Kindlin-2 Is an Essential Component of Intercalated Discs and Is Required for Vertebrate Cardiac Structure and Function

James J. Dowling, Elizabeth Gibbs, Mark Russell, Daniel Goldman, Jeremy Minarcik, Jeffrey A. Golden, Eva L. Feldman

Abstract—Integrins and proteins that associate with integrins are implicated in normal cardiac muscle function and development. UNC-112 is a cytoplasmic adaptor protein required for the proper establishment of integrin junctions in Caenorhabditis elegans muscle. A vertebrate homolog of unc-112, kindlin-2, is an integrin-binding protein that is expressed in cardiac muscle, but its function is unknown. We sought to understand the role of kindlin-2 in the development and function of the mouse and zebrafish heart. In the mouse, we found that kindlin-2 is highly expressed in the heart and is enriched at intercalated discs and costameres. Targeted disruption of the murine kindlin-2 gene resulted in embryonic lethality before cardiogenesis. To better assess the role of kindlin-2 in cardiac muscle development, we used morpholinos to knockdown the kindlin-2 homolog in zebrafish (z-kindlin-2), which resulted in severe abnormalities of heart development. Morphant hearts were hypoplastic and dysmorphic and exhibited significantly reduced ventricular contractility. Ultrastructural analysis of these hearts revealed disrupted intercalated disc formation and a failure in the attachment of myofibrils to membrane complexes. We conclude that kindlin-2 is an essential component of the intercalated disc, is necessary for cytoskeletal organization at sites of membrane attachment, and is required for vertebrate myocardial formation and function. These findings provide the first characterization of the in vivo functions of this novel and critical regulator of cardiogenesis. (Circ Res. 2008;102:423-431.)

Key Words: integrin ■ intercalated discs ■ kindlin-2 ■ cardiomyopathy ■ zebrafish

Integrins are heterodimeric transmembrane proteins that link the extracellular matrix with the cytoskeleton and with various cytoplasmic signaling cascades.1 In the heart, integrin receptors primarily contain β1 integrin and localize to costameres and intercalated discs.2 There they perform critical roles in the development of normal cardiac function and are involved in various cardiac disease processes.3 In mice, loss of β1 integrin function results in disturbance of myocyte morphology and loss of myocardial mechanical integrity.4,5 The overall consequence is the development of cardiac fibrosis and dilated cardiomyopathy. Activation of β1 integrin function, conversely, promotes cardiac remodeling and cardiac hypertrophy.6 In humans and mice, integrin signaling is implicated in both the normal hypertrophic response as well as in the development of pathological cardiac hypertrophy.6

The integrin-linked kinase (ILK) is a critical biochemical regulator of integrin-dependent cardiac development and homeostasis.7 ILK functions both enzymatically as a serine/threonine kinase and as a cytoskeletal adaptor protein. Cardiomyocyte-specific ablation of ILK in mice results in similar phenotypic and pathological abnormalities to those observed in the β1 integrin conditional knockout.8 Recently, mutations in ILK were associated with human dilated cardiomyopathy.9 Taken together, these results suggest that integrins and their associated signaling partners are critical for normal cardiac muscle function.

The nematode Caenorhabditis elegans has provided essential insights into the molecular components required for integrin function in muscle.10 One such critical component is unc-112, so named because mutation in its gene product results in embryonic lethality before cardiogenesis.11 The unc-112 protein directly interacts with the Caenorhabditis elegans homolog of ILK, and is required for pat-4 association with β integrin.12 Through these interactions, unc-112 mediates the attachment of actin-myosin myofilaments to integrin transmembrane complexes, and mutations in unc-112 result in loss of the dense bodies and M-lines, the worm equivalents of costameres and intercalated discs.11

There are 3 vertebrate homologs of unc-112.13,14 These genes, the kindlins, all share a similar structure with unc-112 and contain a pleckstrin homology domain and a FERM
domain. FERM domains are implicated as modulators of integrin and actin cytoskeleton interactions, and the kindlin FERM domain is similar by sequence to the integrin and actin binding protein talin.\textsuperscript{13} Both kindlin-1 and kindlin-2 directly bind to $\beta$1 integrin, and kindlin-2 can associate with ILK.\textsuperscript{16–18} Both proteins regulate integrin-dependent cell spreading and cell adhesion in vitro. Kindlin-1 mediates the mechanical function of adherens junctions in keratinocytes, and mutations in the gene cause Kindler syndrome, a blistering skin disease marked by epidermal fragility.\textsuperscript{14,17,19,20} Kindlin-1, however, is not expressed in cardiac myocytes.\textsuperscript{13} Only kindlin-2, also known as mig-2, is reported to be expressed in cardiac tissue.\textsuperscript{13}

