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

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Abstract

Rationale—The Xin repeat-containing proteins, mXinα and mXinβ, localize to the intercalated disc (ICD) of mouse heart and are implicated in cardiac development and function. The mXinα directly interacts with β-catenin, p120-catenin and actin filaments. Ablation of *mXin*α results in adult late-onset cardiac cardiomyopathy with conduction defects. An up-regulation of the mXinβ in mXinα-deficient hearts suggests a partial compensation.

Objective—The essential roles of mXinβ in cardiac development and ICD maturation were investigated.

Methods and Results—Ablation of *mXin*β led to abnormal heart shape, ventricular septal defects, severe growth retardation and postnatal lethality with no up-regulation of the mXinα. Postnatal up-regulation of mXinβ in wild type hearts, as well as altered apoptosis and proliferation in *mXin*β-null hearts suggest that mXinβ is required for postnatal heart remodeling. The *mXin*β-null hearts exhibited a mis-organized 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 ICDs and the mis-localization of mXinα and N-cadherin. The *mXin*β-null hearts showed up-regulation of active Stat3 (signal transducer and activator of transcription 3) and down-regulations of the activities of Rac1, IGF-1 (insulin-like growth factor 1) receptor, Akt and Erk1/2 (extracellular-signal-regulated kinases 1/2).

Conclusions—These findings identify not only an essential role of mXinβ in the ICD maturation but also mechanisms of mXinβ modulating N-cadherin-mediated adhesion signaling and its crosstalk signaling for postnatal heart growth and animal survival.

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Disclosures
None

Keywords

N-cadherin-mediated adhesion signaling; Xin repeat-containing protein; intercalated disc maturation; diastolic dysfunction; postnatal heart growth

Introduction

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 major force for the heart to evolve new structures.[1,2] However, the addition of new transcription factors can only be 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 mXinα, 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 15~28 copies of the conserved 16-amino acid (aa) Xin repeats, and originated just prior to 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 (β-catBD) 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.[3]

In mammals, however, a pair of paralogous *Xin*α and *Xin*β genes exists. Ablation of the mouse *mXin*α gene does not affect heart development. Instead, the mXinα-deficient mice show cardiac hypertrophy and cardiomyopathy with conduction defects during adulthood. In the mXinα-deficient mice, mXinβ is up-regulated at both message and protein levels, suggesting a compensatory role of mXinβ.[7] Consistent with this idea, both mXin proteins have highly conserved Xin repeats and β-catBD, as well as other functionally undefined domains located in the N-terminals.[6] On the other hand, the C-terminals of both proteins are more diverged, suggesting that they also have distinct functions.[6] Since *mXin*β is more conserved than *mXin*α with the ancestral lamprey *Xin* that demarked the emergence of heart chamber, we hypothesized that *mXin*β might play an essential role in heart morphogenesis. To test this hypothesis, we generated and characterized *mXin*β knockout mice. The *mXin*β-null mice died prior to weaning and showed abnormal heart shape, ventricular septal defects (VSDs), mis-organized myocardium and diastolic dysfunction. The mechanisms underlying these cardiac defects involved dys-regulation of the N-cadherin-mediated signaling pathway and its crosstalks via abnormally activated Stat3 and depressed Rac1, IGF-1 receptor (IGF-1R), Akt and Erk1/2 activities.

Materials and Methods

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

Results

Generation of *mXin*β-null mice

To construct a targeting vector, we cloned full-length *mXin*β cDNAs and the corresponding genomic fragments. Alignment of these sequences revealed that the *mXin*β gene contains nine exons and encodes three mRNA species (*mXin*β-*A*, *mXin*β-*B* and *mXin*β-*C*) in adult heart through alternative splicing of exon 8 and alternative usage of polyA signals (Online Figure IA). Both *mXin*β-*A* and *mXin*β-*B* encode a polypeptide of 3,283 aa residues (termed mXinβ), whereas *mXin*β-*C* is predicted to encode a protein of 3,300 residues (termed mXinβ-a) (Online Figure IB). By sequencing 24 randomly picked transformants generated from 3'-RACE, we found 23 clones representing either *mXin*β-*A* or *mXin*β-*B*, suggesting that mXinβ is the major isoform. Force-expression of the cloned *mXin*β-*B* cDNAs in CHO cells confirmed that *mXin*β-*B* encoded the protein having the same size as endogenous mXinβ and reacting to anti-mXin antibody (Online Figure IC). Furthermore, force-expressed mXinβ co-localized with actin filaments to stress fibers and cell cortex (Online Figure ID).

Using multiple tissue Western blot, mXinβ was detected only in the striated muscles such as tongue, heart and diaphragm (Online Figure IIA). During postnatal heart development, the expression of mXinβ increased at least 3 folds from postnatal day 0.5 (P0.5) to P13.5 (Online Figure IIC). The timing of this up-regulation of mXinβ coincides with the period for intercalated disc maturation.[11,12]

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

All *mXin*β-null mice die before weaning

Northern blot analysis revealed a complete loss of *mXin*β message in homozygote and a reduction in heterozygote (Figure 1D). Western blot analyses with antibody U1013[7] recognizing both mXinα and mXinβ (Figure 1E, top blot) or with antibody U1040 recognizing C-terminal of mXinβ (data not shown) verified a complete loss of mXinβ in homozygotes and a reduced level in heterozygotes. The *mXin*β-null 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 *mXin*β-null pups from heterozygous crosses was smaller than the expected number (Online Table I), however, this reduction was not statistically significant (p=0.17, Chi square test). In contrast, from P3.5 and on, the number of viable *mXin*β−/− mice was significantly lower than the expected one. No viable *mXin*β−/− mice could

be observed at weaning stage. Thus, these observations suggest that *mXin*β is essential for postnatal mouse survival.

Loss of mXinβ leads to severe growth retardation

The *mXin*β-null mice had severely retarded growth and reduced activity. The skin of newborn *mXin*β−/− mice was apparently paler than their littermates, suggesting a systemic circulation defect. Great vessels in the newborn *mXin*β-null mice were normal (Online Figure III). The body weight (BW) of P0.5 *mXin*β−/− mice was about 14.3% lighter than wild type or heterozygous littermates (Figure 2A). From birth to P12.5, the *mXin*β-null mice also gained weight more slowly than their littermates (Figure 2A). At P12.5, *mXin*β-null mice weighed only about 45% of wild type or heterozygous mice. The loss of just one copy of *mXin*βin heterozygotes had neither effect on BW nor on viability. Neonatal *mXin*β−/− 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 (HW) of newborn wild type and *mXin*β-null pups were similar. However, from P3.5 to P12.5, the wild type hearts grew much faster than *mXin*β-null hearts. As a result, both P7.5 and P12.5 wild type hearts were significantly larger than *mXin*β-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 *mXin*β in heterozygotes did not affect their heart size (data not shown). The HW/BW ratio of *mXin*β−/− mice at most of postnatal stage except P3.5 was significantly higher than that of wild type mice (Figure 2C) due to significantly smaller BW in *mXin*β-null mice. Similar to the hearts, mXinβ-non-expressing organ such as liver (Figure 2D) of *mXin*β-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 *mXin*βnull mice remained no difference (Figure 2E). Thus, the loss of mXinβ affected the *mXin*β-expressing and *mXin*β-non-expressing organs differently.

