here). Since SAJ's stability is α -catenin independent, Cavey *et al.* suggested that a factor "X" is responsible for connecting SAJs to the actin patches (185, 186). One candidate for the factor X in mammalian epithelial cells might be the protein EPLIN, which binds to both α -catenin and actin (187). Abe and Takeichi showed that EPLIN directly couples the E-cadherin- β -catenin- α -catenin complex to actin filaments (need to add ref 184 here). Interestingly, after treating the cells with actin depolymerizing drugs, the adhesion belt became fragmented and formed spots resembling the SAJs in *Drosophila*. It was shown that EPLIN stabilized the actin patches underling the cadherin clusters in drug treated cells (187). One difference between Abe and Cavey's studies is that in order to stabilize adherens junctions, EPLIN requires α -catenin but the factor X does not. Potentially, the factor X in *Drosophila* has a partner in the cadherin complex other than α -catenin. Thus, these studies suggest that direct coupling to the actin cytoskeleton is critical for the stability of the cadherin complex and the specific molecular link might be cell type-dependent. In the heart, we have shown that mXin α can stabilize adherens junctions. Because mXin α directly interacts with β -catenin and actin filaments (89), mXin α may realize this function by acting as the factor X in the cardiomyocytes. Considering that mXin β also contains the highly conserved β -catenin interaction domain (16), it is possible that mXin β may also act as a stable link between the adherens junctions and actin filaments.

In addition to regulating adherens junction stability at the lateral surface and in maturing ICDs, cardiomyocytes likely promote adherens junction expansion at the maturing ICDs through active mechanisms. In epithelial cells, Rho family small GTPases have been shown not only to mediate signals initiated by cadherin engagement (177) but also to drive adherens junction initiation and expansion (188). Rac activity promotes lamellipodia formation, which promotes contact formation, whereas RhoA regulates contact expansion

through regulating actomyosin contractility underlining the forming adherens junctions (188). It has been shown in non-muscle cells, that the small GTPase, Rap1, acts upstream of Rac and Rho to regulate adherens junctions (189, 190). Interestingly, Rap1 mediated signals have been shown to enhance adherens junction formation followed by gap junction establishment in cardiomyocytes (191), suggesting that the roles of these small GTPases are likely conserved in cardiomyocytes.

An important question about ICD formation is how do cardiomyocytes establish polarity? Cardiomyocytes are highly polarized cells with distinct lateral and terminal membrane domains. The mechanism that defines these membrane domains is poorly understood. One possibility is that epithelial apical-basal polarity regulators also function in cardiomyocytes. Indeed, the polarity protein Scribble was localized to the entire lateral surface but was excluded from ICDs in adult cardiomyocytes (192). It was shown that mutation of Scribble led to a temporary mis-localization of N-cadherin in embryonic hearts of mice. Unfortunately, the role of Scribble in ICD formation was not studied due to the early lethality of *Scribble* mutants (192).

On the other hand, it was firmly established that mechanical stretch plays critical roles in establishing and maintaining polarized positioning of junctional components in cultured cardiomyocytes and the small GTPase, Rac1, is an important mediator in this process (193-195). Nevertheless, it is still unclear how cardiomyocytes transduce mechanical stretch to localized signaling that dictates the positioning of cell-cell junctions.

3.7.3. Roles of mXin β in ICD formation

3.7.3.1. mXin β is required for ICD formation

Previously, our studies indicate that mXin β is an important factor for ICD maturation (18). In the *mXin β* -null heart at P16.5, ICDs fail to form. In contrast to the terminal localization of N-cadherin in wild-type cardiomyocytes, mutant cardiomyocytes exhibit numerous N-cadherin puncta on the lateral surface. Accompanying the mis-localization of N-cadherin, gap junctions and desmosomes are also scattered on the lateral surface and fail to concentrate at the termini. Our recent time course observation of N-cadherin distribution between P3.5 and P24.5 in the mutant heart showed that N-cadherin localization is indistinguishable from the wild-type heart up to P7.5, at which time cardiomyocytes are characterized by N-cadherin spots all over the surface and patches of diffusely localized N-cadherin. Therefore, mutant cardiomyocytes likely preserve the ability to carry out such process up to P7.5. However, at P13.5 when wild-type cardiomyocytes form morphologically distinguishable ICDs at their termini, very few ICDs can be found in the mutant heart; instead, the N-cadherin spots are still scattered on the surface of the mutant cardiomyocytes. Quantification showed that the proportion of N-cadherin localized to the termini of cardiomyocytes remain largely unchanged in mutant cardiomyocytes from P13.5 to P24.5, while wild-type cardiomyocytes exhibit a continuous increase of N-cadherin at the termini. These observations

suggest that loss of mXin β results in a failure to localize N-cadherin at the termini of cardiomyocytes as well as defects in the reduction of laterally localized N-cadherin spots. However, N-cadherin clustering into spots seems to be independent of mXin β .