Given the essential role of unc-112 for integrin function in \textit{C elegans} muscle and the critical roles of integrins and ILK in cardiac muscle, we hypothesized that a vertebrate unc-112 homolog is required for cardiac muscle structure and function. We focused our investigation on kindlin-2 because it is the only kindlin expressed in the heart. We confirmed that mouse kindlin-2 is abundantly expressed in cardiac muscle and found it enriched at intercalated discs and costameres. Targeted mutation of murine kindlin-2 arrests embryogenesis by embryonic day (E)7.5, revealing a requirement for kindlin-2 during early development but precluding analysis of its role in cardiogenesis. We then used zebrafish as an alternate vertebrate model system for studying cardiac development and demonstrated that knockdown of kindlin-2 causes a severe disruption in cardiac structure and function. In particular, loss of kindlin-2 disrupts the structure of the intercalated disc and affects ventricle morphology, size, and contractility. These results suggest that kindlin-2 is essential for the structural formation of the vertebrate heart and likely is a key mediator of integrin function in the myocardium.

Materials and Methods

Embryonic Stem Cells and Transgenic Mice

Murine embryonic stem cells (129/Ola background) were obtained from the Sanger Institute Gene Trap Resource (Cambridge, UK). Cells were propagated without feeder cells (http://www.sanger.ac.uk/PostGenomics/genetrap/protocols.shtml) and then injected into blastocysts in the Transgenic Core at the Children’s Hospital of Philadelphia. Germline transmission was obtained and resulting heterozygous mice (C57BL6 background) maintained according to Institutional Animal Care and Use Committee rules and regulations at the Children’s Hospital of Philadelphia. Embryos for analysis were generated by timed matings of heterozygous animals.

Zebrafish Morpholino Studies

Zebrafish were maintained in compliance with University of Michigan Institutional Animal Care and Use Committee UCUSA standards. Morpholino antisense oligonucleotides were obtained for the following sequences (GeneTools LLC, Philomath, Ore): ATG MO (ATG translational site morpholino) (z-kindlin-2), GCATCCGTATACCGTCCAGCGCCAT; SPL (splice site) MO (z-kindlin-2), GCATCCGTATACCGTCCAGCGCCAT; SPL (splice site) MO (z-kindlin-2), GCATCCGTATACCGTCCAGCGCCAT.

The control morpholino, which by BLAST search is not homologous to any sequence in the zebrafish genome, was also obtained from Gene Tools LLC: control MO: CCT CTT ACC TCA GTT ACA ATT TAT A.

Morpholinos were diluted to 0.1 mmol/L in Danieau buffer, and $\sim$1 ng was injected into the yolk of 1- to 2-cell stage embryos. For live images, embryos were immobilized in media containing tricine and viewed on a Leica MZFLIII microscope. Images were obtained using an Optronics digital camera and Magnafire software suite. Live image analysis was used for measurements of cardiac function.\textsuperscript{21}

Morpholino Rescue

Mouse kindlin-2 cDNA was subcloned from pcDNA3.1D/V5 (Invitrogen) into pCS2+ (gift of D. Turner, University of Michigan, Ann Arbor). RNA was synthesized using SP6 and the mMessage mMachine kit (Ambion) and injected at the 1- to 2-cell stage independently or in combination with morpholino.

Results

Kindlin-2 Is Expressed in Cardiac Muscle and Enriched at Intercalated Discs

The tissue distribution of kindlin-2 RNA was evaluated using Northern blot analysis on adult mouse tissue. In agreement with previously published data,\textsuperscript{13} Kindlin-2 expression is highest in cardiac muscle, with lower levels of expression in skeletal muscle and testis (Figure 1A, arrow).