Loss of mXinβ results in VSDs, abnormal heart shape and mis-organized myocardium

About 15% (5/33) of newborn *mXin*β-null hearts had abnormal shape (Figure 3B). VSD was detected in 58% (7/12) of newborn *mXin*β-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, since 42% of *mXin*β-null mice without VSD also became small and weak, and died before weaning. Mis-organized myocardium (non-compaction in right ventricle) could be detected in *mXin*β-null hearts as early as embryonic day 14.5 (E14.5) (Figure 3F&F'). Thus, *mXin*β-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, since no significant loss of newborn *mXin*β-null mice was found (Online Table I). Furthermore, all *mXin*β-null neonatal hearts examined showed various degree of mis-organized myofibers within myocardium (an example shown in Figure 3H'&H"). Electron microscopic (EM) analysis of P15.5 *mXin*β-null hearts detected no sarcomere disorganization within each myocytes (Figure 3J), suggesting no myofilament disarray in mutant hearts.

Developing *mXin*β-null hearts exhibit diastolic dysfunction

Since all *mXin*β−/− mice exhibited mis-organized myocardium, we next analyzed chamber size, wall thickness and cardiac function by echocardiography. Because echocardiographic results from wild type and heterozygotes were very similar, we treated them as a control group for the comparison to *mXin*β-null group (Online Table II). We observed a reduction in left ventricular internal dimension and volume of both P3.5 and P12.5 *mXin*β-null hearts during diastole and systole (Online Table II). In contrast, there was no difference between control and *mXin*β-null mice in heart rate, left ventricular posterior wall thickness and interventricular

septum thickness (Online Table II). Furthermore, the *mXin*β-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 *mXin*β-null hearts exhibited abnormal ventricular filling. In *mXin*β−/− 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 due 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*β-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 *mXin*β-null hearts.

The delay in switching off slow skeletal troponin I (ssTnI) also supports diastolic dysfunction associated with *mXin*β-null mice

Apparent preservation of systolic function and presence of diastolic dysfunction in *mXin*β-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 (MHC) to α-MHC (Online Fig. V) and from embryonic cardiac troponin T (ecTnT) to adult cTnT (acTnT) (Figure 5C) largely support that *mXin*β-null hearts having normal systolic function. In contrast, a significant delay in switching off ssTnI was detected in P7.5 and P13.5 *mXin*β-null hearts (Figure 5B). This delay may allow mutant hearts to gain increased Ca^{2+}-activated myofilament tension to compensate for function, as suggested from previous study comparing force generations between ssTnI- and cardiac troponin I (cTnI)-expressing cardiomyocytes.[14] Nonetheless, transgenic mice ectopically expressing ssTnI in the heart exhibit impairments of cardiomyocyte relaxation and diastolic function.15 Together, the delay in switching off ssTnI also supports diastolic dysfunction in *mXin*β-null heart. It should be noted that *mXin*β-null hearts did not up-regulate N-terminal truncated cTnI (cTnI-ND) (Figure 5B), which has been previously shown to enhance ventricular diastolic function in transgenic mice.[16]

Developing *mXin*β-null hearts exhibit an increased apoptosis as well as a decreased proliferation

Apoptosis and proliferation contribute greatly to myocardial remodeling during postnatal development.[17] Thus, we asked whether defects in these processes might contribute to the mis-organization 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[17] (Figure 6A, A' and E). In contrast, the level of apoptosis in P0.5 *mXin*β-null hearts decreased more slowly and remained significantly higher at P3.5 and P7.5 (Figure 6B, B' and E). Using bromodeoxyuridine (BrdU) labeling, we found that there was no difference in proliferation rate in *mXin*β-null and control hearts until P7.5, at which mutant hearts showed slightly reduced cell proliferation (Figure 6C, D and F). Therefore, slightly decreased proliferation and increased apoptosis in *mXin*β-null hearts postnatally may in part account for the smaller HW and the mis-organized 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 *mXin*β-null cardiomyocytes in either cell shape or cell width (Online Figure VIA, VIB, VIE). In contrast, by P12.5, *mXin*β−/− cardiomyocytes became more irregularly shaped (Online Figure VID) and smaller in cell width (Online Figure VIE).

*mXin*β-null hearts fail to develop mature intercalated discs

At the first two weeks of age, mXinα, N-cadherin and β-catenin progressively coalesce to the termini of aligned cardiomyocytes to form mature intercalated discs.[12] We asked whether mXinβ plays a role in the intercalated disc maturation. In P16.5 wild type hearts, majority of mXinβ, N-cadherin and mXinα (Figure 7A, 7C, and 7E) as well as β-catenin and p120-catenin (data not shown) were 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 *mXin*β-null hearts remained as small puncta along the lateral contacts of cardiomyocytes, while p120-catenin (data not shown) and mXinα (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 *mXin*β-null mice from newborn to 2~3 weeks of age appeared to express comparable amounts of N-cadherin and connexin 43 (Cx43) (Online Figure V, Online Figure VIIA, and some data not shown). Both N-cadherin and Cx43 continued to accumulate to the myocyte termini of the wild type mice from P15.5 to P18.5, whereas the terminal distribution of both molecules remained unchanged in mutants (Online Figure VIIB), again suggesting a defect in the maturation of intercalated discs. EM analysis revealed that the developing intercalated disc at the cell termini of P15.5 *mXin*β-null hearts was smaller than the wild type counterparts (arrows in Figure 7G & H). At higher magnification, the membranes at the maturing intercalated disc of *mXin*β-null cardiomyocytes were less convoluted and less wavy (Figure 7J), suggesting a depressed membrane activity at the termini of *mXin*β-null cells. At the lateral membrane contacts, developing T-tubules could be detected in both mutant and control cells (* in Figure 7G and H), and less difference in the membrane activity was observed.

The *mXin*β-null hearts increased Stat3 activity but decreased Rac1, IGF-1R, Akt and Erk1/2 activities

Accumulated lines of evidence suggest that N-cadherin-mediated adhesion signaling is critical for intercalated disc integrity and cardiac function.[18] Cadherin and its associated catenins are also known to interact with many signaling 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, 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 *mXin*β-null hearts was reduced to ~65% of the control, whereas the active RhoA level in *mXin*β-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 #705), persistently in *mXin*β-null hearts starting from P0.5. This Stat3 activation was not correlated to the activation of Jak2 (Janus kinase 2) (one member of non-receptor 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 suppressor of cytokine signaling 3 (SOCS3) or tyrosine phosphatases, may participate in the abnormal activation of Stat3 in mutant hearts. Moreover, the activations/phosphorylations of Akt, GSK3β (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 up-regulation of mXinβ coincides with the maturations of the intercalated disc,[11,12] T-tubule and sarcoplasmic reticulum,[19] as well as diastolic function.[20] Second, ablation of *mXin*β 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, α-catenin, binds β-catenin to organize the adhesion complex that links to actin cytoskeleton.[24] However, this stable linkage role for α-catenin has not been proven; instead, compelling evidence suggests α-catenin being a molecular switch that modulates actin cytoskeleton.[25] Consistent with this notion, two 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.[26] 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 up-regulation and functional hierarchy of mXinβ initiate the formation of mature intercalated discs. The mXinα further reinforces the stability of intercalated discs. In support of this role, loss of mXinβ leads to failure of forming mature intercalated discs and mis-localizations of mXinα and N-cadherin. On the other hand, mature intercalated discs form normally in the *mXin*α-null heart (Online Figure VIII), but eventually lose close membrane apposition between cardiomyocytes at young adult. This structural defect progressively worsens by older age.[7]

Diastolic dysfunction may be responsible for heart failure and lethality in *mXin*β-null mice

The *mXin*β-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 *mXin*β-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[13]. 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 *mXin*β-null cardiomyocytes after P15.5 exhibited a significant reduction in the terminal Cx43 localization (Online Figure VII), which may cause arrhythmic sudden death. However, this spatial Cx43 alteration cannot be the cause for the loss of *mXin*β-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,[23] and for regulating mitogen-activated protein kinases[21] and NADPH oxidase activity[22] for cardiac hypertrophy. Moreover, transgenic mice expressing constitutively active Rac1 in the heart develop dilated myocardium with high postnatal mortality.[27] Most transgenic mice die within 2~3 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-PI3K-Vav2, and Vav2 is a guanine nucleotide exchange factor capable of binding to p120-catenin.[28,29] The loss of mXinβ may dys-regulate this signaling, leading to a down-regulation of Rac1 activity and forming less convoluted, less wavy, and less stable intercalated discs. The loss of mXinβ may also dys-regulate cytokine/AngII/growth hormone-mediated signaling, 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 signalings by regulating the activities of Stat3, Rac1, Erk1/2 and Akt. Ablation of *mXin*β leads to VSDs, cardiac diastolic dysfunction and severe growth retardation. Human ortholog, *cardiomyopathy-associated 3* (*CMYA3*), of *mXin*β 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 conferring susceptibility to premature hypertension.[31] Further studies are warranted to characterize mXinβ's involvement in cardiac development, function and disease.