Distribution of desmoplakin and connexin43 in developing hearts indicated that both desmosomes and gap junctions are scattered on the surface of cardiomyocytes by P24.5. Interestingly, double labeling showed that the associations between desmosomes and adherens junctions as well as between gap junctions and adherens junctions are not different between wild-type and mutant hearts, suggesting that the laterally localized N-cadherin clusters were capable of organizing desmosomes and gap junctions.

Supporting the role of mXin β in the redistribution of N-cadherin to the cell termini and the expansion of ICDs, a process that is most rapid during the second postnatal week, we showed that mXin β expression is significantly upregulated at P7.5 and P13.5 (18). We have further extended this observation with quantitative Western blot analysis and showed that two expression peaks exist for mXin β postnatally. The level of mXin β protein reached the first peak at P13.5, followed by a significant reduction at P15.5 only to increase again to reach adult level. Most importantly, we found that at P7.5, mXin β was highly localized to the cardiomyocytes' termini where it is co-localized with N-cadherin. On the other hand, the lateral surface of cardiomyocytes contains little mXin β . From P7.5 to adulthood, mXin β is always localized to the termini of cardiomyocytes. Taken together, restricted localization of mXin β to the termini of cardiomyocytes proceeds that of N-cadherin, suggesting that mXin β is required for forming ICDs at the cell termini.

3.7.3.2. Potential mechanism by which mXin β mediates ICD formation

How can mXin β mediate the redistribution of N-cadherin to form the ICDs? As described in section 3.5 (Figure 5) and Supplemental Figure 2B in (17), *mXin β* but not *mXin α* messages are preferentially localized to the ICDs, suggesting an efficient way for mXin β protein to initiate ICD localization. This process is further enhanced by the postnatal surge of mXin β expression at the onset of redistribution of N-cadherin clusters/puncta to cell termini (18). Through its Xin repeats and conserved β -catenin binding domain (16), mXin β could directly interact with actin filaments and β -catenin, linking N-cadherin clusters to the underlying actin cytoskeleton. Further stabilization of N-cadherin clusters at the cell termini to form ICDs may require a collaborative role for mXin α , because mXin α , similar to N-cadherin, is mis-localized in *mXin β* -null hearts (18). Previously, we have also shown that mXin α can directly interact with both β -catenin and p120-catenin (89) and both catenins are known to be necessary for assembling and maintaining cadherin on the cell surface (182, 196). Thus, by collaborating with mXin α at the cell termini, mXin β may further enlist the catenins for the localized stabilization of adherens junctions and thus facilitate ICDs to be formed. Moreover, our observations that local activity of Rac1 at the forming ICD was significantly down-

regulated in the *mXin β* -null hearts may suggest an additional role of mXin β in regulating Rac1 activity for ICD expansion (18). In summary, mXin β may mediate ICD formation by initiating the redistribution of N-cadherin to cell termini, collaborating with mXin α to further stabilize the forming adherens junctions, and regulating Rac1 activity to expand the ICD membrane.

3.8. mXin plays important roles in the surface expression of ion channels

mXin proteins are co-localized with N-cadherin and Cx43 in the myocardium of the mouse (148). Loss of mXin α causes a decrease in the expression level of β -catenin, N-cadherin, and desmoplakin in the adult heart, weakening cardiomyocyte adhesion and compromising ICD integrity. Consequently, Cx43 in *mXin α* -null hearts is mis-localized, which may lead to cardiac gap junction remodeling and conduction defects (17).

