Essential Roles of an Intercalated Disc Protein, mXinβ, in Postnatal Heart Growth and Survival

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Rationale: The Xin repeat–containing proteins mXinc and mXinβ localize to the intercalated disc of mouse heart and are implicated in cardiac development and function. The mXinc directly interacts with β-catenin, p120-catenin, and actin filaments. Ablation of mXinc results in adult late-onset cardiomyopathy with conduction defects. An upregulation of the mXinβ in mXinc-deficient hearts suggests a partial compensation.

Objective: The essential roles of mXinβ in cardiac development and intercalated disc maturation were investigated.

Methods and Results: Ablation of mXinβ led to abnormal heart shape, ventricular septal defects, severe growth retardation, and postnatal lethality with no upregulation of the mXinc. Postnatal upregulation of mXinβ in wild-type hearts, as well as altered apoptosis and proliferation in mXinβ-null hearts, suggests that mXinβ is required for postnatal heart remodeling. The mXinβ-null hearts exhibited a misorganized myocardium as detected by histological and electron microscopic studies and an impaired diastolic function, as suggested by echocardiography and a delay in switching off the slow skeletal troponin I. Loss of mXinβ resulted in the failure of forming mature intercalated discs and the mislocalization of mXinc and N-cadherin. The mXinβ-null hearts showed upregulation of active Stat3 (signal transducer and activator of transcription 3) and downregulation of the activities of Rac1, insulin-like growth factor 1 receptor, protein kinase B, and extracellular signal-regulated kinases 1 and 2.

Conclusions: These findings identify not only an essential role of mXinβ in the intercalated disc maturation but also mechanisms of mXinβ modulating N-cadherin–mediated adhesion signaling and its crosstalk signaling for postnatal heart growth and animal survival. (Circ Res. 2010;106:1468-1478.)

Key Words: N-cadherin–mediated adhesion signaling ▪ Xin repeat-containing protein ▪ intercalated disc maturation ▪ diastolic dysfunction ▪ postnatal heart growth

A regulatory network of transcription factors is known to control cardiac morphogenesis. Although the core players in this network are highly conserved, from organisms with simple heart-like cells to those with complex four-chambered hearts, it has been theorized and proven that expansion of this regulatory network by adding new transcription factors is a part of the mechanism underlying the formation of complex hearts. The transcription factors must act through their downstream targets, which are directly involved in cardiac morphogenesis, growth, and function. However, our inventory of such downstream targets remains incomplete.

The Xin repeat–containing proteins from chicken and mouse hearts (cXin and mXinc, respectively) were first identified as a target of the Nkx2.5-Mef2C pathway.3,4 Another mouse Xin protein, mXinβ (or myomaxin), has been subsequently identified as a Mef2A downstream target.5 Evolutionary studies suggest that Xin may be one of the factors that arose when the heart evolved from simple heart-like cells to the complex true-chambered hearts.6 Functional studies reveal that Xin proteins are involved in heart chamber formation and cardiac function in vertebrates.3,4,7 The striated muscle-specific Xin family of proteins are defined by the presence of 15 to 28 copies of the conserved 16-aa Xin repeats and originated just before the emergence of lamprey, coinciding with the appearance of the true-chambered heart.6 The Xin repeats are responsible for binding actin filaments,8-10 whereas a highly conserved β-catenin binding domain overlapping with the Xin repeats is responsible for localizing Xin to the intercalated discs.6,9 Supporting the roles of Xin in heart chamber formation and function, we
have previously shown that knocking down the sole cXin in the chicken embryo collapses the wall of heart chambers and leads to abnormal cardiac morphogenesis.  

In mammals, however, a pair of paralogous Xino and Xinβ genes exists. Ablation of the mouse mXino gene does not affect heart development. Instead, the mXino-deficient mice show cardiac hypertrophy and cardiomyopathy with conduction defects during adulthood. In the mXino-deficient mice, mXinβ is upregulated at both message and protein levels, suggesting a compensatory role of mXinβ. Consistent with this idea, both mXin proteins have highly conserved Xin repeats and β-catenin-binding domain, as well as other functionally undefined domains located in the N termini. On the other hand, the C termini of both proteins are more diverged, suggesting that they also have distinct functions. Because mXinβ is more conserved than mXino with the ancestral lamprey Xin that demarked the emergence of heart chamber, we hypothesized that mXinβ might play an essential role in heart morphogenesis. To test this hypothesis, we generated and characterized mXinβ knockout mice. The mXinβ-null mice died before weaning and showed abnormal heart shape, ventricular septal defects (VSDs), misorganized myocardium, and diastolic dysfunction. The mechanisms underlying these cardiac defects involved dysregulation of the N-cadherin–mediated signaling pathway and its crosstalk via abnormally activated Stat3 (signal transducer and activator of transcription 3) and depressed Rac1, insulin-like growth factor (IGF)-1 receptor (IGF-1R), protein kinase B (Akt), and extracellular signal-regulated kinase (Erk)1/2 activities.

Methods

All animal procedures were approved and performed in accordance with institutional guidelines. The mXinβ-null line has been backcrossed to and maintained in C57BL/6J. All of phenotypes observed earlier remained the same.

Cloning of mXinβ cDNAs and genomic fragments; construction of a targeting vector; Southern, Northern, and Western blot analyses; tissue collection; DNA and RNA isolations; histological staining and immunofluorescence; electron microscopy; echocardiography; proliferation; apoptosis; Rho GTPase assays; and data analysis are described in the Online Data Supplement, available at http://circres.ahajournals.org.

Results

Generation of mXinβ-Null Mice

To construct a targeting vector, we cloned full-length mXinβ cDNAs and the corresponding genomic fragments. Alignment of these sequences revealed that the mXinβ gene contains 9 exons and encodes 3 mRNA species (mXinβ-A, mXinβ-B, and mXinβ-C) in adult heart through alternative splicing of exon 8 and alternative usage of polyA signals (Online Figure I, A). Both mXinβ-A and mXinβ-B encode a polypeptide of 3283-aa residues (termed mXinβ), whereas mXinβ-C is predicted to encode a protein of 3300-aa residues (termed mXinβ-a) (Online Figure I, B). By sequencing 24 randomly picked transformants generated from 3′ rapid amplification of cDNA ends, we found 23 clones representing either mXinβ-A or mXinβ-B, suggesting that mXinβ is the major isoform. Force expression of the cloned mXinβ-B cDNAs in CHO cells confirmed that mXinβ-B encoded the protein having the same size as endogenous mXinβ and reacting to anti-mXin antibody (Online Figure I, C). Furthermore, force-expressed mXinβ colocalized with actin filaments to stress fibers and cell cortex (Online Figure I, D). Using multiple tissue Western blot, mXinβ was detected only in the striated muscles such as tongue, heart, and diaphragm (Online Figure II, A). During postnatal heart development, the expression of mXinβ increased at least 3-fold from postnatal day (P)0.5 to P13.5 (Online Figure II, C). The timing of this upregulation of mXinβ coincides with the period for intercalated disc maturation.

A targeting vector was designed to delete the genomic region that encodes the highly conserved β-catenin–binding domain and Xin repeats (Figure 1A). After electroporation and selection, resistant embryonic stem clones were screened by Southern blot analysis (Figure 1B). The positive embryonic stem clone was used to generate chimeric founders. After confirming germline transmission, the heterozygous progeny were further crossed to obtain mXinβ-null mice. The genotypes of the resulting littermates were determined with tail DNAs by Southern blot and by PCR genotyping (Figure 1C).