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 (ICD) of the heart, and their human orthologs, *CMYA1* and *CMYA3*, co-express with the 13 known cardiomyopathy-associated genes, serving as potential diagnostic markers and drug targets for cardiac diseases.
- Mice lacking mXinα up-regulate 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 up-regulation of mXinβ coincides with ICD and T-tubule maturation during postnatal cardiac remodeling.

- Complete loss of mXinβ in mice leads to mis-organized 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 mis-organized 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 ICD maturation. Mechanistically, mXinβ is involved in N-cadherin-mediated signaling and its crosstalk signaling pathways that are essential for ICD 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 *mXin*β. A genome-wide association analysis has also revealed candidate genes near 2q24.3 for premature hypertension. Moreover, the *CMYA3* co-expresses 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard Abbreviations and Acronyms

ICD	intercalated disc
aa	amino acid
β-catBD	β-catenin-binding domain
VSDs	ventricular septal defects
BrdU	bromodeoxyuridine
P	postnatal day
ES	embryonic stem
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
α-TM	α-tropomyosin
EM	electron microscopy
HW	heart weight
BW	body weight
LW	liver weight
E/A	mitral valve E-wave (early filling) to A-wave (atrial contraction/late filling) ratio
α- and β-MHC	α- and β-myosin heavy chain

cTnT	cardiac troponin T
ecTnT	embryonic cTnT
acTnT	adult cTnT
cTnI	cardiac troponin I
ssTnI	slow skeletal troponin I
cTnI-ND	N-terminal truncated cTnI
fsTnI	fast skeletal troponin I
ssTnT	slow skeletal troponin T
PI3K	phosphatidylinositol 3-kinase
IGF-1	insulin-like growth factor 1
Erk1/2	extracellular-signal-regulated kinase 1/2
Akt	protein kinase B
GSK3β	glycogen synthase kinase 3β
Stat3	signal transducer and activator of transcription 3
Jak2	Janus kinase 2
Grb2	growth factor receptor-bound protein 2
SOCS3	suppressor of cytokine signaling 3
Cx43	connexin 43

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References

1. Olson EN. Gene regulatory networks in the evolution and development of the heart. Science 2006;313:1922–1927. [PubMed: 17008524]
2. Clark KL, Yutzey KE, Benson DW. Transcription factors and congenital heart defects. Annu Rev Physiol 2006;68:97–121. [PubMed: 16460268]
3. Wang DZ, Reiter RS, Lin JL, Wang Q, Williams HS, Krob SL, Schultheiss TM, Evans S, Lin JJ. Requirement of a novel gene, Xin, in cardiac morphogenesis. Development 1999;126:1281–1294. [PubMed: 10021346]
4. Lin JJ-C, Gustafson-Wagner EA, Sinn HW, Choi S, Jaacks SM, Wang DZ, Evans S, Lin JL-C. Structure, expression, and function of a novel intercalated disc protein, Xin. J Med Sci 2005;25:215–222. [PubMed: 16708114]
5. Huang HT, Brand OM, Mathew M, Ignatiou C, Ewen EP, McCalmon SA, Naya FJ. Myomaxin is a novel transcriptional target of MEF2A that encodes a Xin-related alpha-actinin-interacting protein. J Biol Chem 2006;281:39370–39379. [PubMed: 17046827]
6. Grosskurth SE, Bhattacharya D, Wang Q, Lin JJ. Emergence of Xin demarcates a key innovation in heart evolution. PLoS ONE 2008;3:e2857. [PubMed: 18682726]

7. Gustafson-Wagner EA, Sinn HW, Chen YL, Wang DZ, Reiter RS, Lin JL, Yang B, Williamson RA, Chen J, Lin CI, Lin JJ. Loss of mXinα, an intercalated disk protein, results in cardiac hypertrophy and cardiomyopathy with conduction defects. Am J Physiol Heart Circ Physiol 2007;293:H2680–H2692. [PubMed: 17766470]
8. Pacholsky D, Vakeel P, Himmel M, Lowe T, Stradal T, Rottner K, Furst DO, van der Ven PFM. Xin repeats define a novel actin-binding motif. J Cell Sci 2004;117:5257–5268. [PubMed: 15454575]
9. Choi S, Gustafson-Wagner EA, Wang Q, Harlan SM, Sinn HW, Lin JL, Lin JJ. The intercalated disc protein, mXinα, is capable of interacting with β-catenin and bundling actin filaments. J Biol Chem 2007;282:36024–36036. [PubMed: 17925400]
10. van der Ven PFM, Ehler E, Vakeel P, Eulitz S, Schenk JA, Milting H, Micheel B, Furst DO. Unusual splicing events result in distinct Xin isoforms that associate differentially with filamin c and Mens/VASP. Exp. Cell Res 2006;312:2154–2167. [PubMed: 16631741]
11. Perriard JC, Hirschy A, Ehler E. Dilated cardiomyopathy: a disease of the intercalated disc? Trends Cardiovasc Med 2003;13:30–38. [PubMed: 12554098]
12. Sinn HW, Balsamo J, Lilien J, Lin JJ. Localization of the novel Xin protein to the adherens junction complex in cardiac and skeletal muscle during development. Dev Dyn 2002;225:1–13. [PubMed: 12203715]
13. Ishiwata T, Nakazawa M, Pu WT, Tevosian SG, Izumo S. Developmental changes in ventricular diastolic function correlate with changes in ventricular myoarchitecture in normal mouse embryos. Circ Res 2003;93:857–865. [PubMed: 14551244]
14. Westfall MV, Metzger JM. Troponin I isoforms and chimeras: tuning the molecular switch of cardiac contraction. News Physiol Sci 2001;16:278–281. [PubMed: 11719605]
15. Fentzke RC, Buck SH, Patel JR, Lin H, Wolska BM, Stojanovic MO, Martin AF, Solaro RJ, Moss RL, Leiden JM. Impaired cardiomyocyte relaxation and diastolic function in transgenic mice expressing slow skeletal troponin I in the heart. J Physiol 1999;517:143–157. [PubMed: 10226156]
16. Barbato JC, Huang QQ, Hossain MM, Bond M, Jin JP. Proteolytic N-terminal truncation of cardiac troponin I enhances ventricular diastolic function. J Biol Chem 2005;280:6602–6609. [PubMed: 15611140]
17. Fernandez E, Siddiquee Z, Shohet RV. Apoptosis and proliferation in the neonatal murine heart. Dev Dyn 2001;221:302–310. [PubMed: 11458390]
18. Li J, Patel VV, Radice GL. Dysregulation of cell adhesion proteins and cardiac arrhythmogenesis. Clin Med Res 2006;4:42–52. [PubMed: 16595792]
19. Hirakow, R.; Gotoh, T. Ontogenetic implication of the myocardial ultrastructure in the development of mammalian heart. In: van Praagh, R.; Takao, A., editors. Etiology and morphogenesis of congenital heart disease. Mount Kisco, NY: Futura Publishing Co.; 1980. p. 99-108.
20. Zhou YQ, Foster FS, Parkes R, Adamson SL. Developmental changes in left and right ventricular diastolic filling patterns in mice. Am J Physiol Heart Circ Physiol 2003;285:H1563–H1575. [PubMed: 12805021]
21. Clerk A, Pham FH, Fuller SJ, Sahai E, Aktories K, Marais R, Marshall C, Sugden PH. Regulation of mitogen-activated protein kinases in cardiac myocytes through the small G protein Rac1. Mol Cell Biol 2001;21:1173–1184. [PubMed: 11158304]
22. Satoh M, Ogita H, Takeshita K, Mukai Y, Kwiatkowski DJ, Liao JK. Requirement of Rac1 in the development of cardiac hypertrophy. Proc Natl Acad Sci U S A 2006;103:7432–7437. [PubMed: 16651530]
23. Yamane M, Matsuda T, Ito T, Fujio Y, Takahashi K, Azuma J. Rac1 activity is required for cardiac myocyte alignment in response to mechanical stress. Biochem Biophys Res Commun 2007;353:1023–1027. [PubMed: 17207463]
24. Pokutta S, Weis WI. The cytoplasmic face of cell contact sites. Curr. Opin. Struc. Biol 2002;23:255–262.
25. Drees F, Pokutta S, Yamada S, Nelson WJ, Weis WI. Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. Cell 2005;123:903–915. [PubMed: 16325583]
26. Cavey M, Rauzi M, Lenne PF, Lecuit T. A two-tiered mechanism for stabilization and immobilization of E-cadherin. Nature 2008;453:751–756. [PubMed: 18480755]

