Heart Failure

Targeted Ablation of PINCH1 and PINCH2 From Murine Myocardium Results in Dilated Cardiomyopathy and Early Postnatal Lethality

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Background—PINCH proteins are 5 LIM domain–only adaptor proteins that function as key components of the integrin signaling pathway and play crucial roles in multiple cellular processes. Two PINCH proteins, PINCH1 and PINCH2, have been described in mammals and share high homology. Both PINCH1 and PINCH2 are ubiquitously expressed in most tissues and organs, including myocardium. Cardiac-specific PINCH1 knockout or global PINCH2 knockout mice exhibit no basal cardiac phenotype, which may reflect a redundant role for these 2 PINCH proteins in myocardium. A potential role for PINCH proteins in myocardium remains unknown.

Methods and Results—To define the role of PINCH in myocardium, we generated mice that were doubly homozygous null for PINCH1 and PINCH2 in myocardium. Resulting mutants were viable at birth but developed dilated cardiomyopathy and died of heart failure within 4 weeks. Mutant hearts exhibited disruptions of intercalated disks and costameres accompanied by fibrosis. Furthermore, multiple cell adhesion proteins exhibited reduced expression and were mislocalized. Mutant cardiomyocytes were significantly smaller and irregular in size. In addition, we observed that the absence of either PINCH1 or PINCH2 in myocardium leads to exacerbated cardiac injury and deterioration in cardiac function after myocardial infarction.

Conclusions—These results demonstrate essential roles for PINCHs in myocardial growth, maturation, remodeling, and function and highlight the importance of studying the role of PINCHs in human cardiac injury and cardiomyopathy. (Circulation. 2009;120:568-576.)

Key Words: cardiomyopathy ■ cell adhesion molecules ■ heart failure ■ pinch protein, mouse ■ remodeling

Cells communicate with their microenvironment and neighbors by several specialized cell membrane–associate structures. In myocardium, these include costameres and intercalated disks, which provide mechanical and electric coupling between myocytes and enable myocardium to function as a syncytium. Dysfunction of cell adhesions in both animal models and human diseases is implicated in the pathogenesis of cardiomyopathy, heart failure, and injury repair.1–5 Engagement of integrin with extracellular matrix leads to recruitment and formation of a cytoplasmic focal adhesion complex composed of integrin-linked kinase (ILK), Parvin, and PINCH, which links integrins to the actin cytoskeleton and other intracellular pathways.6 In contrast to Drosophila and Caenorhabditis elegans, which have only 1 PINCH protein, 2 PINCH proteins, PINCH1 and PINCH2, have been described in mammals and share high sequence and structural similarity.11 PINCH1 is expressed in the blastocyst, whereas PINCH2 expression starts on embryonic day 14.5,11,12 From embryonic day 14.5 to adulthood, both are expressed ubiquitously in all tissues, although they may diverge within different cell types of some organs.11 Furthermore, PINCH1 and PINCH2 may play redundant roles in most organs and compensate for each other under certain circumstances.12–15

Received March 12, 2009; accepted June 1, 2009.
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The online-only Data Supplement is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.109.864686/DC1.

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Circulation is available at http://circ.ahajournals.org DOI: 10.1161/CIRCULATIONAHA.109.864686
PINCH proteins contain 5 LIM domains that are involved in mediating protein-protein interactions. The LIM1 domain of PINCH binds the N-terminal ankyrin-repeat domain of ILK, and the interaction of PINCH and ILK is obligatory for targeting the ILK, Parvin, and PINCH complex to integrin membrane sites and for the stability of each component of the complex.6 Deletion of one of the components leads to the degradation of the others and impaired cell mobility and survival.12–14,16