All mXinβ-Null Mice Die Before Weaning

Northern blot analysis revealed a complete loss of mXinβ message in homozygote and a reduction in heterozygote (Figure 1D). Western blot analyses with antibody U10137 recognizing both mXinα and mXinβ (Figure 1E, top blot) or with antibody U1040 recognizing C terminus of mXinβ (data not shown) verified a complete loss of mXinβ in homozygotes and a reduced level in heterozygotes. The mXinβ-null

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>BrdUrd</td>
<td>bromodeoxyuridine</td>
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<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>cTn</td>
<td>cardiac troponin</td>
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<tr>
<td>E/A</td>
<td>mitral valve E-wave (early filling) to A-wave (atrial contraction/late filling) ratio</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
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<tr>
<td>Erk</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth factor receptor-bound protein 2</td>
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<tr>
<td>HW</td>
<td>heart weight</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>IGF-1R</td>
<td>insulin-like growth factor 1 receptor</td>
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<tr>
<td>Jak2</td>
<td>Janus kinase 2</td>
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<tr>
<td>LW</td>
<td>liver weight</td>
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<tr>
<td>P</td>
<td>postnatal day</td>
</tr>
<tr>
<td>ssTn</td>
<td>slow skeletal troponin</td>
</tr>
<tr>
<td>Stat3</td>
<td>signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TM</td>
<td>tropomyosin</td>
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<tr>
<td>VSD</td>
<td>ventricular septal defect</td>
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hearts expressed the similar amounts of mXinα-a, mXinα, α-actinin, and α-tropomyosin (α-TM) as their age-matched counterparts (Figure 1E).

At birth (P0.5), the number of the mXinβ-null pups from heterozygous crosses was smaller than the expected number (Online Table I); however, this reduction was not statistically significant (P=0.17; χ² test). In contrast, from P3.5 and on, the number of viable mXinβ⁻/⁻ mice was significantly lower than expected. No viable mXinβ⁻/⁻ mice could be observed at weaning stage. Thus, these observations suggest that mXinβ is essential for postnatal mouse survival.

**Loss of mXinβ Leads to Severe Growth Retardation**

The mXinβ-null mice had severely retarded growth and reduced activity. The skin of newborn mXinβ⁻/⁻ mice was apparently paler than their littermates, suggesting a systemic circulation defect. Great vessels in the newborn mXinβ-null mice were normal (Online Figure III). The body weight (BW) of P0.5 mXinβ⁻/⁻ mice was ~14.3% lighter than wild-type or heterozygous littermates (Figure 2A). From birth to P12.5, the mXinβ-null mice also gained weight more slowly than their littermates (Figure 2A). At P12.5, mXinβ-null mice weighed only ~45% of wild-type or heterozygous mice. The loss of just one copy of mXinβ in heterozygotes had neither effect on BW nor on viability. Neonatal mXinβ⁻/⁻ pups apparently breathed normally, and milk was always visible in their stomach, suggesting that a weakness in skeletal muscles is unlikely to be the major cause for the growth retardation and lethality.

The heart weights (HWS) of newborn wild-type and mXinβ-null pups were similar. However, from P3.5 to P12.5, the wild-type hearts grew much faster than mXinβ-null hearts. As a result, both P7.5 and P12.5 wild-type hearts were significantly larger than mXinβ-null counterparts (Figure 2B), suggesting that mXinβ is required for postnatal heart growth. Similar to its effects on BW, the loss of one copy of mXinβ in heterozygotes did not affect their heart size (data not shown). The HW/BW ratio of mXinβ⁻/⁻ mice at most of postnatal stage except P3.5 was significantly higher than that of wild-type mice (Figure 2C) because of significantly smaller BW in mXinβ-null mice. Similar to the hearts, non-mXinβ-expressing organ such as liver (Figure 2D) of mXinβ-null mice also became significantly smaller between P3.5 and P7.5. However, the liver weight (LW) to BW (LW/BW) ratios of wild-type and mXinβ-null mice remained no difference (Figure 2E). Thus, the loss of mXinβ affected the mXinβ-expressing and non–mXinβ-expressing organs differently.

**Loss of mXinβ Results in VSDs, Abnormal Heart Shape, and Misorganized Myocardium**

Approximately 15% (5/33) of newborn mXinβ-null hearts had abnormal shape (Figure 3B). VSD was detected in 58% of P0.5 mice were normal (Online Figure III). The body weight (BW) apparently paler than their littermates, suggesting a systemic growth retardation. Great vessels in the newborn mXinβ-expressing and non–mXinβ-expressing organ such as liver (Figure 2D) of mXinβ-null mice at most of postnatal stage except P3.5 was significantly higher than that of wild-type mice (Figure 2C) because of significantly smaller BW in mXinβ-null mice. Similar to the hearts, non-mXinβ-expressing organ such as liver (Figure 2D) of mXinβ-null mice also became significantly smaller between P3.5 and P7.5. However, the liver weight (LW) to BW (LW/BW) ratios of wild-type and mXinβ-null mice remained no difference (Figure 2E). Thus, the loss of mXinβ affected the mXinβ-expressing and non–mXinβ-expressing organs differently.

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(7/12) of newborn mXinβ-null hearts analyzed by serial section analysis (arrow in Figure 3D). The VSD could be found in any locations within the muscular septum, and could be small, large or multiple. However, the VSD could not be the cause of postnatal lethality, because 42% of mXin/H9252-null mice without VSD also became small and weak and died before weaning. Misorganized myocardium (noncompaction in right ventricle) could be detected in mXin/H9252-null hearts as early as embryonic day 14.5 (Figure 3F and 3F/H11032). Thus, mXin/H9252-null embryo may already have a defect in heart function, leading to a slight but significant reduction in BW at birth (Figure 2A). However, this defect may not be enough to cause embryonic lethality, because no significant loss of newborn mXinβ-null mice was found (Online Table I). Furthermore, all mXinβ-null neonatal hearts examined showed various degrees of misorganized myofibers within myocardium (an example shown in Figure 3H and 3H/H11033). Electron microscopic (EM) analysis of P15.5 mXinβ-null hearts detected no sarcomere disorganization within each myocyte (Figure 3J), suggesting no myofilament disarray in mutant hearts.

**Developing mXinβ-Null Hearts Exhibit Diastolic Dysfunction**

Because all mXinβ−/− mice exhibited misorganized myocardium, we next analyzed chamber size, wall thickness, and cardiac function by echocardiography. Because echocardiographic results from wild types and heterozygotes were very similar, we treated them as a control group for the comparison to mXinβ-null group (Online Table II). We observed a reduction in left ventricular internal dimension and volume of both P3.5 and P12.5 mXinβ-null hearts during diastole and systole (Online Table II). In contrast, there was no difference between control and mXinβ-null mice in heart rate, left ventricular posterior wall thickness and interventricular septum thickness (Online Table II). Furthermore, the mXinβ-null hearts had normal or slightly higher systolic function, as determined by the ejection fraction and the fraction shortening (Online Table II). Using pulsed wave Doppler recordings, we found that mXinβ-null hearts exhibited abnormal ventricular filling. In mXinβ−/− mice, the mitral inflow E-wave (early filling) but not A-wave (atrial contraction) peak velocity was reduced (Figure 4), and the E/A ratios were also significantly smaller (Online Table II). These results suggest a diastolic dysfunction in mutant hearts as early as P3.5. However, this diastolic dysfunction was not attributable to increased fibrosis that could stiffen the myocardium, because trichrome staining detected no increase in fibrosis at P11.5 (Online Figure IV).

Developmental changes in ventricular diastolic function correlate well with changes in myoarchitecture (compact versus trabecular areas in ventricles).13 In general, the peak
E-wave velocity is exponentially correlated with the area of compact region per unit myocardium, whereas the peak A-wave velocity is correlated with the area of trabecular region per unit myocardium. Using similar measurement in newborn mXin/H9252-null mice, we found a significant reduction in the area of left ventricle compact myocardium and a trend of increase in the area of left ventricle trabecular myocardium in mutant hearts (Online Table III). Similar trends of decrease in compact area and increase in trabecular area were also observed for right ventricle (Online Table III). These results again support diastolic dysfunction associated with newborn mXinβ-null hearts.