27. Sussman MA, Welch S, Walker A, Klevitsky R, Hewett TE, Price RL, Schaefer E, Yager K. Altered focal adhesion regulation correlates with cardiomyopathy in mice expressing constitutively active rac1. J Clin Invest 2000;105:875–886. [PubMed: 10749567]

28. Fukuyama T, Ogita H, Kawakatsu T, Inagaki M, Takai Y. Activation of Rac by cadherin through the c-Src-Rap1-phosphatidylinositol 3-kinase-Vav2 pathway. Oncogene 2006;25:8–19. [PubMed: 16170364]

29. Noren NK, Liu BP, Burridge K, Kreft B. p120 catenin regulates the actin cytoskeleton via Rho family GTPases. J Cell Biol 2000;150:567–580. [PubMed: 10931868]

30. Honsho S, Nishikawa S, Amano K, Zen K, Adachi Y, Kishita E, Matsui A, Katsume A, Yamaguchi S, Nishikawa K, Isoda K, Riches DW, Matoba S, Okigaki M, Matsubara H. Pressure-mediated hypertrophy and mechanical stretch induces IL-1 release and subsequent IGF-1 generation to maintain compensative hypertrophy by affecting Akt and JNK pathways. Circ Res 2009;105:1149–1158. [PubMed: 19834007]

31. Kalmyrzaev B, Aldashev A, Khalmatov M, Polupanov A, Jumagulova A, Mamanova L, Wilkins MR, Town M. Genome-wide scan for premature hypertension supports linkage to chromosome 2 in a large Kyrgyz family. Hypertension 2006;48:908–913. [PubMed: 17000929]

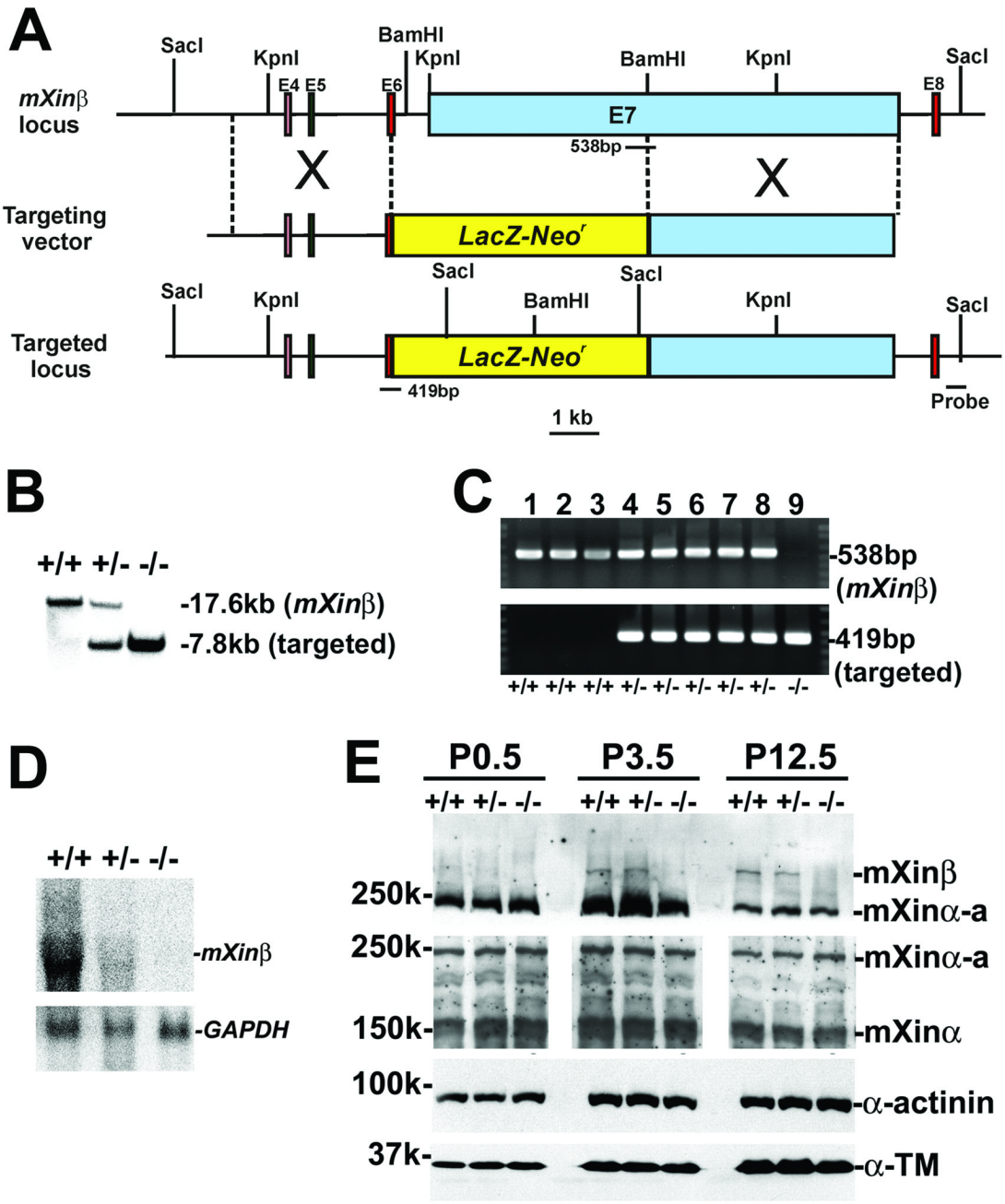

Figure 1.