To determine the localization of kindlin-2 within cardiomyocytes, adult mouse heart was examined using immunofluorescence and antibodies to kindlin-2. Kindlin-2 staining was detected along the long axis of the sarcolemmal membrane (arrowhead) and at the junctions between myocytes (arrow) (Figure 1B). Examination at higher magnification revealed that kindlin-2 is expressed at intercalated discs (arrow) and at costameres (arrowheads) (Figure 1C). Double labeling with the Z-band marker $\alpha$-actinin shows that kindlin-2 is expressed adjacent to Z-bands, consistent with costameric localization (arrowhead). Localization to the intercalated disc was verified by costaining with an antibody to vinculin (arrows, Figure 1D). In contrast to these results for kindlin-2, no cardiac staining was seen by immunofluorescence with an antibody to kindlin-1 (data not shown). The lack of kindlin-1 protein expression in the heart is consistent with published data of kindlin-1 RNA expression;\textsuperscript{13} in addition, we used real-time PCR to verify that kindlin-2 is the only unc-112 homolog appreciably expressed in cardiac tissue (Figure I in the online data supplement, available at http://circres.ahajournals.org).

Kindlin-2 Is Required for Murine Embryonic Development

We were interested in determining the role of kindlin-2 in cardiogenesis in vivo. We first attempted to study kindlin-2 function in the mouse using a gene targeting strategy. We derived mice from ES cells with an insertional mutation at intron 11 of the kindlin-2 gene locus (Figure 2A). Mice heterozygous for the mutation were phenotypically normal; however, matings of the mice yielded no live born $\sim$/-- offsprings. Therefore, kindlin-2 $^{-/-}$ mice likely died during embryogenesis.

Litters from heterozygous crosses were subsequently examined at multiple ages during embryogenesis. No kindlin-2 $^{-/-}$ embryos were detected at E8.5 or later. We found 3 embryos at E7.5 with a $\sim$/-- genotype (Figure 2B). We examined and attempted to genotype a total of 76 embryos at E7.5 (Figure 2B). Instead of the predicted 25% (19/76) of embryos having the $\sim$/-- genotype, only 2.6% (2/76) were homozygous for the targeted allele (supplemental Table I). In addition, we found 18 resorptions that were unable to
Genotype (Figure 2B). We assume that the majority of these resorptions are dead \(-/–\) embryos. Based on these observations, we concluded that this mutation in kindlin-2 results in embryonic lethality at or before E7.5. This very early lethality precluded analysis of heart development in our mice.

**Kindlin-2 Is Required for Zebrafish Development**

We next explored kindlin-2 function in the developing zebrafish as an alternative model for studying cardiac muscle development. Using BLAST analysis of the zebrafish genome, a single gene of high-sequence homology to mammalian kindlin-2 was uncovered (88% similar by amino acid sequence). This gene (im:7145859; http://www.zfin.org), which we call z-kindlin-2, is expressed in both cardiac and skeletal muscle in the developing zebrafish (Figure 3A and previously published\(^{22}\)). To study z-kindlin-2 function, we performed gene knockdown using an antisense morpholino designed to the putative translational start site (ATG MO). As expected, injection of this morpholino resulted in greatly decreased z-kindlin-2 protein expression (Figure 3B).

We compared ATG and control-injected embryos at various developmental time points. ATG MO knockdown of z-kindlin-2 resulted in a severe and reproducible phenotype. Morphological changes were apparent by visual inspection at 24 hours after fertilization (hpf) (Figure 3C) and became even more pronounced at 48 hpf (Figure 3D). These abnormalities continued to progress, and by 96 hpf, z-kindlin-2 morphant fish had severe, widespread defects (Figure 4D). All phenotypically abnormal fish died by 7 days after fertilization. The z-kindlin-2 morphant phenotype was highly penetrant. Ninety-six percent of ATG MO injected embryos exhibited abnormalities by 48 hpf, as compared with 2% of control injected embryos (n = 100). The most common morphological change was pericardial edema, occurring in 95% of embryos. In addition, the majority of morphant embryos had abnormal-
ities in their body structure. This included an overall reduction in size, a bent midbody, and a fore-shortened tail (Figure 3C and 3D). Fifty percent of embryos were nonmotile at 48 hpf. The combination of an aberrant body shape with impaired motility is consistent with skeletal muscle dysfunction (see Figure 8). Ocular and central nervous system abnormalities were also noted but not investigated (Figure 3C and 3D, arrows).