The Delay in Switching off Slow Skeletal Troponin I Also Supports Diastolic Dysfunction Associated With mXinβ-Null Mice

Apparent preservation of systolic function and presence of diastolic dysfunction in mXinβ-null hearts led us to examine the expression levels and isoform switches of contractile and regulatory proteins. The observations of normal expression levels of α-actinin and α-TM (Figure 1E), as well as normal timing of switching from β-myosin heavy chain to α-myosin heavy chain (Online Figure V) and from embryonic cardiac troponin (cTn)T to adult cTnT (Figure 5C) largely support that mXinβ-null hearts having normal
systolic function. In contrast, a significant delay in switching off slow skeletal troponin (ssTnI) was detected in P7.5 and P13.5 mXinβ-null hearts (Figure 5B). This delay may allow mutant hearts to gain increased Ca\textsuperscript{2+}-activated myofilament tension to compensate for function, as suggested from a previous study comparing force generations between ssTnI- and cardiac troponin I (cTnI)-expressing cardiomyocytes.\textsuperscript{14} Nonetheless, transgenic mice ectopically expressing ssTnI in the heart exhibit impairments of cardiomyocyte relaxation and diastolic function.\textsuperscript{15} Together, the delay in switching off ssTnI also supports diastolic dysfunction in mXin/H9252-null heart. It should be noted that mXin/H9252-null hearts did not upregulate N-terminal truncated cTnI (Figure 5B), which has been previously shown to enhance ventricular diastolic function in transgenic mice.\textsuperscript{16}

**Developing mXinβ-Null Hearts Exhibit an Increased Apoptosis and a Decreased Proliferation**

Apoptosis and proliferation contribute greatly to myocardial remodeling during postnatal development.\textsuperscript{17} Thus, we asked whether defects in these processes might contribute to the misorganization of mutant myocardium. The wild-type hearts had high apoptosis only at P0.5, as detected by anti-active caspase 3, which then rapidly declined to a minimal level at P7.5, similar to that of adult heart (Figure 6A, 6A’, and 6E).\textsuperscript{17} In contrast, the level of apoptosis in P0.5 mXinβ-null hearts decreased more slowly and remained significantly higher at P3.5 and P7.5 (Figure 6B, 6B’, and 6E). Using bromodeoxyuridine (BrdUrd) labeling, we found that there was no difference in proliferation rate in mXinβ-null and control hearts until P7.5, at which mutant hearts showed slightly reduced cell proliferation (Figure 6C, 6D, and 6F). Therefore, slightly decreased proliferation and increased apoptosis in mXinβ-null hearts postnatally may in part account for the smaller HW and the misorganized myocardium.

Cardiomyocyte organization was compared from cross-sections of individual cardiomyocytes of similar regions of littermate hearts. The cTnT-positive cardiomyocytes were outlined by anti-laminin for shape and width comparison. At P3.5, there was no detectable difference between wild-type and mXinβ-null cardiomyocytes in either cell shape or cell width (Online Figure VI, A, B, and E). In contrast, by P12.5, mXinβ-/- cardiomyocytes became more irregularly shaped (Online Figure VI, D) and smaller in cell width (Online Figure VI, E).

**mXinβ-Null Hearts Fail to Develop Mature Intercalated Discs**

At the first 2 weeks of age, mXinα, N-cadherin, and β-catenin progressively coalesce to the termini of aligned cardiomyocytes to form mature intercalated discs.\textsuperscript{12} We asked whether mXinβ plays a role in the intercalated disc maturation. In P16.5 wild-type hearts, the majority of mXinβ, N-cadherin, and mXinα (Figure 7A, 7C, and 7E), as well as β-catenin and p120-catenin (data not shown), was already localized to the mature intercalated discs. In contrast, most N-cadherin (Figure 7D) and β-catenin (data not shown) found in the P16.5 mXinβ-null hearts remained as small puncta along the lateral contacts of cardiomyocytes, whereas p120-catenin (data not shown) and mXina (Figure 7F) puncta became dispersed throughout the cardiomyocytes. These results suggest that mXinβ is essential for promoting and...
maintaining the localization of adherens junctional components and mXinα to the mature intercalated discs.

The wild-type and mXinβ-null mice from newborn to 2 to 3 weeks of age appeared to express comparable amounts of N-cadherin and connexin 43 (Online Figure V; Online Figure VII, A; and some data not shown). Both N-cadherin and connexin 43 continued to accumulate to the myocyte termini in the maturation of intercalated discs. EM analysis revealed that the developing intercalated disc at the cell termini of wild-type mice from P15.5 to P18.5, whereas the terminal distribution of both molecules remained unchanged in mutants (Online Figure VII, B), again suggesting a defect in the lateral distribution of both molecules, providing the ability to cross-talk with other signaling pathways such as receptor tyrosine kinase–, cytokine receptor–, and G protein coupled receptor–mediated signaling. We asked whether impairing intercalated disc maturation by the loss of mXinβ could lead to abnormal activities of Rho GTPase, Stat, protein kinase B (Akt), and Erk, important effectors in relaying signaling for postnatal heart development.

Using GST-Pak PBD and GST-Rhotekin RBD beads to pull-down active forms of Rac1 and RhoA, respectively, we found that relative GTP-bound Rac1 in P7.5 mXinβ-null hearts was reduced to ~65% of the control, whereas the active RhoA level in mXinβ-null hearts did not change significantly (Figure 8A). A reduction of Rac1 activity may result in less dynamic membranes at the termini of mutant cells, which was indeed suggested by the EM observation. Using phospho-specific antibodies to assess the activation of key signaling molecules, we found an increased Stat3 activity, as suggested by increased level (Figure 8B) and nuclear localization (data not shown) of p-Stat3(Y705) (tyrosine-phosphorylated Stat3 at no. 705), persistently in mXinβ-null hearts starting from P0.5. This Stat3 activation was not correlated to the activation of Jak2 (Janus kinase 2) (one member of nonreceptor tyrosine-protein kinases upstream of Stat3) (Figure 8B), suggesting that other Jaks and/or c-Src may be involved in the activation of Stat3. Alternatively, defects in negative regulators of Stat3, such as SOCS3 (suppressor of cytokine signaling 3) or tyrosine phosphatases, may participate in the abnormal activation of Stat3 in mutant hearts. Moreover, the activations/phosphorylations of Akt,
Glycogen synthase kinase 3β (a downstream target of Akt), Erk1/2, and IGF-1R were significantly depressed in mutant hearts starting from P7.5, whereas the total proteins of Akt and Grb2 (growth factor receptor-bound protein 2) in mutant and control hearts remained the same (Figure 8B). The persistent activation of Stat3, although not 100% penetrant, precedes the reductions in the activations of growth-related signaling molecules.

**Discussion**

In this study, we demonstrate that an intercalated disc–associated and Xin repeat–containing protein, mXinβ, is required for postnatal heart development. First, the postnatal upregulation of mXinβ coincides with the maturation of the intercalated disc,11,12 T-tubule, and sarcoplasmic reticulum,19 as well as diastolic function.20 Second, ablation of mXinβ leads to abnormal heart shape, VSD, diastolic dysfunction, severe growth retardation, and postnatal lethality. Third, loss of mXinβ results in failure of forming mature intercalated disc. Our data further identify that the proper clustering of N-cadherin to form intercalated disc regulates the Stat3 activity and activates the Rac1, IGF-1R, Akt, and Erk1/2 activities, which are required for postnatal heart growth/hypertrophy.21–23

**How Does the Intercalated Disc Mature?**

Postnatal maturation of intercalated discs is characterized by gradual clustering of N-cadherin complexes/puncta from lateral localization to termini of aligned cardiomyocytes. Such a clustering process likely involves modulating the interaction between cadherins and underlining actin cytoskeleton. In a classic view, the actin bundling protein