Generation of *mXin*β-null mice. (A) Targeting strategy. A restriction map of the relevant genomic region of *mXin*β is shown at the top (*mXin*β locus). The targeting vector (in the middle) contains the genomic region with a *LacZ-Neo*^{r} cassette to replace portion of Exon 6 (E6)-intron 6-portion of E7. The targeted locus is shown at the bottom. The probe used for Southern blot is located downstream of E8. (B) Southern blot analysis of SacI-digested genomic DNAs. (C) PCR genotyping. The locations of the PCR products for endogenous *mXin*β (538bp) and targeted locus (419bp) are shown in (A). (D) Northern blot analysis. A ~12kb *mXin*β message was detected in both wild type and heterozygous but not homozygous samples. The same membrane was hybridized with *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) probe

to show RNA loading. (E) Western blot analysis on developing heart extracts from each genotype with U1013 anti-mXin, anti-α-actinin and anti-α-TM antibodies. A ~340 kDa mXinβ was detected in the developing wild type and heterozygous but not homozygous extracts (the top panel). There are no apparent changes in the expression of mXinα-a, mXinα, α-actinin, or α-TM in the *mXin*β-null hearts.

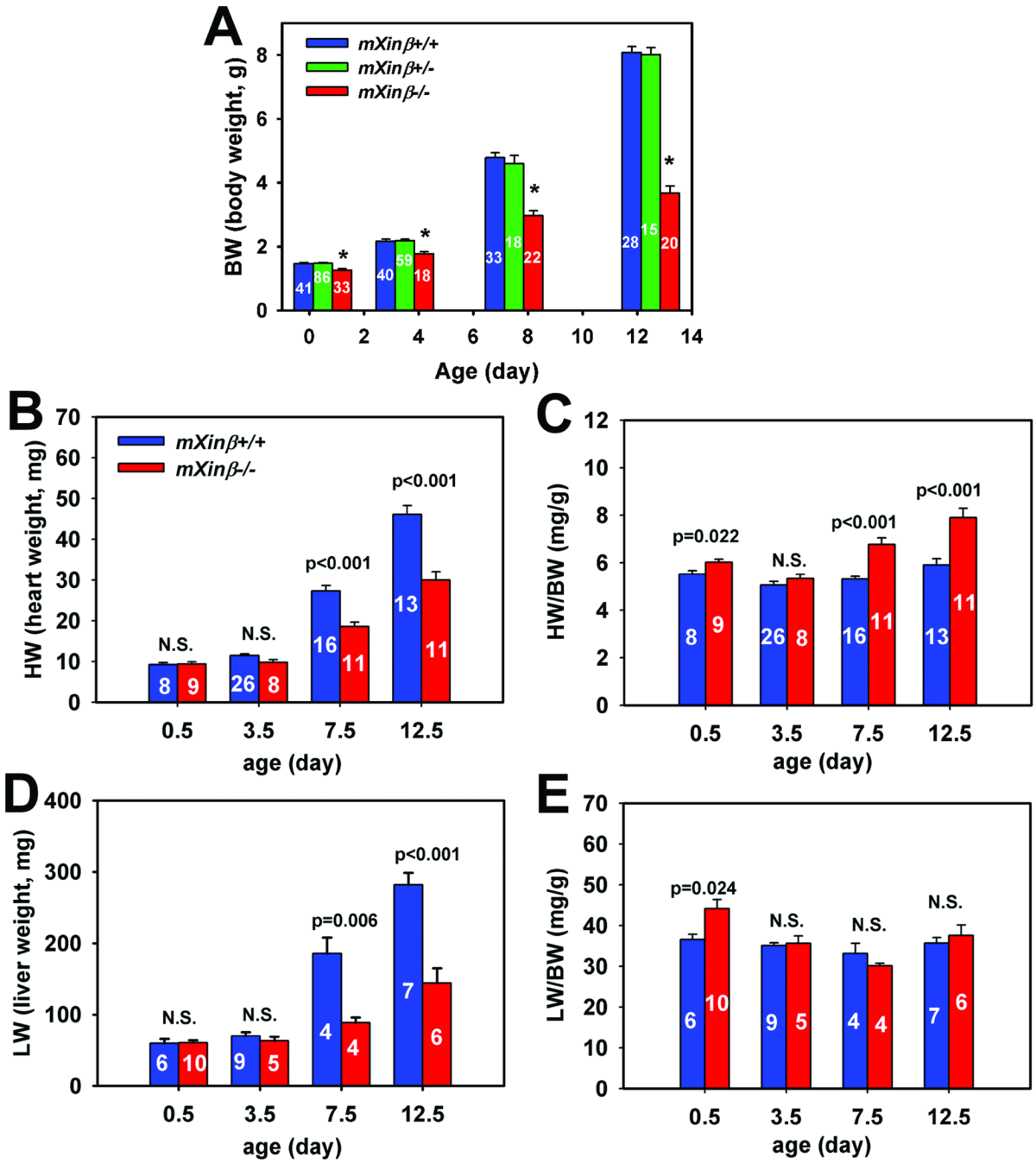

Figure 2.

Loss of mXinβ results in severe growth retardation. (A) Body weight (BW) comparison. * p<0.01, significant difference between *mXin*β−/− and control (*mXin*β+/+ or *mXin*β+/−), ANOVA. (B, C) Heart weight (HW) and HW/BW comparisons. (D, E) Liver weight (LW) and LW/BW comparisons. The numbers of animals measured are indicated within each bar. The means±SEM are displayed graphically. t-test for (B)~(E). N.S., p>0.05 no significant difference.