We verified the specificity of the morpholino phenotype using 2 experimental approaches. First, we assayed the ability of kindlin-2 RNA to rescue the morphant phenotype. Using mRNA derived from the murine kindlin-2 gene, we performed coinjections with either control or ATG morpholinos and analyzed 25 embryos per condition at 72 hpf. Coinjection of control MO and kindlin-2 RNA resulted in normally developing fish (Figure 4A), whereas injection of ATG MO gave the expected morphological abnormalities (Figure 4B). Coinjection of ATG MO with kindlin-2 RNA resulted in no detectable phenotype and thus complete rescue (Figure 4C). In addition to verifying morpholino specificity, by using mouse kindlin-2, this rescue experiment suggests that kindlin-2 function is conserved across vertebrate species.

Next we determined whether a second kindlin-2 morpholino produced similar abnormalities to our ATG morpholino. This morpholino (SPL MO) was designed to the splice donor site of exon 3 and is predicted to cause exclusion of exon 3 and produce a premature stop codon. Injection of SPL MO resulted in the same constellation of phenotypic changes as the ATG MO (Figure 4D and 4E). Quantitative RT-PCR revealed an 82% reduction in exon 3 expression.

**z-Kindlin-2 Knockdown Results in Abnormal Ventricular Contractility**

The presence of pericardial edema in morphant embryos by 48 hpf is highly suggestive of a primary cardiac defect. We therefore examined cardiac function in more detail. Live image analysis at 48 and 72 hpf revealed that z-kindlin-2-MO hearts have abnormal, wave-like contractions with limited ventricular movement (supplemental Videos 1 and 2). We
used these live images to measure 2 elements of cardiac function, heart rate, and ventricular contractility.

We measured heart rate at 48 and 72 hpf (Figure 5A). At 48 hpf, control zebrafish had heart rates of 120 bpm, while ATG morphant zebrafish had heart rates of 108 bpm. At 72 hpf, heart rates were 134 bpm for controls and 135 bpm for ATG morphants (n=10).

Figure 5. z-Kindlin-2 is required for cardiac size and function. A, Heart rate, as measured in beats per minute (bpm), for control (CTL) and ATG morpholino–injected embryos. Heart rates at 48 hpf were 120 bpm vs 108 bpm (CTL vs ATG) (**P<0.0005; n=10). Heart rates at 72 hpf were 134 bpm vs 135 bpm (CTL vs ATG) (P=0.76; n=25). B, Shortening fraction was measured by comparing ventricular area at end systole to that at end diastole. Values are expressed as percentages and were obtained in triplicate for each embryo tested. Shortening fractions at 48 hpf were 34.4% vs 12.3% (CTL vs ATG) (**P=0.01; n=4) and at 72 hpf were 32.2% vs 11.8% (CTL vs ATG) (***P<0.001; n=5). C, End diastolic mean ventricular area. Each embryo was analyzed in triplicate. At 48 hpf, mean areas (in millimeters squared) were 74.2 vs 48.5 (CTL vs ATG) (*n=4; P=0.002). At 72 hpf, mean areas were 201.5 vs 54.7 (**n=5; P<0.0001).

The defect in ventricular contraction was accompanied by a substantial reduction in ventricular size. We studied this observation quantitatively by comparing end-diastolic ventricular areas (Figure 5C). At 48 hpf, ATG MO embryos were only 48.5 mm², as compared with 74.2 mm² for controls. At 72 hpf, ventricles from ATG morphants increased only minimally in size to 54.7 mm² and were markedly smaller than control ventricles (201.5 mm²).

A possible explanation for reduction in ventricular size is an increase in cardiomyocyte cell death. To address this, we performed TUNEL assays on sections from control and z-kindlin-2 morphant embryos (n=3). No difference in number of TUNEL-positive cells was observed (data not shown). We also examined whether cardiomyocyte proliferation was altered by z-kindlin-2 knockdown. As determined by 5-bromodeoxyuridine labeling and immunohistochemistry with phospho-histone H3 (a marker for proliferation), no change was detected (data not shown).