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**Figure 7. Mislocalization of N-cadherin and mXinα, as well as structural alteration in developing intercalated disc of mXinβ-null hearts.** Frozen heart sections from P16.5 mice were immunofluorescently stained with U1040 anti-mXin (A and B), anti-cadherin (C and D), and R1697 anti-mXin (E and F). In wild-type hearts, mXinβ (A), N-cadherin (C), and mXinα (E) all localized to the mature intercalated discs. In contrast, the loss of mXinβ (B) in mXinβ-null heart led to mislocalization of N-cadherin (D) and mXinα (F). Scale bar: 30 μm. G and H, EM images of P15.5 wild-type and mXinβ-null cardiomyocytes. Arrows indicate intercalated discs; *, T-tubules. Scale bar: 1 μm. I and J, High-magnification images of maturing intercalated discs of P15.5 wild-type and mXinβ-null cells. The closely apposite membranes of mutant intercalated disc were less convoluted and less wavy. des indicates desmosome; lig, 2 membranes in the process of ligation together to form intercalated disc. Scale bar: 0.2 μm.
α-catenin binds β-catenin to organize the adhesion complex that links to actin cytoskeleton. However, this stable linkage role for α-catenin has not been proven; instead, compelling evidence suggests α-catenin as a molecular switch that modulates actin cytoskeleton. Consistent with this notion, 2 types of cadherin-mediated intercellular contacts are recently detected in the adherens junctions of epithelia: a mobile and α-catenin–dependent contact associated with a dynamic actin network, as well as a stable and α-catenin–independent contact associated with a stable actin patch. The existence of this stable contact suggests that an unidentified X protein has to link the cadherin/catenin complex to actin patches. In the heart, the role of this unidentified X protein may be served by the Xin repeat–containing proteins. We propose that developmental upregulation and functional hierarchy of mXin initiate the formation of mature intercalated discs. The mXin further reinforces the stability of intercalated discs. In support of this role, loss of mXin leads to failure of forming mature intercalated discs and mislocalizations of mXinα and N-cadherin. On the other hand, mature intercalated discs form normally in the mXinα-null heart (Online Figure VIII) but eventually lose close membrane apposition between cardiomyocytes at young adult. This structural defect progressively worsens by older age.

Diastolic Dysfunction May Be Responsible for Heart Failure and Lethality in mXinβ-Null Mice

The mXinβ-null hearts have normal systolic function and heart rate but exhibit a significant delay in switching off ssTnI and significant reductions in mitral early filling (E-wave) peak velocity and E/A ratio, suggesting diastolic dysfunction. Impaired diastolic function was also suggested by the left ventricle internal dimension and left ventricle volumes being smaller in mXinβ-null mice. The detection of a significant reduction in the compact areas of ventricles in newborn mutant hearts (Online Table III) further supported a reduction in E-wave velocity. The diastolic dysfunction would lead to diminished cardiac output (stroke volume×heart rate) of mutant hearts and could contribute in part to heart failure and postnatal lethality. The mXinβ-null cardiomyocytes after P15.5 exhibited a significant reduction in the terminal connexin 43 localization (Online Figure VII), which may cause arrhythmic sudden death. However, this spatial connexin 43 alteration cannot be the cause for the loss of mXinβ-null mice at earlier age (Online Table I).

mXinβ Regulates Postnatal Cardiac Growth

In the heart, the Rac1 activation is essential for rearranging cytoskeleton to align cardiomyocytes and for regulating mitogen-activated protein kinases and NADPH oxidase activity for cardiac hypertrophy. Moreover, transgenic mice expressing constitutively active Rac1 in the heart develop dilated myocardium with high postnatal mortality. Most transgenic mice die within 2 to 3 weeks after birth, suggesting that postnatal heart development requires an intricate regulation of Rac1 activity. It is also known that classic cadherin engagement activates Rac1 through c-Src–
phosphatidylinositol 3-kinase–Vav2, and Vav2 is a gua-
nine nucleotide exchange factor capable of binding to
p120-catenin.28,29 The loss of mXinβ may dysregulate
this signaling, leading to a downregulation of Rac1 activity and
forming less convoluted, less wavy, and less stable inter-
calated discs. The loss of mXinβ may also dysregulate
cytokine/angiotensin II/growth hormone-mediated signal-
ning, leading to a persistent activation of Stat3 (Online
Figure IX). The activation of Stat can promote IGF-1
production,30 which would facilitate postnatal heart
growth. However, the lack of mature intercalated discs in
mutant hearts reduced the activities of IGF-1R, Akt, and
Erk1/2, resulting in severely retarded growth.

In summary, we have identified that mXinβ, as a critical
component for the intercalated disc maturation, is essential
for postnatal heart development. Our findings provide the
first insights into its function of transducing the N-
cadherin-mediated adhesion and crosstalk signaling by
regulating the activities of Stat3, Rac1, Erk1/2, and Akt.
Ablation of mXinβ leads to VSDs, cardiac diastolic dys-
function, and severe growth retardation. The human or-
tholog, cardiomyopathy-associated 3 (CMYA3), of
mXinβ is mapped to 2q24.3. Human patients with chromosome
band 2q24 deletion also exhibit severe growth retardation
and VSDs (http://www.orpha.net/data/patho/GB/uk-2q24.
pdf). The genome-wide linkage analysis of a large Kyrgyz
family also reveals candidate genes on 2q24.3-q31.1 con-
ferring susceptibility to premature hypertension.31 Further
studies are warranted to characterize the involvement of
mXinβ in cardiac development, function, and disease.

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Disclosures
None.

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Novelty and Significance

What Is Known?

- The origin of Xin repeat–containing proteins coincides with the genesis of true heart chambers.
- The mouse Xin (mXinα and mXinβ) proteins localize to the intercalated disc of the heart, and their human orthologs, CMYA1 and CMYA3, coexpress with the 13 known cardiomyopathy-associated genes, serving as potential diagnostic markers and drug targets for cardiac diseases.
- Mice lacking mXinα upregulate mXinβ, develop adult-onset cardiac hypertrophy and cardiomyopathy with conduction defects, and attenuate the induction of atrial fibrillation in their left atrial-pulmonary vein tissues.

What New Information Does This Article Contribute?

- Developmental upregulation of mXinβ coincides with intercalated disc and T-tubule maturation during postnatal cardiac remodeling.
- Complete loss of mXinβ in mice leads to misorganized myocardium, abnormal heart shape, ventricular septal defect, cardiac diastolic dysfunction, severe growth retardation, and postnatal lethality.
- The mXinβ in a functional hierarchy plays essential roles for the terminal localization of mXinα, N-cadherin, and connexin 43 and for mediating N-cadherin and its crosstalk signaling pathways for postnatal heart growth and animal survival.

Early evolutionary and functional studies reveal the critical requirement of Xin repeat–containing proteins in cardiac chamber formation and cardiac function. Here, we report for the first time that mice lacking mXinβ exhibit abnormal heart shape, ventricular septal defect, and misorganized myocardium. Impaired left ventricular relaxation and filling, as well as smaller diastolic volumes, may be responsible for severe growth retardation and premature death of all mutant mice. We also demonstrate for the first time that postnatal increase in the expression of mXinβ is required for the intercalated disc maturation. Mechanistically, mXinβ is involved in N-cadherin–mediated signaling and its crosstalk signaling pathways that are essential for intercalated disc formation and postnatal heart growth. Importantly, postnatal growth retardation, ventricular septal defects, progressive heart failure, and lethality have been reported in human infants missing chromosome band 2q24.3, which contains the human ortholog (CMYA3) of mXinβ. A genome-wide association analysis has also revealed candidate genes near 2q24.3 for premature hypertension. Moreover, the CMYA3 coexpresses with many known cardiomyopathy-associated genes and may serve as a useful diagnostic marker and therapeutic target for cardiac diseases. Thus, the mXinβ-deficient mice represent a novel model for studying heart development and diseases.
Essential Roles of an Intercalated Disc Protein, mXinβ, in Postnatal Heart Growth and Survival
Qinchuan Wang, Jenny Li-Chun Lin, Benjamin E. Reinking, Han-Zhong Feng, Fu-Chi Chan, Cheng-I. Lin, Jian-Ping Jin, Elisabeth A. Gustafson-Wagner, Thomas D. Scholz, Baoli Yang and Jim Jung-Ching Lin