Myocyte cell-cell interactions are mediated by intercalated disks,17 which are composed of 3 distinct junctional components: adherens junctions, desmosomes, and gap junctions. Adherens junctions are mediated by N-cadherin and its associated catenin family proteins (α, β, γ-catenin) and serve as anchors for myofibrils at cell-cell contacts.2,18 Gap junctions are formed by a family of connexins. Connxin 43 is critical for electric coupling of myocardium.10,19 In addition, crosstalk between cell–extracellular matrix connections and cell-cell junctions has been reported, and integrin-mediated signaling pathways have been found to regulate the formation of cell-cell junctions and vice versa.20

Postnatal myocardial maturation and remodeling is a complex developmental process during which cardiomyocytes undergo physiological hypertrophic growth, realign cytoskeletal components, and acquire a mature, adult cytoarchitecture to meet a dramatically increased hemodynamic load. One feature of postnatal myocardial remodeling and maturation is the redistribution and segregation of distinct cell-matrix and cell-cell adhesions. During embryonic development, cardiomyocytes interact with each other extensively via cadherin-mediated adherens junctions, which appear to play dominant roles in mediating myocyte adhesion and function.21 During perinatal development, the myocytes continue to elongate and interact with each other only at the bipolar ends. Intercalated disks are disassembled from lateral cell membranes and reassembled at the bipolar ends of the cells, whereas costameres remain in the lateral cell membranes. This process occurs postnatally.22,23 However, molecular mechanisms regulating the segregation and redistribution of cell adhesions during postnatal myocardium remodeling and maturation remain largely unknown.

Integrin signaling pathways also have been shown to be important for the maintenance of cardiac structure and function.1,4,5 Genetic studies in Drosophila and C elegans, as well as in the mouse epiblast, have confirmed that the PINCH-ILK complex plays an essential role in the integrin-actin network at cell-matrix adhesions.1,4,5,24–28 Deletion of integrin-β1 in mouse cardiomyocytes leads to dilated cardiomyopathy and cardiac dysfunction.1,5 Similarly, ablation of ILK in myocardium results in disruption of the focal adhesion complex and disaggregation of cardiomyocytes, leading to cardiomyopathy and heart failure.4 In addition, it has been shown that PINCH1 forms a functional complex with thymosin-β4 and ILK that plays a role in promoting cardiomyocyte migration and survival after cardiac injury.3 Whether PINCH is required in normal myocardium or for postinjury healing of myocardium has not been addressed.

To explore the functional role of PINCH in cardiomyocytes, we generated several mouse lines in which PINCH1 and PINCH2 are singly or doubly ablated in myocytes. Analysis of these mouse models has revealed that PINCH1 and PINCH2 play essential roles in myocardial remodeling and maturation and in maintaining myocardial integrity.

Methods

Histological Analysis

Perfused hearts fixed with 4% paraformaldehyde were processed for paraffin-embedded sections and subsequently analyzed by hematoxylin and eosin staining12,29 and by Masson trichrome stain according to the manufacturer’s protocol (Sigma-Aldrich, St Louis, Mo).

Mouse Neonatal Cardiomyocyte Isolation

Neonatal cardiomyocytes were prepared by the use of a Percoll gradient as described.50

In Situ Hybridization and Immunostaining

Whole-mount in situ hybridization and immunostaining was performed as previously described.29 The following primary antibodies were used: α-actinin (Sigma-Aldrich), Alexa Fluor 568 phalloidin (F-actin) (Molecular Probes, Carlsbad, Calif), α-E-catenin (Sigma-Aldrich), Akt (Cell Signaling, Danvers, Mass), β-catenin (BIOMOL, Plymouth Meeting, Pa), cadherin (Sigma-Aldrich), connexin 43 (Zymed Laboratory, San Francisco, Calif), GAPDH (Santa Cruz Biotechnology Inc, Santa Cruz, Calif), integrin-β1(Sigma-Aldrich), ILK (Sigma-Aldrich), Parvin, paxillin (Abcam, Cambridge, Mass), phospho-Akt (serine 473; Cell Signaling), PINCH1,12 Vinculin (Abcam), Alexa Fluor 568 wheat germ agglutinin (WGA; Invitrogen), and ZO-1 (Zymed Laboratory). The sections were incubated with fluorescently labeled secondary antibodies except phalloidin and WGA staining and analyzed under the confocal microscope.