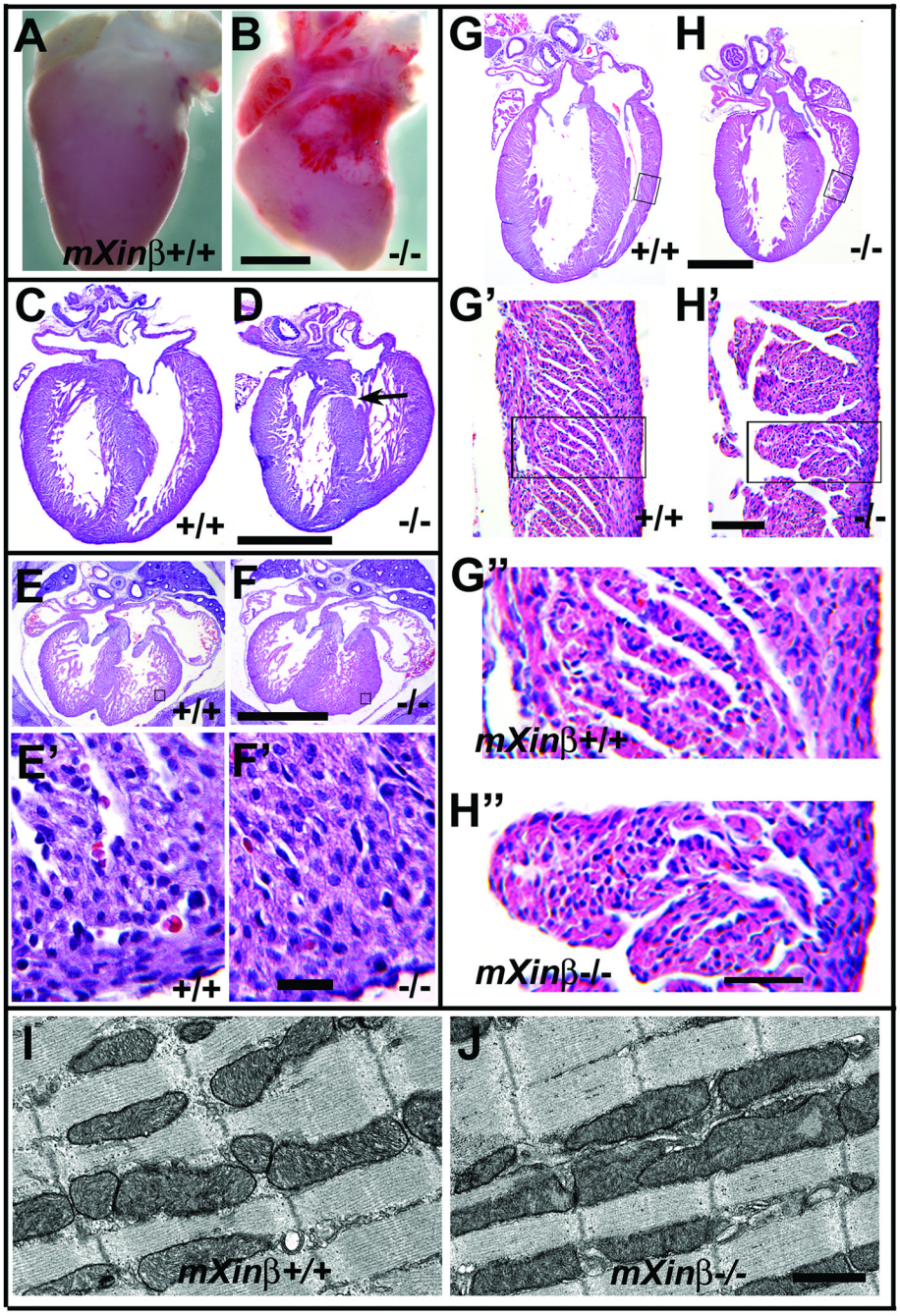

Figure 3.

Structural analyses of *mXin*β+/+ and *mXin*β−/− hearts. (A&B) Gross morphology showing abnormal heart shape in P0.5 *mXin*β-null mice. Bar=1mm. (C&D) H&E-stained sections demonstrating the VSD (arrow in D) in P0.5 *mXin*β-null heart. Bar = 1 mm. (E&F) H&E-stained sections of E14.5 wild type and *mXin*β-null embryos. Bar=0.5mm. (E'&F') higher-magnification images of the boxed areas in E&F. Bar=50μm. (G&H) H&E-stained sections from P3.5 wild type and *mXin*β-null hearts. Bar=1mm. (G'&H') higher-magnification images of the boxed areas in G&H. Bar=0.1mm. (G"&H") higher-magnification images of the boxed areas in G'&H', showing mis-organized myocytes in mutant ventricle. Bar=50μm. (I&J) EM

micrographs showing no alteration in sarcomere organization of P15.5 *mXin*β-null hearts. Bar=1μm.

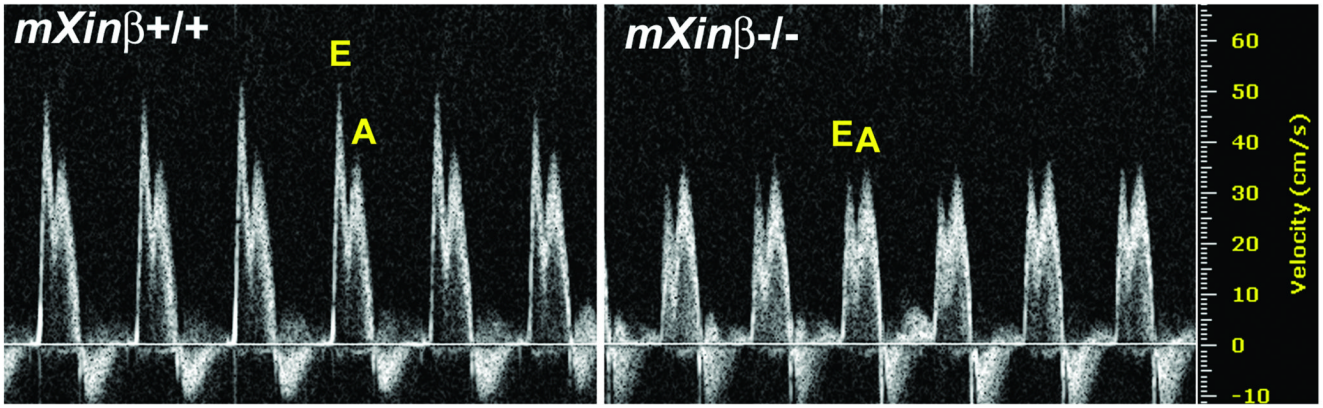

Figure 4.
Doppler flow spectra recorded from the mitral valvular orifices of P12.5 wild type and *mXin*β-null mice. E-wave represents early filling velocity, whereas A-wave is late filling (atrial contraction) velocity.

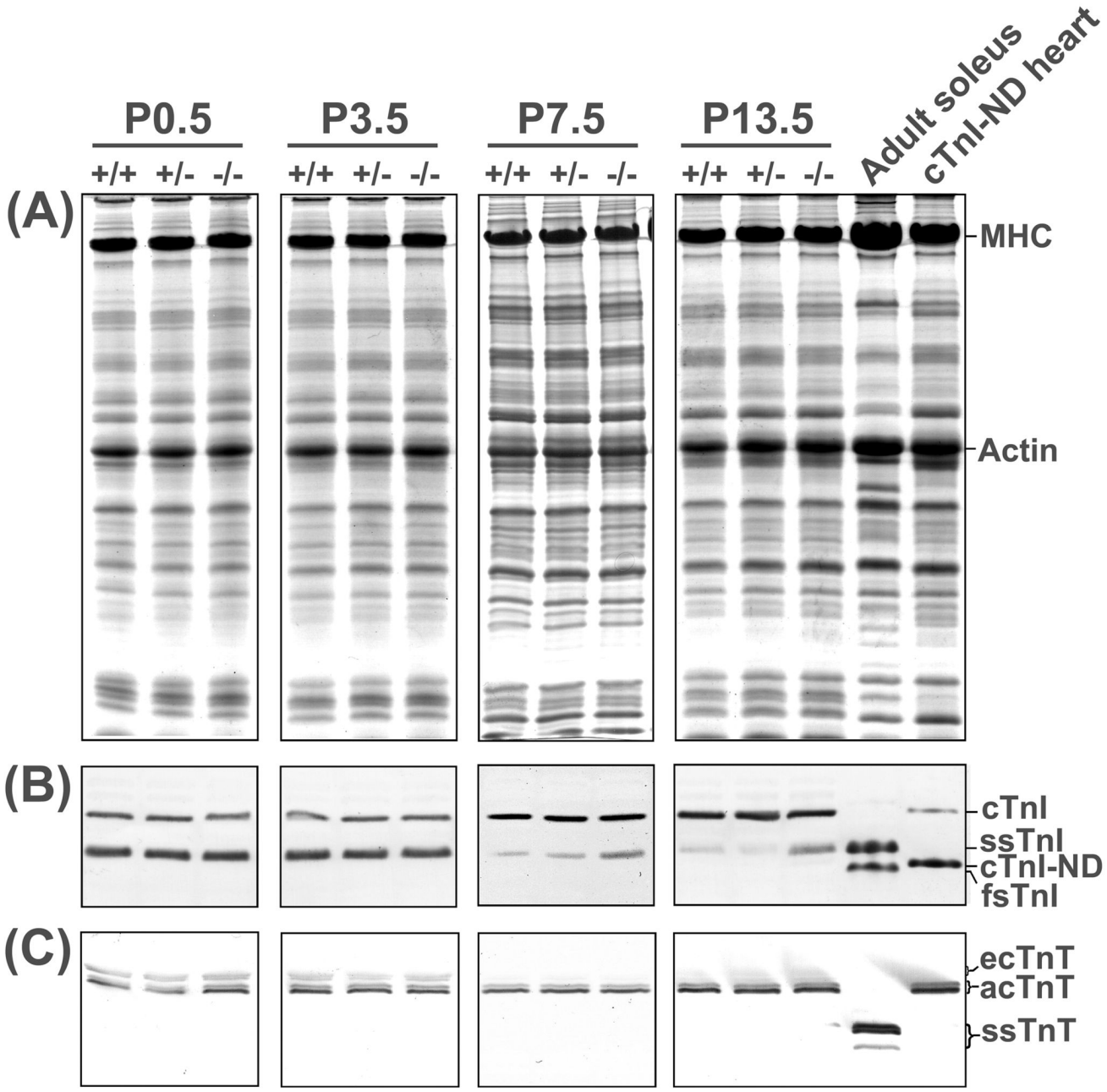

Figure 5.