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Materials and Methods

5' and 3'-RACE (rapid amplification of cDNA ends) of mXinβ cDNAs

We have previously obtained several overlapping cDNA clones from a custom-made cDNA library constructed from poly(A)+ RNAs prepared from adult mXinα-null mouse hearts. The composite sequence of these pBKX cDNA clones has 4,382 bp covering 9 bp of exon 1, exon 2–6 and partial exon 7. Since exon 7 was predicted to be a very large exon from genomic sequence, we PCR amplified from the mXinβ genomic β14 clone to construct our cDNA plasmid containing 9,985bp. The Northern blot analysis revealed that mRNA size of mXinβ was ~12 kb, and a stop codon had not been reached in our composite cDNA sequence, indicating that the full length mXinβ cDNA had not yet been obtained. Therefore, 5' and 3'-RACE cloning were further performed using Marathon cDNA amplification kit (Clontech) as previously described to obtain additional cDNA sequence information. For 5'-RACE, two antisense primers 5'-TGCCTCCTGCTCAGCTCTGCTCTCATGTCG-3' (nucleotide #463~434 relative to mXinβ-A) 5'-GGGACAGCGCCTCCAGGAGATCCGACTG-3' (nucleotide #251~224) located in exon 5 and exon 3, respectively, were used in different experiments. For 3'-RACE, primers 5'-ACACCACCTTCCCCACCAAGGAGTCGTTCA-3' (nucleotide #8,990 ~ 9,019), and 5'-CTTTGACTTCAAGCATGCCCCACCGACC-3' (nucleotide #9,808~9,835) located within the predicted exon 7, as well as 5'GGCCGCTGAAGTGACCATCCGTCC-3' (nucleotide #10,515~10,542) in exon 9 were used. The 5' and 3' sequences of mXinβ messages obtained from RACE were submitted to GenBank (accession no. EU286528~286531). Accordingly, three different full-length cDNAs were further constructed from the 9,985bp fragment and these RACE products (Online Figure IA). To characterize these full-length cDNAs, the constructs were subcloned into pcDNA1.1 (Invitrogen) and pEGFP-C2 (Clontech) vectors and transfected to Chinese Hamster Ovary (CHO) cells. Immunofluorescence microscopy and Western blot analysis were performed on the transfected cells to verify protein products.

Generation of mXinβ isoform-specific antibodies

To generate isoform-specific antibodies that recognize either mXinβ or mXinβ-a, we subcloned the cDNA fragments encoding the isoform-specific regions of mXinβ (encoding aa#3,255-3,278) and mXinβ-a (aa#3,256-3,300) (Online Figure IB) into the pGEX4-T2 vector (GE Healthcare Life Sciences). GST-fused recombinant proteins were produced in BL21(DE3)pLysS bacteria and affinity purified with Glutathione Sepharose 4B beads (Amersham). The purified proteins were used to immunize rabbits to produce mXinβ and mXinβ-a specific antisera (Cocalico Biologicals, Inc.). To affinity-purify antibodies specific to mXinβ (U1040) and mXinβ-a (U1043), the antisera were first passed through GST-conjugated Sepharose 4B column to deplete the anti-GST antibodies. U1040 and U1043 were then affinity-purified with their respective antigen-conjugated Sepharose 4B column.

Construction of mXinβ targeting vector and generation of mXinβ-null mice

Using previously cloned pBKX-2 cDNA (accession no. AY775570) as a probe, we screened λfix II genomic library constructed from mouse strain 129SVJ genomic DNA (Stratagene) and obtained 8 overlapping clones including β14 containing portion of intron 3 to exon 8 of the mXinβ gene. Subclones of β14 genomic fragments were used to construct targeting vector for inactivating mXinβ gene by replacing portions of exon 6, intron 6 and portion of exon 7 with a LacZ-Neo' cassette, since these regions encode highly conserved DNA-binding domain (DBD), β-catenin-binding domain (β-catBD) and Xin repeats (Online Figure IB). The linearized targeting vector was electroporated into R1 embryonic stem (ES) cells at the University of Iowa Gene Targeting Facility. After selection, G418-resistant ES clones were screened for the presence of the targeted locus by Southern blot analysis. Positive clones were expanded and microinjected into C57BL6 blastocysts to generate chimera. Chimeric male mice were
analyzed for germ-line transmission by Southern blot analysis and PCR genotyping. For PCR genotyping, genomic DNA extracted from the toes or tails of mice was used. The primer pairs used were 5’-GACAGGCTGGCCATACTCAA-3’ and 5’-ACATTTTTCTAAGGCTTTTCTCAA-3’ for the endogenous mXinβ locus, and 5’-CCTGGGCCCTACTCTACCTTTTT-3’ and 5’-CGGGCCCTTCTCGTATTACG-3’ for the targeted locus. The heterozygous mice were back-crossed to C57BL6 for at least 7 generations, maintained in C57BL6 background, and used for obtaining most results reported here. All phenotypes observed in mXinβ-null mice earlier in a mixed C57BL6/129SVJ background essentially remained the same in the mutants with C57BL6 background.

**Histological staining, immunofluorescence, and assessment of ventricular myoarchitecture**
Hearts excised from wild-type, heterozygous and homozygous littermates were fixed in 10% formalin in phosphate buffered saline (PBS) for one to two days at 4 °C. Tissue processing, Hematoxylin and Eosin (H&E) staining, and Masson’s trichrome staining were carried out as previously described. For immunofluorescence microscopy, a pair of hearts from both wild type and mXinβ-null littermates were arranged in the same orientation and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc.) in the same Peel-A-Way mold (Thermo Scientific) and frozen in liquid nitrogen for 30 seconds. Frozen sections were cut at 4 μm thickness and mounted onto Superfrost PLUS slides (Thermo Scientific). Subsequent immunofluorescence microscopy was carried out as previously described. The primary antibodies used included rabbit polyclonal U1697 anti-mXino,1 U1040 anti-C-terminus of mXinβ, anti-pan cadherin (Sigma), mouse monoclonal anti-β-catenin (Zymed Laboratories Inc.), anti-p120-catenin (Zymed), anti-N-cadherin (Zymed), anti-connexin 43 (Cx43) (Chemicon International, Inc.), CT3 anti-cardiac troponin T (cTnT),4 as well as rat monoclonal LAM-1 anti-laminin (ICN Biochemical, Inc.).

For measuring ventricular myoarchitecture, ventricles from P0.5 wild type and mXinβ-null mice were processed according to the method described previously.5 The area of the myocardial layer was measured at the lateral free wall in both left and right ventricles using sections obtained at the levels of major papillary muscles. The compact and trabecular regions were calculated as areas per 1 μm myocardium as defined previously.

**Transmission electron microscopy**
P15.5 littermates were anesthetized and dissected. The major blood vessels entering and exiting the heart were clamped with a hemostat, and the right atrium was cut open for drainage of blood. Using an AutoMate In Vivo manual gravity perfusion system (Braintree Scientific, Inc.), the heart was infused from the left ventricle with 2 ml of Locke’s solution (146 mM NaCl, 5.63 mM KCl, 2.38 mM NaHCO3, 1.63 mM CaCl2, 0.1% lidocaine and 50 USP units/ml Heparin, pH7.4), and then perfusion-fixed with 5 ml of primary fixative (2.5% glutaraldehyde, 4% paraformaldehyde, 30 mM CaCl2, 0.1 M sodium cacodylate, pH7.4). After perfusion-fixation, the left ventricle of dissected heart was diced into small blocks and kept in fixative for overnight followed by sequential treatments with osmium tetraoxide and uranyl acetate. The tissue blocks were then processed and embedded in epon resin. Ultrathin sections were cut, mounted, post-stained and observed under a JEOL1230 electron microscope (Central Microscopy Research Facility, University of Iowa).