Northern Blotting and Western Blotting

Total RNA was isolated with TRIZOL reagent (GIBCO, BRL). Gel separation, blotting, and hybridization were performed. The protein assay was performed as described.31 Expression levels of proteins and RNA were quantified by Photoshop.

Myocardial Infarction

Mice were anesthetized with a mixture of ketamine and xylazine. Mice were placed in the supine position, and a midline cervical incision was made to expose the trachea and carotid arteries by microsurgical techniques. After endotracheal intubation, the cannula was connected to a volume-cycled rodent ventilator. The chest cavity was entered through the second intercostal space at the left upper sternal border through a small incision. The left anterior descending branch was ligated permanently in the mouse heart. Cardiac function and infarct size were measured by echocardiography.

Electron Microscopy

Cardiac ventricles were processed for electron microscopy analysis as described.50

Statistical Analysis

Data are presented as mean±SEM. Student t test was used for 2-group comparisons. Differences were considered statistically significant at P<0.05.

Results

Expression of PINCH1 and PINCH1 in Mouse Myocardium

To determine the role of the 2 PINCHs in the heart, we first analyzed in detail their developmental expression pattern in heart. In situ hybridization revealed that PINCH1 is expressed in heart from embryonic day 9.5 on, whereas PINCH2 is not detected until embryonic day 14.5 (Figure 1A and 1C,
Northern blotting revealed that, among tissues analyzed, heart showed the highest level of expression of both PINCH1 and PINCH2 (Figure 1B and 1D). To further determine whether PINCHs are expressed in cardiomyocytes, we performed immunostaining on purified cardiomyocytes from neonatal (Figure 1E and 1F) and adult (Figure 1G and 1H) hearts and found that both PINCHs are abundantly expressed in myocytes.

**Ablation of PINCH1 and PINCH2 in Myocardium Leads to Cardiomyopathy, Heart Failure, and Early Postnatal Lethality**

To test whether PINCH1 and PINCH2 may play a redundant role in myocardium, we generated a mouse line in which both PINCH1 and PINCH2 were ablated in cardiomyocytes. To further determine whether PINCHs are expressed in cardiomyocytes, we performed immunostaining on purified cardiomyocytes from neonatal (Figure 1E and 1F) and adult (Figure 1G and 1H) hearts and found that both PINCHs are abundantly expressed in myocytes.

**PINCH knockout alleles and PINCH2-null alleles (CDKO; cTNT-Cre;PINCH1<sup>+/−</sup>; PINCH2−/−)** (Figure 1B of the online-only Data Supplement). The effective excision of PINCH1 from myocardium was confirmed by Western blot on purified cardiomyocytes (Figure 1C of the online-only Data Supplement). The resulting CDKO mice were born at expected mendelian ratios and appeared morphologically normal at birth. However, with postnatal development, CDKO pups gradually developed severe growth retardation (Figure 2A), exhibited progressive signs of heart failure, and eventually died within 4 weeks (Figure 2B). Examination of multiple organs and tissues before death of mutants revealed extensive edema (pulmonary edema/increased lung weight, enlarged and congested liver) and ascites, suggesting that CDKO mutants died of heart failure.