Cardiac Morphology Is Affected by z-Kindlin-2 Knockdown

In addition to the ventricular hypoplasia, hearts from z-kindlin-2 morphant embryos appeared to have abnormal cardiac morphology. Specifically, they were misshapen, appeared to lack the normal looped structure and had indistinct demarcations between atria and ventricles (Figure 6A).

Higher-resolution analysis on sections of cardiac muscle confirmed the presence of dysmorphic, thin-walled, dilated chambers (Figure 6B). Because of the apparent lack of distinction between atrium and ventricle in the morphant heart, we examined whether z-kindlin-2 knockdown altered differentiation of the cardiac chambers. Whole-mount in situ hybridization with cmlc-2, a marker of both atrium and ventricle, revealed 2 discrete chambers in the ATG MO embryo (Figure 6C). Whole-mount immunohistochemistry with Zn-5, a ventricle specific marker, gave ventricle restricted expression (Figure 6D). Taken together, these results suggest that cardiac differentiation, at least to the stage of chamber specification, was undisturbed by z-kindlin-2 knockdown.

z-Kindlin-2 Knockdown Disrupts the Structure of the Intercalated Disc

Because defects in cardiac morphology and function are often associated with structural abnormalities, we examined the ventricular myocytes using electron microscopy (Figure 7A). Our analysis focused on the intercalated discs, which are enriched in kindlin-2, and on the underlying sarcomeres. The
Intercalated discs from z-kindlin-2 morphants appeared abnormal (arrows, Figure 7B, versus control, Figure 7A), being thin, less electron dense, and less numerous. Their submembranous compartments are essentially undetectable (Figure 7B1). Control intercalated discs, on the other hand, had the expected enrichment of electron dense material at the membrane and in the space below it (Figure 7A1, arrows). In addition, whereas well-formed sarcomeric units were present in the z-kindlin-2 knockdown, they were not observed directly adjacent to the intercalated discs, and instead appear disorganized in the immediate perisarclemmal space.

**z-kindlin-2 Is Required for Sarcomere-Membrane Association in Skeletal Muscle**

We examined the structure of skeletal muscle myofibrils and costameres in the z-kindlin-2 morphants to see whether there were ultrastructural abnormalities comparable to those observed in cardiac muscle. At the light microscopic level, muscle in the kindlin-2 knockdown embryos was disorganized and showed evidence of vacuolization (Figure 8A). Electron microscopy revealed a failure of actin-myosin myofibers to associate with the myocyte membrane (Figure 8B and 8C). This observation is analogous to our data in cardiac muscle and strongly supports a requirement for kindlin-2 in membrane–cytoskeleton attachment.

**Discussion**

In this study of kindlin-2 localization and function in vivo, we identify kindlin-2 as a novel component of costameres and intercalated discs and demonstrate that kindlin-2 is required for embryonic development in the mouse and the zebrafish. Loss of kindlin-2 in zebrafish causes abnormalities in cardiac development and function, with specific effects on ventricular size and contractility. Our data further demonstrate that kindlin-2 is required in the establishment of the intercalated disc and in the attachment of myofibrils to membrane junctions in both cardiac and skeletal muscle. Kindlin-2 is the first integrin-linked protein to be associated with this function at the intercalated disc.

**Kindlin-2 and Embryonic Development**

Our data suggest an essential role for kindlin-2 in normal vertebrate embryonic development. The early lethality observed in the mouse knockout underscores the importance of kindlin-2 function. Of note, the age of lethality in our mice is similar to that reported for the β1 integrin and ILK knockouts.25,26 These embryos die between E4.5 and E6.5 from defects in epiblast polarity and epiblast adhesion. Given the known associations between these molecules and kindlin-2, loss of kindlin-2 may cause similar defects. Downregulation of z-kindlin-2 expression in zebrafish also causes premature
lethality, but development in the morphant zebrafish proceeds far enough to enable examination of cardiogenesis.