Studies have revealed a more detailed mechanism by which mXin α affects the electrophysiology of the heart. Whole-cell patch-clamp recordings on ventricular myocytes obtained from 10~20-week-old mice reveal that *mXin α* -null cells have an increased inward Na⁺ current (I_{Na}), a reduced transient outward K⁺ current (I_{TO}), a weaker L-type Ca²⁺ current ($I_{Ca,L}$) and a smaller inward rectifier K⁺ current (I_{K1}) densities (140, 197, 198). In addition, the amplitude of the intracellular Ca²⁺ transient decreases significantly in *mXin α* -null myocytes prepared from ventricles and left atrial-pulmonary veins (LA-PV) (140). Optical mapping analyses reveal that the conduction velocity is significantly slower in *mXin α* -null ventricles and LA-PV as compared to wild-type (198, 199). These data suggest that mXin α plays an important role in regulation of channel activity in both ventricular and atrial myocytes.

mXin α interacts with different proteins that regulate the surface expression of ion channels, which may account for the altered electrophysiological properties of *mXin α* -null cardiomyocytes. Yeast two-hybrid interaction assays reveal that mXin α interacts with KChIP2, an auxiliary subunit of the I_{TO} channel (140). It is also known that KChIP2 interacts with Kv4.2, the pore-forming α -subunit of the I_{TO} channel, and maintains the surface expression of I_{TO} (200, 201). Consistently, loss of mXin α decreases the expression of KChIP2 protein and *KChIP2* message, leading to significant reduction in the surface expression of the I_{TO} channel (140). Furthermore, mXin α has been shown to bind to filamins and actin filaments (89, 149); thus, mXin α affects the I_{TO} current activity by interacting with and stabilizing the KChIP2 and filamin proteins.

The role of mXin α in regulating ion channel surface expression and function may be analogous to that of the actin-binding protein ankyrins. The ankyrins interact with actin filaments and spectrin, and together with their associated proteins form a membrane cytoskeleton, which plays an important role in the targeting of ion channels, transporters and cell adhesion molecules to specialized

compartments within the plasma membrane. Mutations affecting the association between ankyrins and ion channels alter the functions of ion channels and result in cardiac arrhythmia. For example, the human *SCN5A* missense mutation which is defective in binding to ankyrin-G leads to reductions of I_{Na} at T-tubes and ICDs and results in Brugada syndrome (202). mXin α interacts with actin filaments and maintains the structure of ICDs; thus, the increase of I_{Na} current density in mXin α -null ventricular myocytes may result from an altered cytoskeletal structure of the ICDs.

Alterations of channel activity may induce cardiac electrical remodeling. Our previous studies using optical mapping (198) found that the mXin α -deficient mice had a hypertrophied ventricular myocardium with reduced conduction velocity. Similarly, the conduction velocity was also reduced in LA-PV tissues from *mXin α* -null hearts as compared to wild-type hearts (199). The latter study also found that *mXin α* -null LA-PV have a larger area of conduction block than the control (defined as the area with a conduction velocity ≤ 10 cm/s; (203)). Finally, when we used isoproterenol to activate the β -adrenergic receptors, *mXin α* -null LA-PV tissues were less responsive to the adrenergic induction of atrial fibrillation as compared to wild-type LA-PVs.

It is known that loss of Cx43 or N-cadherin decreased the degree of coupling and conduction characteristics. Thus in *mXin α* -null mice, decreased cardiac conduction velocity may partly result from decreased expression of N-cadherin and Cx43 proteins.

4. XIN AND CARDIAC DISEASES

ICDs are essential structures unique to cardiac muscle, which enable mechanical coupling and chemical communication between cardiomyocytes (3). It is known that mutations or deficiencies in its components give rise to many types of cardiomyopathy and other fatal heart diseases (40, 102, 204-207). However, the molecular mechanisms for most of the proteins responsible for normal cardiac function remain to be elucidated. The human ortholog, *hXin α* or *Cardiomyopathy-associated 1* (*CMYA1*), of *mXin α* has been mapped to chromosome 3p21.2-22.2 by radiation hybrid analysis (145) or to 3p22.2 by DNA sequence, which is near the loci for dilated cardiomyopathy with conduction defect-2 (3p22-25) (208) and ARVD-5 (3p23) (209). Genome-wide association studies suggest a locus in or adjacent to *ULK4* (3p22.1) associated with diastolic blood pressure (210). *mXin α* -null mice are viable and fertile, but develop adult late-onset cardiomyopathy with conduction defects as well as up-regulation of mXin β (17). Despite being viable, mXin α -deficient mouse hearts exhibit progressive ICD structure defects, which may account for the late-onset cardiac hypertrophy and cardiomyopathy. However, juvenile *mXin α* -null mouse hearts show neither hypertrophy nor cardiomyopathy, but electrophysiological studies on these mutant cardiomyocytes detect alterations in their channel properties, particularly for the depression of the I_{TO} current density (140). The underlying mechanism for this