**Proliferation and apoptosis**
To study proliferation, littermates were intraperitoneally injected with 50μg bromodeoxyuridine (BrdU, Invitrogen) per gram of body weight. Four hours later, hearts were dissected from the injected mice and processed for frozen sections. At least five sections that cut through both mitral and tricuspid valves were collected from each heart. The sections were fixed in methanol for 20 minutes, denatured with 2N HCl for 2 hours and neutralized with 0.1 M Sodium Borate. Immunofluorescence staining was then performed on the neutralized sections with mouse monoclonal anti-BrdU (Developmental Studies Hybridoma Bank, University of Iowa). Nuclei were counter-stained with 4’,6’-diamidino-2-phenylindole (DAPI) as
described previously. To quantify the frequency of BrdU-positive nuclei in the sections, 5 fluorescence images were taken with a 10x objective from comparable regions of each section. The number of BrdU positive nuclei and total nuclei were counted with the analyze particle function of ImageJ (http://rsbweb.nih.gov/ij/). Student’s t-test was used to compare the frequency of BrdU-positive cells between wild type and mXinβ-null hearts. For each stage, at least three wild type and three mXinβ-null hearts were used.

To study apoptosis, immunofluorescence staining with ab13847 anti-active caspase 3 antibody (Abcam, Inc.) was performed on frozen sections of wild type and mXinβ-null hearts. The cardiomyocytes and nuclei were counter-stained with CT3 anti-cTnT and DAPI, respectively. Image collection and analysis were performed as described above.

Cardiomyocyte width measurement
Frozen sections of postnatal day 3.5 (P3.5) and P12.5 wild type and mXinβ-null hearts were immunostained with anti-laminin to outline cardiomyocytes, anti-cTnT to label cardiomyocyte cytoplasm and DAPI to label nuclei. Images were taken from comparable area of the heart sections of each genotype. To quantify the width of cardiomyocytes, cross sections of cardiomyocytes that cut through the nuclei were identified. Then the length of the shorter axis that run through the nuclear center of the cross-sectioned cardiomyocytes was measured with Openlab 4.03 (Improvision Inc.).

Body weight, heart weight and liver weight measurements
Body weight was measured just before the mice were dissected. For heart and liver weights, heart and liver were dissected out from mice, rinsed in cold PBS or Tris buffered saline (TBS, pH7.5), blotted dry and then weighed.

Echocardiography
The mice were anesthetized and placed on a warming platform, and an appropriately sized nose cone was placed over the pup nose. Anesthesia was maintained at a minimum to suppress spontaneous movements. Heart rate was maintained between 350 and 600 beats per minute. Temperature was monitored with a rectal thermometer and maintained between 35 and 36 °C.

Echocardiograms were performed using the Visual Sonics Vevo 770 High Resolution Imaging System and software (Visual Sonics, Inc.), as described. The 704 (center frequency 40 MHz, focal length 10 mm), 707B (center frequency 30 MHz, focal length 12.7 mm), and 710B (center frequency 25 MHz, focal length 15 mm) RMV scan heads were used. Scan heads were interchanged during each study to allow for optimal image acquisition. Parasternal long axis, parasternal short axis and apical four chamber views were obtained in all animals. Pulsed wave Doppler recordings were made across the left ventricular outflow tract, right ventricular outflow tract and mitral valve when the Doppler sample volume was thought to be parallel to flow. Doppler tracings could not be obtained in every animal and those that were more than 20 degrees from parallel were not used for data analysis. M-mode recordings were obtained of the right and left ventricles in the parasternal short axis view at the level of the left ventricular papillary muscles. Measurements were made of the interventricular septum thickness in diastole and systole (IVSd, IVSs), left ventricular internal dimension in diastole and systole (LVIDd, LVIDs), and left ventricular posterior wall thickness in diastole and systole (LVPWd, LVPWs). These measurements were then used to calculate the left ventricular ejection fraction (EF) and fraction shortening (FS), left ventricular diastolic and systolic volumes (LVVd, LVVs), and left ventricular mass. Calculations were made by the Vevo 770 software. Measurements were made in accordance with the American Society for Echocardiography Guidelines.

Western blot analysis
For Western blot, tissues were dissected from anesthetized mice, rinsed in cold TBS, blotted dry, weighed and then homogenized by a Pro200 homogenizer with Multi-Gen 7 generators (Pro Scientific Inc.) in
buffer (40 µl buffer per mg heart weight) containing 20 mM HEPES (pH 7.2), 25 mM NaCl, 2 mM EGTA, 2 mM Na3VO4, 25 mM β-glycerophosphate, 50 mM NaF, 1% Triton X-100 and complete protease inhibitor cocktail (cat#11836145001, Roche Applied Sciences). An equal volume of 2X SDS-PAGE gel sample buffer was added to the homogenate. After heating at 100 °C for 5 minutes, the homogenate was stored at -70 °C as aliquots until use. Western blot analysis was carried out as described previously. The primary antibodies used included rabbit polyclonal U1013 anti-mXin, α-actinin (a generous gift from Dr. K. Burridge, UNC Chapel Hill); mouse monoclonal CH1 anti-α-tropomyosin, TnI anti-tropomin I (Tnl), FA2 anti-α-myosin heavy chain (MHC), anti-β-MHC (Sigma), anti-plakoglobin (BD Biosciences), ARC03 anti-Rac1 (Cytoskeleton, Inc.), and ARH03 anti-RhoA (Cytoskeleton, Inc.); as well as rabbit monoclonal antibodies (Cell Signaling Technology, Inc.) including C67E7 anti-pan Akt, D9E anti-p-Akt(S473), C31E5E anti-p-Akt(T308), DA7A8 anti-p-IGF-1R, D3A7 anti-p-Stat3(Y705), 79D7 anti-Stat3, and C80C3 anti-p-Jak2(Y1007) and rabbit polyclonal antibodies (Cell Signaling Technology, Inc.) including anti-p-GSK3β(S9) and anti-p-Erk1/2.

**Analysis of immunolocalization of N-cadherin and Cx43 during postnatal heart development**

Frozen 4-µm sections of hearts from wild type and mXinβ-null littermates were processed for immunofluorescence labeling with anti-N-cadherin or anti-Cx43 as described above. The proportion of label at the myocyte termini during postnatal development was determined by the previously described method with a slight modification. Briefly, micrographs were taken from comparable regions of the ventricular myocardia and analyzed in ImageJ. To facilitate analysis, images of individual cardiomyocytes that had been longitudinally sectioned were extracted from the micrographs. Differential interference contrast micrographs were used to aid this process. The termini of individual cardiomyocytes were defined by making a rectangular box at both ends of the cardiomyocytes. The width of each box was 10% of the longitudinal axis of the cardiomyocyte. The percentage of terminally localized N-cadherin or Cx43 was calculated by dividing the number of fluorescence pixels in the termini with the number of fluorescence pixels in the whole cardiomyocytes.

**Rac1 and RhoA activity assay**

Active Rac1 and RhoA pull-down assays were carried out essentially as described with slight modifications. For active Rac1 pull-down assay, P7.5 mouse heart of each genotype was quickly dissected, rinsed in cold TBS, weighed and then homogenized in ice cold Rac1 lysis buffer (40 µl buffer per mg heart weight) containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% TritonX-100, 10 mM MgCl2, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin, and 1 mM phenylmethyl sulfonyl fluoride (PMSF). The homogenate was cleared by centrifugation at 20,000 x g for 15 minutes. Then, 200 µl Rac1 lysis buffer containing 100 µg GST-Pak PBD (GST-Pak pre-bound to Glutathione sepharose) beads were added to 400 µl cleared homogenate to pull-down active Rac1. After washing the beads, GTP-bound (active) Rac1 was recovered in SDS-PAGE gel sample buffer, followed by Western blot analysis.