Kindlin-2, Cytoskeletal Attachments, and Intercalated Discs

Knockdown of z-kindlin-2 in both cardiac and skeletal muscle results in the failure of myofibrils to attach to transmembrane adhesion complexes, suggesting that kindlin-2 is required at intercalated discs and costameres for attachment of the cytoskeleton. A similar function has been demonstrated for unc-112, the homolog of kindlin-2 in *C. elegans*.27 How kindlin-2 participates in cytoskeletal organization is unknown. Although kindlin-2 does not have a canonical actin binding motif, it does have a FERM domain. FERM domains in other proteins have been shown to bind actin.15 Thus, it is possible that kindlin-2 directly interacts with myofibrils via its FERM domain. In addition, there is evidence to suggest that kindlin-2 functions as an adaptor protein, physically linking the membrane with downstream biochemical mediators. To date, 2 cytosolic proteins are known to interact with kindlin-2: migfilin and ILK, a critical regulator of cardiogenesis.7 Interestingly, mutations in zebrafish ILK cause similar cardiac functional abnormalities to those resulting from kindlin-2 knockdown.⁹,²⁸ Thus, evidence from both zebrafish and *C. elegans* suggests that ILK and kindlin-2 may function together as regulators of cell membrane signaling. Some aspects of kindlin-2 function are likely to be independent of ILK, however. Zebrafish ILK mutations neither disrupt intercalated disc structure nor cause significant skeletal muscle abnormalities. Migfilin, instead, is a candidate for mediating these functions. It is expressed in cardiomyocytes,²⁹ found in vitro at both integrin and cadherin junctions,³⁰ and known to associate with the actin binding proteins filamin and vasodilator-stimulated phosphoprotein (VASP).³⁰ Kindlin-2 may therefore link the intercalated disc to the cytoskeleton by recruiting migfilin, which in turn can bind filamin and/or VASP and initiate an association with actin. VASP localizes to the intercalated disc, and disruption of this localization causes dilated cardiomyopathy in mice.³¹

Figure 8. z-Kindlin-2 is required for skeletal myocyte organization. A, Light microscopic analysis of semithin sections from control (CTL) and ATG morpholino–injected embryos at 72 hpf. Arrow indicates an area of vacuolization. B and C, Electron microscopic analysis of skeletal muscle from CTL and ATG morphants. Arrows point to myofibrils, which associate with the membrane in the CTL but not in the ATG-MO muscle (B, ×25 000; C, ×64 000).

Kindlin-2, Ventricular Morphology, and Cardiac Function

Kindlin-2 knockdown results in abnormalities in ventricular morphology and contractility. Many aspects of the kindlin-2 phenotype are similar to those reported in zebrafish with mutations in the intercalated disc protein N-cadherin. Thus, these abnormalities may be the structural consequences of intercalated disc dysfunction.²⁴ One feature of the morphological phenotype that seems unique to kindlin-2 is ventricular hypoplasia. This was not reported for the N-cadherin mutants, and similarly has not been shown with mutations in ILK or costamere-associated proteins like ZASP.²⁴,²⁸,³² The marked ventricular hypoplasia suggests that kindlin-2 may perform function(s) in addition to those related to organization of the intercalated disc. As we observed no increase in cell death, this function may instead be related to regulation of myocyte differentiation, proliferation or myocyte size. Kindlin-2 interaction with migfilin may also be involved in these functions, as migfilin has been shown to shuttle between the cytoplasm and the nucleus, interact with Nkx2.5, and regulate cardiac differentiation in vitro.²⁹

Although the constellation of morphological abnormalities noted with z-kindlin-2 knockdown are relatively unique, they bear a strong resemblance to those recently described with morpholino inhibition of obscurin.²¹ Obscurin morphant fish have hypoplastic ventricles, poor ventricular contractility, and disorganized myofibrils in both cardiac and skeletal muscle. Interestingly, in *C. elegans* the orthologs of obscurin and kindlin-2, unc-89, and unc-112, respectively, are part of a multiprotein complex that links myofibrils via integrins to the extracellular matrix. Our data suggest this that this relationship may be conserved in vertebrates.