A significant delay in switching off ssTnI in *mXin*β-null hearts. (A) Protein profiles from developing hearts of each genotype, adult soleus and transgenic mouse hearts over-expressing cTnI-ND. (B and C) Western blot analyses with anti-TnI and anti-cTnT antibodies, respectively. By P7.5~13.5, the control hearts (+/+ and +/−) almost switched off ssTnI, whereas the *mXin*β−/− heart still expressed significant amounts of ssTnI. In contrast, the cTnT isoform switch from ecTnT to acTnT in homozygous hearts was normal. fsTnI, fast skeletal TnI.

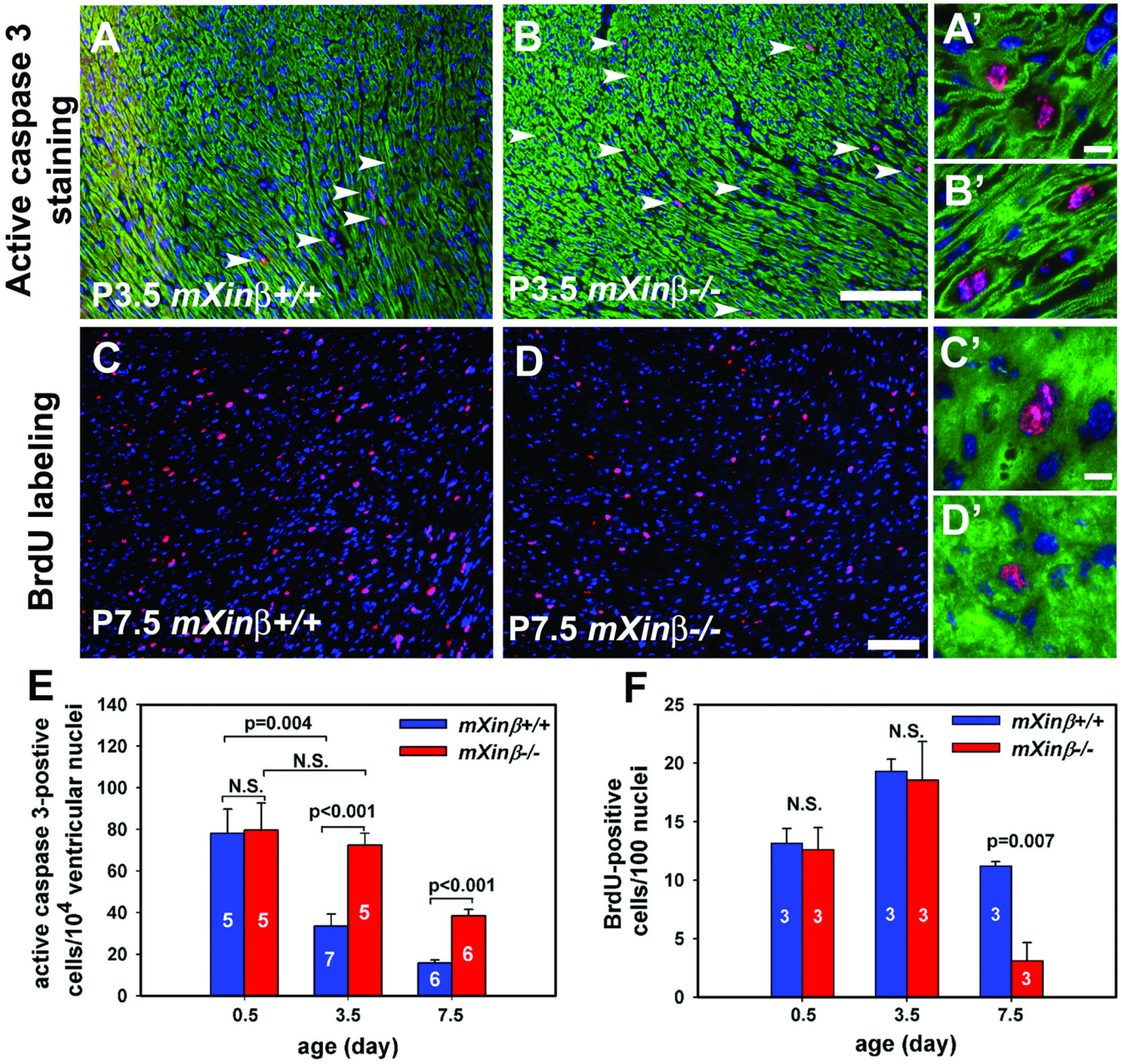

Figure 6.

Increased apoptosis and decreased proliferation in developing *mXin*β-null hearts. (A&B) Representative images from P3.5 heart sections, stained with anti-active caspase 3 for apoptotic cells (red, arrowheads), anti-cTnT for cardiomyocytes (green), and DAPI for nuclei (blue). Bar=100μm. (A'&B') higher-magnification images of A&B. Bar=10μm. (C&D) Representative images from heart sections of P7.5 BrdU-labeled mice, stained with anti-BrdU for proliferative cells (red) and counterstained with DAPI (blue). Bar=100μm. (C'&D') higher-magnification images of heart sections triple-stained with anti-BrdU (red), anti-α-TM (green) and DAPI (blue). Bar=10μm. (E&F) Apoptotic and proliferative cell populations, respectively, in developing wild type and *mXin*β-null hearts. The numbers of animals measured are indicated within each bar. The means±SEM are displayed graphically. N.S., p>0.05 no significant difference (t-test).