Cardiac morphology was examined in detail. At postnatal day 1, control hearts exhibited an elongated shape along the apical-to-basal axis, and trabeculae were rearranged into an apical-to-basal orientation and compressed within the ventricular wall to result in a significantly increased ventricular compact myocardium (Figure 2C). In contrast, CDKO hearts were abnormally shaped, with dilated atria and largely disorganized trabeculae that failed to fuse and incorporate into compact myocardium, resulting in a significantly thinner compact myocardium (Figure 2C). At day 20, control myocardium exhibited a matured, striated appearance with well-oriented myocardial fibers. Hearts of CDKO mutants that survived to this stage were abnormally shaped, with dilated atria and left ventricle and a bulge near the base of the right ventricle (Figure 2D). Histological examination showed dilated left ventricle, irregular thickness of the ventricular wall

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**Figure 1.** The expression pattern of PINCH1 and PINCH2. A, In situ hybridization detects PINCH1 in the embryonic day (E) 9.5. B, Northern blotting (quantification shown on bottom) reveals highest expression of PINCH1 in the adult mouse heart. C, In situ hybridization detects PINCH2 in cardiomyocytes isolated from the newborn (E) and adult (G) mice, and control section without PINCH1 antibody added shows negative staining. D, Northern blotting (quantification shown on bottom) reveals highest expression of PINCH2 in the adult mouse heart. E and G, Immunostaining shows expression of PINCH1 in cardiomyocytes isolated from the newborn (E) and adult (G) mice, and control section without PINCH1 antibody added shows negative staining. F and H, Immunostaining shows expression of PINCH2 in cardiomyocytes isolated from the newborn (F) and adult (H) mice, and control section without PINCH2 antibody added shows negative staining. α-Actinin marks the myocytes. H indicates heart; B, brain; Sp, spleen; Lu, lung; Li, liver; Sk, skeletal muscle; K, kidney; T, thymus; RA, right atria; RV, right ventricle; LA, left atria; LV, left ventricle; and VS, ventricular septum.

**Figure 2.** General analyses of PINCH CDKO. A, Growth retardation of the double knockouts. B, The survival curve shows that the double knockouts died within 1 month. C, Morphological and histological analyses show a dilated heart of the newborn double knockouts vs control littermate. D, Morphological and histological analyses show cardiomyopathy with extensive fibrosis in the double knockouts at postnatal day (PN) 20 compared with the wild-type littermate (WT). Arrows indicate fibrosis; arrowheads, thrombosis.
with abnormally thickened right ventricular free wall, but significantly thinner left ventricular free wall and septum compared with control hearts (Figure 2D). Atria of mutant hearts were dilated with abnormalities in pectinate muscles in atrial appendages. A large intracardiac thrombus was frequently found in the left atria (Figure 2D). Trichome staining of sections from CDKO heart at postnatal day 20 revealed fibrosis in CDKO hearts, particularly in the ventricular septum and within thinned regions of ventricular myocardium (Figure 2D).

Ultrastructural Abnormalities in PINCH CDKO Hearts
To investigate cell–extracellular matrix and cell-cell adhesions at the ultrastructural level, we examined ventricular myocardium of the CDKO and wild-type mouse hearts by electron microscopy. At postnatal day 1, intercalated disk structures remained largely similar between control and mutant samples. However, in a few focal regions, gaps of intercalated disks were widened, with disarrayed sarcomeric structure and distorted Z lines (Figure 3A and 3B), which became more apparent at postnatal day 10 (Figure 3C through 3F, arrows). At postnatal day 10, intercalated disks of control samples were clearly observed, connecting adjacent myocytes end to end (Figure 3C, arrows). However, in CDKOs, intercalated disk structures were variably affected (Figure 3D). In mutants, membranes of the intercalated disk were highly convoluted, and gaps of the intercalated disks were significantly widened and, in some cases, filled with extracellular material (Figure 3D, arrows). The numbers of gap junctions in CDKO mutant hearts were significantly reduced throughout the sections (Figure 3C and 3D, arrowheads). Laterally, the distance between sarcolemma and sarcomeres in the CDKO myocytes was significantly increased (Figure 3F, arrowheads), and the intercellular space was significantly enlarged and filled with vesicles of variable size (Figure 3E and 3F, double-head arrows).