Kindlin-2 and Human Cardiac Disease

Given the association between other intercalated disk proteins and cardiac disease and the critical function of kindlin-2 in the zebrafish intercalated disc, it is possible that kindlin-2 mutations may be involved in human cardiac disease. Dysfunctions of intercalated disc proteins are implicated in...
human cardiac diseases. Mutations in several intercalated disc proteins are reported in patients with dilated cardiomyopathy and arrhythmogenic right ventricular cardiomyopathy, and dysregulation of intercalated disc connections are implicated in acquired heart disease.\(^3,^3,^3^4\) Moreover, mutations in ILK were recently uncovered in patients with dilated cardiomyopathy.\(^3\) Although further studies are needed to explore the role of kindlin-2 in mammalian cardiac function, the zebrafish has shown to be a valid model for mammalian cardiac dysfunction in numerous studies.\(^9,^{23,32,35,36}\) The hypothesis that kindlin-2 disruption may be involved in human cardiac disease could feasibly be tested through a candidate gene screen of kindlin-2 in patients with dilated cardiomyopathy.

In summary, we have identified kindlin-2 as a critical component of the intercalated disc and found that it is essential for normal vertebrate heart development and function. Our findings provide the first insights into its function as a cytoskeletal organizer and suggest that disruption of kindlin-2 can lead to severe developmental and cardiac defects. Further studies are warranted to characterize its involvement in the development of other organ systems and its role in mammalian cardiac function and disease.

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**Disclosures**

None.

**References**


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http://circres.ahajournals.org/content/102/4/423

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/01/03/CIRCRESAHA.107.161489.DC1
Supplemental Materials and Methods:

Northern Blot Analysis

Northern blot analysis was performed using a mouse multi-tissue blot (Clontech) and a P-32 labeled PCR fragment from the 3’ UTR of mouse kindlin-2 (Prime-It kit; Stratagene).

Section Immunofluorescence

Juvenile mice were euthanized by gas inhalation and hearts were immediately removed and frozen in supercooled isopentane. 8 µm sections were obtained using a Leica cryostat, allowed to air dry for 30 minutes, and then incubated overnight at 4°C in primary antibody. Slides were washed 3x10 minutes in PBS, incubated for 2 hours at room temperature in secondary antibody, washed 3x10 minutes in PBS and mounted with ProLong Gold plus DAPI (Invitogen). Samples were analyzed on an Olympus IX-71 inverted confocal microscope and captured using the FluoView v4.3 software. cryosections from fresh frozen adult mouse heart as previously described1. The following primary antibodies were used: goat anti-kindlin-2 (1:25; Santa Cruz); mouse anti-α-actinin (1:800; Sigma); mouse anti-vinculin (1:25; Sigma); rabbit anti-kindlin-1 (1:25; Abcam). Alexafluor conjugated secondary antibodies were used at 1:250 (Invitrogen).

Real Time PCR (qPCR)

Juvenile mice were euthanized as above and heart and kidney were isolated. RNA was extracted using the RNeasy kit from Qiagen. First strand cDNA was synthesized from 1µg RNA using the iScript kit (Bio-Rad), and qPCR was performed using the SYBR-Green reagent and an iCycler (Bio-Rad). qPCR
validated primers to kindlins 1-3 and to GAPDH were obtained from Qiagen. For qPCR on zebrafish, embryos were dechorionated at 48 hpf, euthanized with tricaine, and then homogenized in RLT buffer using a motorized homogenizer. RNA was then extracted using the RNeasy kit and qPCR performed as above. Primers were generated to z-kindlin-2 that spanned exons 3 and 4. Primers to control for RNA levels were to the zebrafish KLF7 gene.

**Gene Trap Embryo Analysis**

Embryos were isolated from timed crosses of heterozygous mice, photographed using a Leica dissecting microscope, and then processed for genotyping. Our PCR genotyping strategy was as follows:

1. **+/+ embryos**: positive band with a primer set to mkindlin-2 exon 11-intron 11 (spanning the insertion breakpoint), negative band with primer set to β-geo.
2. **+/- embryos**: positive band with primer set to mkindlin-2 exon 11-intron 11, positive band with primer set to β-geo.
3. **-/- embryos**: negative band with primer set to mkindlin-2 exon 11-intron 11, positive band with primer set to β-geo.

PCR protocol and β-geo primer sequences were obtained from Bay Genomics (baygenomics.ucsf.edu/protocols/index.html).