depression likely involves the ability of mXin α to interact with KChIP2, an auxiliary subunit of the transient outward potassium channel, and filamin, an actin-crosslinking protein (140). Therefore, the *mXin α* knockout mouse may represent a model for human dilated cardiomyopathy with conduction defect-2.

Ablation of *mXin β* can lead to ventricular septal defects, cardiac diastolic dysfunction, severe growth retardation and postnatal lethality (18). The human ortholog, *CMYA3* (*XIRP2*), of *mXin β* is mapped to 2q24.3, whose nearby disease-associated loci (Online Mendelian Inheritance in Man) include dilated cardiomyopathy 1H (2q14-q22), 1G (2q24.3) (211) and ARVD-4 (2q32.1-32.3) (212). Human patients with monosomy 2q24 (chromosome band 2q24 deletion) also exhibit severe growth retardation and heart anomalies, including ventricular septal defects (<http://www.orpha.net/data/patho/GB/uk-2q24.pdf>). The genome-wide linkage analysis of a large Kyrgyz family with premature hypertension reveals candidate localization on 2q24.3-q31.1 for a gene conferring susceptibility to this disorder (213). Further studies are warranted to characterize the involvement of mXin β in cardiac development, function and disease.

5. CONCLUSIONS AND PERSPECTIVES

The Xin proteins are modular in nature, contain many copies of a 16-amino acid “Xin” repeating unit, which defines a novel actin binding domain, and localize to the ICDs. Together with their ability to interact with Mena/VASP, filamin b & c, gelsolin, tropomyosin, and vinculin (89), the Xin proteins likely provide an effective way to regulate actin dynamics underneath the ICD membrane. The highly conserved β -catenin-binding domain maps within the Xin repeat region (16, 89) and the ability to bind and bundle actin filaments can be enhanced by the presence of β -catenin (89). These findings further suggest that the Xin proteins play a novel role in linking the actin cytoskeleton to N-cadherin-mediated adhesions as well as in regulating ICD stability in the heart. The existence of a p120-catenin interacting domain distinct from the β -catenin binding domain on mXin α provides another level of regulation for the Xin proteins in N-cadherin turnover and signaling pathways through ICDs (unpublished observations). In addition, mXin α is capable of interacting with KChIP2, filamin and actin filaments (89, 140) and then influencing surface expression of the I_{TO} current (140). I_{TO} depression detected in mXin α -deficient ventricular myocytes appears to be independent of cardiac hypertrophy and/or ICD structural defects (140). Therefore, the effects of mXin α on the channel activity of I_{TO} and I_K likely provide a reasonable account for the conduction defects observed in the mXin α -deficient mice. Interestingly, a complete loss of mXin α prevents the induction of atrial fibrillation in the LA-PV tissues (199). A slower conduction velocity and many conduction blocks observed in the *mXin α* -null LA-PV tissues (199) may account for the lack of re-entrance rhythms and thus atrial fibrillation. However, the molecular mechanism of how mXin α is involved in the genesis of atrial fibrillation

remains to be elucidated. Accumulated lines of evidence suggest the existence of a functional hierarchy between mXin α and mXin β . Hearts without mXin β fail to form mature ICDs and result in a mis-localization of mXin α . On the other hand, hearts without mXin α develop late-onset ultrastructural ICD defects, but mXin β is up-regulated and localized normally to ICDs. The molecular basis of this functional hierarchy remains to be determined.