For active RhoA pull-down assay, the heart homogenate was prepared as described for the Rac1 activity assay, except that the lysis buffer for RhoA assay contained 50 mM Tris-HCl (pH 7.2), 500 mM NaCl, 10 mM MgCl2, 1% TritonX-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin, and 1 mM PMSF. For 400 µl cleared homogenates, 50 µg of GST-Rhotekin RBD (GST-Rhotekin pre-bound to Glutathione sepharose) beads were used to pull-down GTP-bound RhoA. After washing the beads, active RhoA was recovered in gel sample buffer, followed by Western blot analysis.

**References**


Online Table I. Genotypes of progenies of \( mXin\beta^{+/-} \) intercrosses

<table>
<thead>
<tr>
<th>Age</th>
<th>( mXin\beta^{+/-} )</th>
<th>( mXin\beta^{+/-} )</th>
<th>( mXin\beta^{-/-} )</th>
<th>Total number</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0.5</td>
<td>46 (45.5)</td>
<td>101 (91)</td>
<td>35 (45.5)</td>
<td>182</td>
<td>0.17</td>
</tr>
<tr>
<td>P3.5</td>
<td>57 (38)</td>
<td>72 (76)</td>
<td>23 (38)</td>
<td>152</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>P7.5</td>
<td>50 (38.25)</td>
<td>79 (76.5)</td>
<td>24 (38.25)</td>
<td>153</td>
<td>0.01</td>
</tr>
<tr>
<td>P12.5</td>
<td>37 (29.25)</td>
<td>68 (58.5)</td>
<td>12 (29.25)</td>
<td>117</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>P17.5</td>
<td>12 (10)</td>
<td>28 (20)</td>
<td>0 (10)</td>
<td>40</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Age: at which genotype was determined.
The number of mice observed is indicated for each genotype and the number of mice expected from Mendelian frequency is shown in parenthesis.
p value: determined from Chi square test.
Online Table II. Echocardiographic analysis of control (mXinβ+/+ & mXinβ+/−) and mXinβ-null mice at P3.5 and P12.5

<table>
<thead>
<tr>
<th>Parameters</th>
<th>P3.5</th>
<th>P12.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=7)</td>
<td>mXinβ/- (n=5)</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>458±48</td>
<td>473±59</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.44±0.08</td>
<td>0.47±0.03</td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>0.73±0.09</td>
<td>0.77±0.14</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.48±0.13</td>
<td>0.42±0.03</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>0.66±0.10</td>
<td>0.74±0.15</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>1.63±0.10</td>
<td>1.45±0.21</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>0.83±0.11</td>
<td>0.60±0.13*</td>
</tr>
<tr>
<td>LVVd (μl)</td>
<td>7.65±1.18</td>
<td>5.76±2.01</td>
</tr>
<tr>
<td>LVVs (μl)</td>
<td>1.36±0.57</td>
<td>0.60±0.26*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>83.22±4.68</td>
<td>88.37±6.15</td>
</tr>
<tr>
<td>FS (%)</td>
<td>49.34±5.65</td>
<td>56.69±11.19</td>
</tr>
<tr>
<td>Mitral valve E/A ratio</td>
<td>0.83±0.03</td>
<td>0.73±0.06*</td>
</tr>
</tbody>
</table>

Two dimensional images were recorded in parasternal long- and short-axis projections with guided M-mode recordings at the midventricular level in both views. Left ventricle (LV) chamber size and wall thickness are measured in at least three beats from each projection and averaged.

bpm: beats per minutes
IVSd and IVSs: interventricular septum thickness at diastole and systole, respectively.
LVPWd and LVPWs: LV posterior wall thickness at diastole and systole, respectively.
LVIDd and LVIDs: LV internal dimension at diastole and systole, respectively.
LVVd and LVVs: LV volume at diastole and systole, respectively.
EF: ejection fraction
FS: fraction shortening
E/A: mitral valve E-wave (early filling) to A-wave (atrial contraction/late filling) ratio
* p ≤ 0.05 significant difference between mXinβ/- and control mice (Student’s t-test)
Online Table III. Assessment of ventricular myoarchitecture

<table>
<thead>
<tr>
<th>Area/length (µm²/µm)</th>
<th>mXinβ+/+ (n=5)</th>
<th>mXinβ-null (n=4)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left ventricle compact region</td>
<td>240.4±15.2</td>
<td>188.5±7.5</td>
<td>0.026*</td>
</tr>
<tr>
<td>Left ventricle trabecular region</td>
<td>41.1±4.9</td>
<td>51.3±9.1</td>
<td>0.332</td>
</tr>
<tr>
<td>Right ventricle compact region</td>
<td>168.3±16.8</td>
<td>120.0±18.6</td>
<td>0.096</td>
</tr>
<tr>
<td>Right ventricle trabecular region</td>
<td>67.3±6.1</td>
<td>114.0±23.7</td>
<td>0.070</td>
</tr>
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</table>

Compact and trabecular regions per unit myocardium within lateral free walls of left and right ventricles of newborn wild type and mXinβ-null mice were determined from H&E stained sections according to the previously described method. This analysis using in developing mouse embryos has previously revealed that the developmental changes in ventricular myoarchitecture correlate very well with the changes in ventricular diastolic function. In general, peak E-wave velocity (active relaxation) is exponentially correlated with the area of compact region per 1µm myocardium, while peak A-wave velocity (passive compliance/atrial contraction) is correlated with the area of trabecular region per 1 µm myocardium. A significant decrease in the left ventricle compact region detected in P0.5 mXinβ-null hearts is consistent with a smaller E-wave velocity and thus diastolic dysfunction. *, significant difference between mXinβ+/+ and mXinβ/- hearts (Student’s t-test)
Online Figure Legends

Online Figure I. Genomic structure, mRNA and protein isoforms of mXinβ. (A) The mXinβ gene contains 9 exons (E1-E9), capable of generating 3 distinct mRNAs (mXinβ-A, mXinβ-B and mXinβ-C), as identified by 5’ and 3’ RACE. Both mXinβ-A and mXinβ-B mRNAs include E8 but use different poly(A) addition signals in E9. These represent the major species, whereas the minor mXinβ-C mRNA specifically splices out the E8. (B) Both mXinβ-A and mXinβ-B use the same stop codon (TAA) in E8 and translate into the same mXinβ protein with 3,283 amino acid (aa) residues. The mXinβ-C uses the stop codon (TAG) in E9 and codes for mXinβ-a protein with 3,300 aa residues. Both mXinβ and mXinβ-a proteins contain actin-binding motifs (Xin repeats, aa#308-1,307), within which predicted β-catenin-binding domain (β-catBD) locates. They also possess consensus sequences for Myb DNA-binding domain (DBD), nuclear export signal (NES), nuclear localization signal (NLS), 3 proline-rich regions (PR1, PR2, and PR3) and ATP/GTP-binding domain (ATP/GTP-BD). (C) Western blot analysis shows that force-expressed mXinβ protein in CHO cells has a similar mobility in SDS-PAGE gel as endogenous mXinβ found in mouse heart extract. (D) Immunofluorescence microscopy reveals that force-expressed mXinβ, detected by rabbit anti-mXin (U1013) and Rhodamine-conjugated goat anti-rabbit IgG (red color), co-localizes with actin filaments to stress fibers, labeled by fluorescein-conjugated phalloidin (green color).

Online Figure II. Spatial and temporal expression patterns of mXinβ in mice. (A) Western blot analysis of total protein extracts prepared from various tissues of a postnatal day 7.5 (P7.5) mXinα-null mouse with U1013 anti-mXin antibody reveals that mXinβ is specifically expressed in striated muscles such as tongue, heart and diaphragm. (B) The Coomassie Blue-stained protein profile from the same total protein extracts used in (A) shows the protein loading in each lane. (C) Western blot analysis with anti-mXin on total protein extracts prepared from 3 individual hearts of wild type mice at P0.5, P3.5, P7.5 and P12.5 reveals a significant up-regulation of mXinβ in postnatal hearts. (D) The Coomassie Blue-stained protein profile from the same total protein extracts used in (C) shows the relative protein loading in each lane.