Disrupted Integrin-ILK signaling in PINCH CDKO Hearts
Deletion of one of the component proteins of the ILK, Parvin, and PINCH complex leads to degradation of the others and impaired cell mobility and survival.13,16,32 Therefore, we examined the expression of a number of key proteins in the integrin pathway in PINCH CDKO mutant myocardium. Western blotting revealed a dramatic reduction in expression of ILK and Parvin in CDKO cardiomyocytes at both postnatal days 1 and 10 compared with controls (Figure 4A and 4B; quantification shown on bottom). Integrin-β1 was expressed initially at normal levels at postnatal day 1 but was significantly reduced at day 10 (Figure 4A and 4B). Immunostaining also confirmed that ILK in CDKO myocardium was significantly reduced compared with controls (Figure 4C and 4D). In addition, increased and disorganized fibronectin expression was observed in mutant myocardium compared with controls (Figure 4E and 4F). However, expression of another integrin-binding protein, Talin, was not changed (Figure 4B).

Phosphorylation of Akt/PKB, one of the key downstream events of integrin signaling, is required for cell growth and survival. Deletion of PINCH1 and PINCH2 resulted in a significant reduction in phosphorylation of PKB/Akt at Ser473 at both postnatal days 1 and 10 (Figure 4G; quantification shown on right), with a concomitant increase in cell death as revealed by increased caspase-3 activation (Figure 4H and 4I).
Disruption of Cell-Cell Adhesions in PINCH CDKO Hearts

Ablation of PINCH1 and PINCH2 in myocardium resulted in disruption of intercalated disk structures. Therefore, we examined the abundance and localization of proteins within these complexes, including cadherins, catenins, ZO-1, and vinculin in adherens junctions and connexin 43 in gap junctions. Western blotting of hearts at day 10 revealed that levels of connexin 43, β-catenin, and ZO-1 were reduced in mutant hearts (Figure 5A). However, levels of cadherin, α-catenin, and vinculin were not significantly reduced (Figure 5A).

To investigate the cellular distribution of intercalated disk proteins, immunostaining was performed on cardiac samples from CDKO and control mice at postnatal day 10, when most CDKO pups were still viable. This stage was chosen because fully mature and compact intercalated disks are clearly observed around day 10, although gap junctions are not yet fully matured. As shown in Figure 5Ba, gap junction protein connexin 43 in control myocardium displayed a punctate pattern in both termini and lateral sides of myocytes. In contrast, connexin 43 in PINCH CDKO myocardium was found in a dispersed pattern across the lateral cell membranes but not in intercalated disks (Figure 5B-a'). Similarly, adherens junctional proteins α-E-catenin, cadherin, and β-catenin were mislocalized to the lateral cell membranes in mutant myocytes rather than within intercalated disks as observed in control myocytes.
Role of PINCH1 and PINCH2 in Focal Adhesion Formation and Spreading and Growth of Cardiomyocytes

To further investigate whether PINCH1 and PINCH2 are essential for cardiomyocyte adhesion and cytoskeletal organization, we isolated neonatal cardiomyocytes and tested their ability to attach and spread on different substrates in culture, including fibronectin, laminin, and collagen. Results with all 3 substrates were comparable. A majority (90%) of control cardiomyocytes had attached and spread out 12 to 24 hours after plating. However, markedly fewer (5%) PINCH CDKO cardiomyocytes were attached 12 to 24 hours after plating. Neonatal cardiomyocytes were stained with antibodies to α-actinin that mark the cytoskeleton and to paxillin, which is associated with focal adhesion complexes and the cytoskeleton. As shown in Figure 6A through 6C, control myocytes displayed a striated sarcomeric structures and punctate paxillin staining, indicative of focal adhesion structures. However, mutant cardiomyocytes did not exhibit striated sarcomeric structures and displayed diffuse or disorganized staining for cytoskeletal structures that were localized mainly to the cell periphery. Paxillin displayed a pattern similar to α-actinin in mutant myocytes, suggesting that it remained associated with the cytoskeleton. A majority of mutant myocytes did not exhibit paxillin staining in focal adhesions (Figure 6D through 6F, arrows). The attached PINCH CDKO cardiomyocytes were strikingly smaller than control myocytes (Figure 6A through 6H). Over a 72-hour period in culture, control cardiomyocytes continued to spread out and grow, leading to a 2-fold increase in cell size, whereas the size of PINCH CDKO myocytes remained the same. Consequently, the difference in cell size, which was initially ∼4.5-fold, had increased to 9-fold after 72 hours in culture (Figure 6H). WGA staining of cell membranes on cardiac sections from control and PINCH CDKO also demonstrated significantly reduced cell size and increased size variability of mutant cardiomyocytes compared with controls, which were visible at postnatal day 1 (not shown) but more dramatic at day 10 (Figure 6I through 6K).