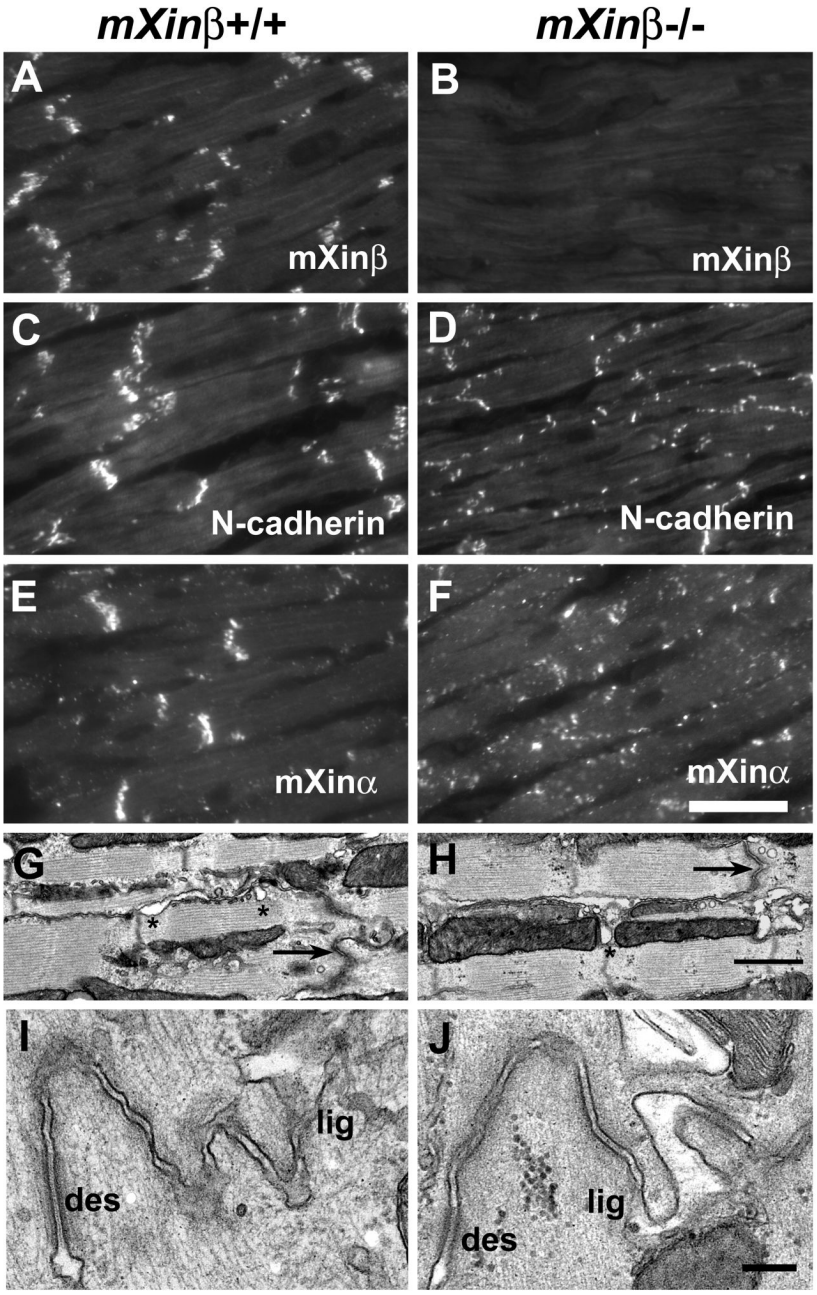

Figure 7.

Mis-localization of N-cadherin and mXinα as well as structural alteration in developing intercalated disc of *mXin*β-null hearts. Frozen heart sections from P16.5 mice were immunofluorescently stained with U1040 anti-mXinβ (A&B), anti-cadherin (C&D) and R1697 anti-mXinα (E&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 *mXin*β-null heart led to mis-localization of N-cadherin (D) and mXinα (F). Bar=30μm. (G&H) EM images of P15.5 wild type and *mXin*β-null cardiomyocytes. Arrows, intercalated discs; *, T-tubules. Bar=1μm. (I&J) high magnification images of maturing intercalated discs of P15.5 wild type and *mXin*β-null cells. The closely apposite membranes of mutant intercalated disc were less

convoluted and less wavy. des, desmosome; lig, two membranes in the process of ligation together to form intercalated disc. Bar=0.2μm.

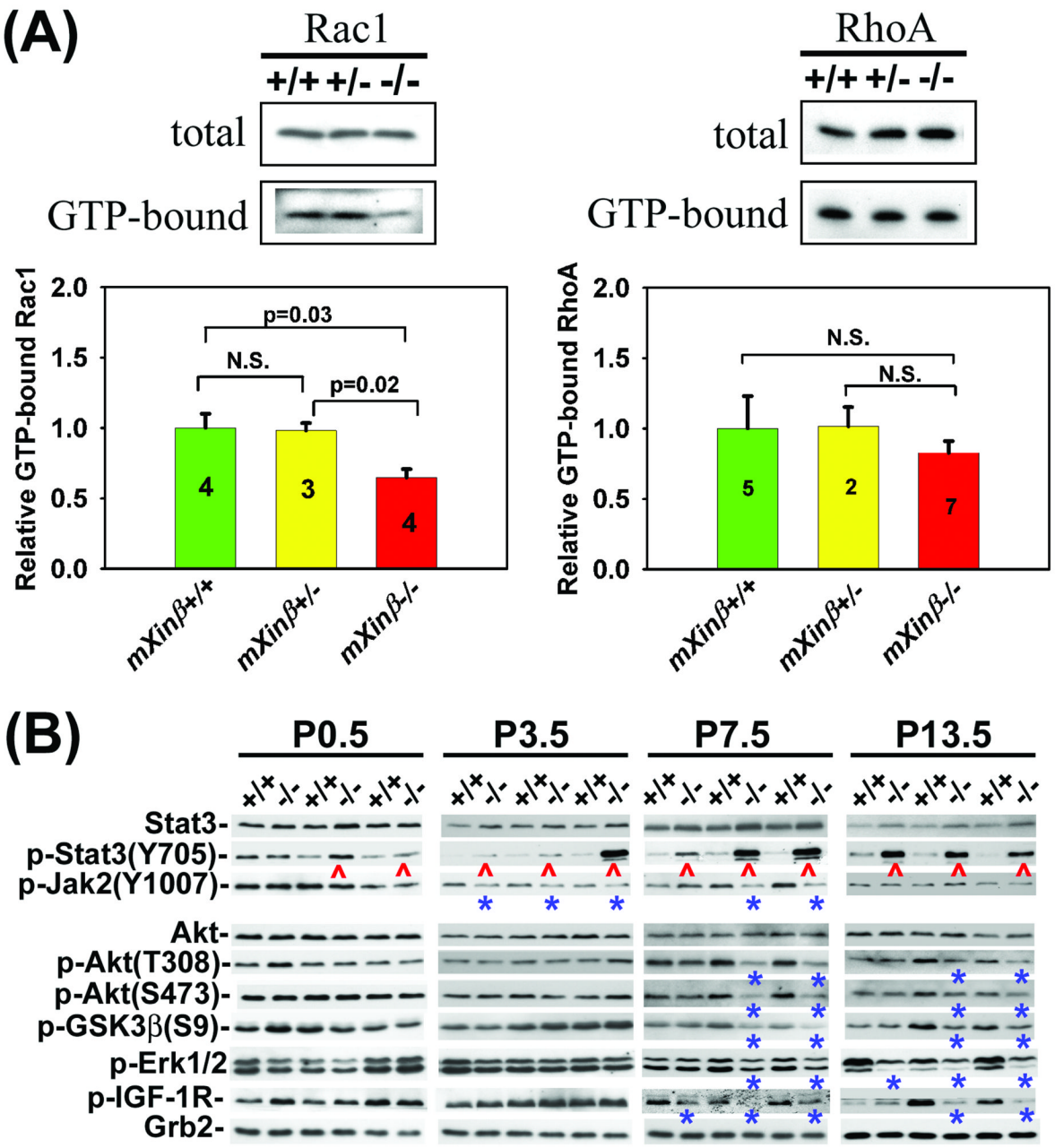

Figure 8.

Increased Stat3 activity and decreased Rac1, IGF-1R, Akt and Erk1/2 activities in *mXin*β-null hearts. (A) The relative GTP-bound Rac1 but not GTP-bound RhoA was significantly reduced in P7.5 *mXin*β-null hearts. N.S., p>0.05 no significant difference (ANOVA). The numbers of animals measured are indicated within each bar. (B) Western blot analyses with phospho-specific antibodies against key signaling molecules. Up-regulation of active Stat3 (p-Stat3) (^) can be detected in some of *mXin*β-null hearts as early as P0.5. However, this Stat3 activation is not parallel to the activation of Jak2 (p-Jak2). Total Akt and Grb2 protein levels remain unchanged; however, the activation/phosphorylation of Akt, GSK3β, Erk1/2 and IGF-1R were significantly reduced beginning from P7.5 in *mXin*β-null hearts (*).