**Whole Mount Immunohistochemistry**

Zebrafish embryos were dechorionated at 24 hpf, exposed to PTU, and euthanized by incubation in tricaine at either 48 or 72 hpf. Embryos were fixed overnight at 4°C in 4% paraformaldehyde, dehydrated into 100% methanol, and
left overnight in 100% methanol at -20°C. Embryos were rehydrated into PBS, washed 3x in PBS-Tween (PBS-Tw), blocked in 10% goat serum, 1% BSA, PBS-Tw for 1 hour at room temperature, and then incubated overnight in primary antibody at 4°C. Embryos were washed 6x 15 minutes in PBS-Tw, incubated overnight at 4°C in secondary antibody, washed 6x 15 minutes in PBS, quenched in 5% H2O2/methanol for 30 minutes, and washed 3x 5 minutes in block. Lastly, embryos were incubated in ABC reagent (Vector Laboratories, Burlingame, CA) for 2 hours at room temperature, washed 4x 15 minutes in PBS-Tw, presoaked in DAB without H2O2, and then developed in DAB plus H2O2. Embryos were washed several times in PBS-Tw and photographed using a Leica MZ FLIII dissecting scope. Primary antibodies were to Zn-5 (1:500; DM-GRASP; ZIRC, Portland, Oregon) and kindlin-2 (1:50; ProteinTech, Chicago, IL). Biotinylated secondary antibodies were used at 1:200 (Vector Laboratories).

**Zebrafish Section Immunofluorescence**

Zebrafish were euthanized and fixed as above. Fixed embryos were sunk in 30% sucrose/PBS overnight at 4°C and then embedded in OCT. 14 µM cryosections were cut and allowed to air dry. Slides were incubated 10 minutes in 0.1% Triton plus BSA/DMSO/PBS, blocked 20 minutes in 2% Normal Donkey Serum plus BSA/DMSO/PBS, then incubated overnight in primary antibody (Zn-5, 1:500). Slides were washed 3x 10 minutes in PBS, incubated 1 hour in secondary antibody (Alexa 594 donkey anti mouse; Invitrogen), washed 3x 10 minutes in PBS, and mounted using ProLong Gold plus DAPI (Invitrogen). Photomicrographs were obtained using an Olympus BX-51 microscope.
Whole Mount In Situ Hybridization

Zebrafish embryos were processed as for immunohistochemistry. In situ hybridization was performed using the Whole Mount In Situ Hybridization kit and protocol from R&D Systems. The cmlc2 probe was kindly provided by D. Stainier (UCSF). Probe was labeled with DIG and T7 (Roche Diagnostics).

Zebrafish Histologic Analysis

Zebrafish embryos were euthanized in tricaine solution, fixed overnight at 4°C in 4% paraformaldehyde/2% glutaraldehyde and then processed for light or electron microscopy by the Microscopy and Image Analysis Lab at the University of Michigan (MIL).

References

Supplemental Figure 1

Supplemental Figure 1: Confirmation of anti-Kindlin-2 Specificity
C2C12 cells were transfected with either scrambled (si scr) or kindlin-2 specific (siKind-2) siRNA and protein was extracted 48 hours later. Samples were processed by Western blotting. Using anti kindlin-2 antibody, a band at approximately 77 kDa was detected in the scrambled lane but not in the si kindlin-2 lane (arrow). Equal loading was verified by blotting with GAPDH. Knockdown was verified by qPCR on RNA extracted from plates transfected in parallel (80% reduction of kindlin-2 mRNA levels).

Supplemental Figure 2

Supplemental Figure 2: Kindlin-2 is the Sole Kindlin Expressed in Mouse Heart
RNA was extracted from heart muscle from 8 week mice and processed for qPCR. Levels were normalized to GAPDH and to expression levels in the kidney. Kindlin-1 is 0.00004 as abundant as Kindlin-2, while Kindlin-3 is 0.0037 as abundant. Experiment was performed in triplicate.
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</thead>
<tbody>
<tr>
<td>Actual</td>
<td>24</td>
<td>31</td>
<td>3*</td>
<td>18</td>
</tr>
<tr>
<td>Expected</td>
<td>19</td>
<td>38</td>
<td>19</td>
<td>0</td>
</tr>
</tbody>
</table>

Online Table I: Table depicting results of genotyping of 76 embryos at E7.5. 18 deciduas had essentially no embryonic tissue and could not be genotyped (UTD, or unable to determine) (*p < 0.0001 for expected vs observed number of -/- embryos using Chi Square test)