Based on our studies of *mXin* knockout mouse lines and other molecular characterizations of mXin proteins, we hypothesize that mXin β initiates the formation of ICDs, whereas mXin α further stabilizes the ICD. Future detailed analysis of the binding/interacting domains on Xin proteins should advance our understanding toward the mechanisms by which mXin proteins function. For example, in addition to binding to β -catenin and p120-catenin (89), mXin α may interact with and recruit p0071, another member of the p120-catenin subfamily of armadillo related proteins found in the heart, to facilitate the ICD maturation and the stability of the N-cadherin-based adhesion. The potential interactions of Xin proteins with p120-catenin and/or p0071 may modulate effectors such as Vav2 (73, 74) and Ect2 (69) to regulate Rac1 and Rho activity, respectively. Further studies of whether mXin α directly interacts with Cx43 and/or ZO-1 may explain the gap junction remodeling (decrease in Cx43 amounts and altered localization) observed in the *mXin α* -null hearts.

Generation and characterization of conditional *mXin β* knockout mice will allow us to further examine the role of mXin β in maintaining ICD integrity in the adult heart. Considering that conditional deletion of N-cadherin in the adult heart leads to total dissolution of ICDs (102), and that postnatal hearts without mXin β fail to form ICDs (18), we anticipate compromised ICD integrity in cardiac-specific *mXin β* conditional knockout mice. The information obtained may also reveal the molecular mechanisms underlying the adult ICD (and/or gap junction) remodeling often associated with cardiomyopathy and heart failure.

mXin α -deficient mice exhibit similar cardiac phenotypes to human dilated cardiomyopathy with conduction defect-2, whereas *mXin β* -null mice die around weaning and exhibit congenital heart defects and severe growth retardation. The future study of single nucleotide polymorphisms on *CMYA1* and *CMYA3* from human populations with cardiomyopathy and conduction defects or with congenital heart defects may potentially define these genes as disease-causing genes. Therefore, it is therefore conceivable that the knowledge gained on the role of Xin proteins in cardiac development and function will provide new insights for improved therapeutic strategies for human cardiomyopathy, arrhythmias and heart failure.

6. ACKNOWLEDGEMENTS

This work was supported, in part, by NIH grants HL075015 and HL107383 (to JJCL) and by National Science council (Taiwan) grants NSC96-2320-B016-013 and NSC98-2320-B016-010-MY3 (to CIL).

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Intercalated disc and Xin repeat-containing protein

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Abbreviations: ICD: intercalated disc, mXin α and mXin β : mouse Xin α and Xin β , PKP2/4: plakophilin 2/4, Cx43: connexin 43, ARVC(D): arrhythmogenic right ventricular cardiomyopathy (dysplasia), Tcf/Lef: T cell factor/lymphoid enhancer factor, siRNA: small interference RNA, GEF: guanine nucleotide exchange factor, ZO-1: zonula occludens-1, EMT: epithelial-mesenchymal transition, BMP: bone morphogenetic protein, FGF: fibroblast growth factor, I_{TO}: transient outward potassium current, KChIP2: Kv channel interacting protein 2, HH st.: Hamberger and Hamilton stage, AVC: atrioventricular canal, hXin α /CMYA1/XIRP1: human Xin α /cardiomyopathy associated 1/Xin actin-binding repeat containing 1, hXin β /CMYA3/XIRP2: human Xin β /cardiomyopathy associated 3/Xin actin-binding repeat containing 2, cXin: chicken Xin, Ang II: angiotensin II, GAPDH: glyceraldehyde 3 phosphate dehydrogenase, D, posthatch day, P, postnatal day, E, embryonic day, lv and rv: left and right ventricles, la and ra: left and right atria, ivs: interventricular septum, ao: aorta, β -catBD: β -catenin-binding domain, PR: proline-rich region, E/V BD (PR1): Mena/VASP-binding domain, DBD: DNA-binding domain, NLS: nuclear localization signal, NES: nuclear export signal, ATP_GTP_A loop: ATP/GTP-binding domain, SAJ, spot adherens junction, I_{Na}: inward sodium current, I_{Ca,L}: L-type Ca²⁺ current, I_{K1}: inward rectifier potassium current, LA-PV: left atrial-pulmonary vein

Key Words: Xin Repeats, Intercalated Disc Formation, Severe Growth Retardation, Postnatal Lethality, Cardiomyopathy With Conduction Defects, Review

Send correspondence to: Jim J-C. Lin, Department of Biology, University of Iowa, 210 E. Iowa Ave. Iowa City, IA 52242-1324, USA, Tel: 319-335-1075, Fax: 319-353-2275, E-mail: jim-lin@uiowa.edu