Online Figure III. Neither persistent truncus arteriosus (PTA) nor patent ductus arteriosus (PDA) was detected in newborn mXinβ-null mouse heart. Ao, aorta; PA, pulmonary artery; DA, ductus arteriosus. Bar = 1mm

Online Figure IV. Masson’s trichrome-stained heart sections from P11.5 wild type and mXinβ-null mice demonstrating no apparent cardiac fibrosis in the mXinβ-null heart. Bar = 1mm

Online Figure V. Western blot analysis on total protein extracts prepared from developing hearts of each mXinβ genotype with anti-myosin heavy chain (MHC) antibodies, anti-N-cadherin, anti-β-catenin, anti-p120-catenin and DM1B anti-β-tubulin. The MHC switch from embryonic β-MHC to adult α-MHC in mXinβ-null hearts occurred normally. Most of the adherens junctional components examined here was expressed normally, except that p120-catenin may be significantly reduced in P12.5 mXinβ-null heart.

Online Figure VI. Representative heart sections from wild type (A, C) and mXinβ-null (B, D) mice at P3.5 and P12.5. Cardiomyocytes outlined by anti-laminin (green color), cytoplasm labeled with anti-cTnT (red color) and nuclei labeled with DAPI (blue color) were used for comparison of cell shape difference. Irregular cell shape was readily observed in P12.5 mXinβ/- heart, suggesting a mis-organization in myocardium. Bar = 10 μm. (E) The cardiomyocyte width was measured by the length of the shortest axis ran through the nuclear center of the cross-sectioned cardiomyocytes. At P3.5, there was no apparent difference in cell width and cell shape between wild type and mXinβ-null cardiomyocytes. In contrast, at P12.5, mXinβ-null cardiomyocytes showed a significant reduction in cell width.
Online Figure VII. The proportion of N-cadherin and connexin 43 localized to the termini of developing cardiomyocytes of wild type and mXinβ-null mice. (A) Images of individual cardiomyocytes immunofluorescently labeling for N-cadherin (a, b) or connexin 43 (c, d). These images were extracted from micrographs taken from comparable regions of P15.5 wild type and mXinβ-null ventricular myocardia. The termini of cardiomyocytes were defined by making a rectangular box at both ends of the cardiomyocytes. The width of each box was 10% of the longitudinal axis of the cardiomyocyte as shown in (a). Both N-cadherin and connexin 43 showed higher level of terminal localization in the wild type cardiomyocytes (a, c) than in the mXinβ-null cardiomyocytes (b, d). (B) Quantification of terminally localized N-cadherin and connexin 43 in P15.5 and P18.5 cardiomyocytes. In both wild type and mXinβ-null hearts, the assembly of Cx43 to the myocyte termini was well behind the assembly of N-cadherin at both time points (p<0.02). The percentages of terminally localized N-cadherin and connexin 43 in the wild type cardiomyocytes at both time points were significantly higher than that in the mXinβ-null counterparts. In addition, the terminal localization of N-cadherin and connexin 43 increased significantly from P15.5 to P18.5 in the wild type cardiomyocytes, whereas the terminal localization of both proteins in the mXinβ-null cardiomyocytes remain unchanged during the same developmental stage. The number of cardiomyocytes measured was indicated in each bar. * p<0.05. N.S., no significant difference.

Online Figure VIII. No mis-localization of mXinβ in mXina-null mouse heart. Immunofluorescence microscopy was performed on frozen heart section from adult mXina-null mice with affinity-purified rabbit U1040 anti-mXinβ (red color) and mouse monoclonal anti-β-catenin (green color). The nuclei were labeled with DAPI (blue color). Bar = 10μm

Online Figure IX. Proposed roles of mXinβ in postnatal heart growth. In the wild type heart, N-cadherin and its associated proteins mediate bi-directional signaling and cross-talks, because these proteins are shown to interact with many signaling molecules such as receptor tyrosine kinases (e.g., IGF-1R), non-receptor tyrosine kinases (e.g., c-Src, Jak), tyrosine phosphatases, phosphatidylinositol-3 kinase (PI3K) and adaptors.15-19 The pleiotropic effects caused by deletion of mXinβ suggest that mXinβ is a pivotal factor for both N-cadherin-mediated bi-directional and cross-talk signalings. Similar to mXina,20 the mXinβ containing several conserved binding domains may also interact with β-catenin, p120-catenin and actin filaments. Together, mXina and mXinβ may play important role in the Rac1 activation through c-Src-PI3K and Vav2 (a guanine nucleotide exchange factor capable of binding to p120-catenin), similar to the signaling found in epithelial cells.21-23 Postnatal heart growth requires an intricate regulation of Rac1 activity,24 and the Rac1 activation is essential for rearranging actin cytoskeleton to align cells in response to mechanical stretch25 and for modulating mitogen-activated protein kinase activity and myocardial oxidative stress (cross-talk signaling) in response to various hypertrophic stimuli.26, 27 In the mXinβ-null heart, the loss of mXinβ impairs the engagement and clustering of N-cadherin, down-regulates the Rac1 activity, and subsequently mis-localizes mXina. These impairments would in turn dys-regulate hormone-, cytokine-, and growth factor-mediated signalings for postnatal heart growth. Although the mechanism remains to be determined, mXinβ-null hearts exhibit a persistent activation of Stat3 and a down-regulation of IGF-1R activity. Furthermore, the up-regulation of Stat3 activity in mutant hearts appears to precede the reductions in the activities of growth-related signaling molecules. Since the stat3 activity is auto-regulated by many positive (such as Jak, c-Src, IGF-128) and negative (such as suppressor of cytokine signaling protein 3, SOCS3,29, cytoplasmic tyrosine phosphatases) regulators,30, 31 it should be worthy to determine which of these regulators, including the ATP/GTP-binding domain-containing mXinβ, are responsible for the up-regulation of Stat3 activity. The defect in the clustering of N-cadherin in mXinβ-null hearts may also impair the IGF-1R organization during postnatal heart growth, leading to the reduced activities of IGF-1R, Akt and Erk-1/2, and the severely retarded growth.
Online Figure II
Online Figure IV

$mXin_{\beta+/-}$  $mXin_{\beta-/-}$
Online Figure VI

A. P3.5 mXinβ+/+
B. P3.5 mXinβ-/-
C. P12.5 mXinβ+/+
D. P12.5 mXinβ-/-

laminin/cardiac troponin T/nuclei (DAPI)

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<th>Cell width (μm)</th>
<th>p value</th>
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<td>P12.5</td>
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<td>8.0±0.1 (n=253)</td>
<td>&lt;0.001</td>
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Online Figure VII

(A) N-cadherin and Connexin 43 expression in different genotypes:
- a. mXinβ+/+
- b. mXinβ-/-
- c. mXinβ+/+
- d. mXinβ-/-

(B) Bar graph showing percentage of myocyte termini:
- N-cadherin:
  - P15.5: 37, 58
  - P18.5: 25, 32
- Connexin 43:
  - P15.5: 38, 51
  - P18.5: 34, 37

Significance levels:
- * indicates statistical significance
- N.S. indicates non-significant differences
Online Figure IX

Wild type heart

Outside-in signal

N-cadherin

Inside-out signal

Hormones Cytokines Growth factor

Cell alignment Adhesion

mXinβ-null heart
Defective in the engagement and clustering of N-cadherin
Up-regulated Stat3 activity
Down-regulated IGF-1R, Akt and Erk1/2 activities
Down-regulated Rac1 activity
Mis-localized mXinα