Loss of PINCH1 or PINCH2 Adversely Affects Infarct Size and Cardiac Function After Infarct

Previous studies have demonstrated that the protective function of thymosin-β4 in myocardium is dependent on activation of ILK and subsequent phosphorylation of Akt. However, the potential involvement of PINCH in this process has not been addressed. To investigate the role of PINCH during infarct, PINCH1 cardiogenic-specific knockout mutants and control littermates and PINCH2-null mutants and control littermates were subjected to myocardial infarction by ligation of the left anterior descending coronary artery at 20 weeks of age. Mice within each group that survived for 12 days were assessed for myocardial function and postmortem histological analysis. No significant functional differences were observed between PINCH single mutants versus controls at 24 hours after infarct. However, by 12 days after infarct, echocardiographic analysis demonstrated a significant reduction in fractional shortening in PINCH1 or PINCH2 single mutants compared with controls (n=6; P<0.001; Figure 7). Consistent with
deterioration in left ventricular function in single PINCH mutants, trichrome staining revealed a significantly larger infarct area and scar tissue with visible hemorrhage in PINCH1 or PINCH2 single mutants compared with controls (Figure 7).

Discussion
Consistent with previous reports,12 we found that PINCH1 is expressed early during development, whereas PINCH2 expression in myocardium was observed from embryonic day 14.5 on. In mice, neither cardiac-specific ablation of PINCH1 nor germline deletion of PINCH2 resulted in any basal cardiac phenotype12,14 (present study), suggesting that PINCH1 and PINCH2 may play a redundant role in mouse heart. In this study, we generated mice that were doubly homozygous null for PINCH1 and PINCH2 in myocardium. The resultant PINCH CDKO mice developed cardiomyopathy and died of cardiomyopathy and heart failure within 4 weeks, suggesting that PINCH1 and PINCH2 play largely redundant yet essential roles in myocardium.

Postnatal myocardial maturation and remodeling is a complex developmental process, during which cardiomyocytes continue to grow, realign cytoskeletal components, and acquire a mature, adult cytoarchitecture to meet the demands of the dramatically increased hemodynamic load. These processes lead to well-aligned and striated myocardium and significantly increased thickness of the myocardial wall. Results of this study suggest that both PINCHs are essential in postnatal myocardial remodeling and maturation and that these processes appear to be blunted in their absence. Hearts of CDKO mutants were shaped abnormally, with dilated atria and left ventricles. Ventricular wall thickness was highly irregular with abnormal trabeculation. The upper free wall of the left ventricle and the ventricular septum were significantly thinner and fibrotic. The upper free wall of the right ventricle was dramatically thickened, leading to narrowing of the right ventricular cavity. Approximately 10% of PINCH CDKO mutants died within 3 to 5 days after birth. However, those that survived this period succumbed to death within 4 weeks with classic signs of heart failure, including cardiac dilation, ascites, and pulmonary and liver congestion.

The observed changes in myocardial architecture may result from abnormal cardiomyocyte adhesion/attachment, cell growth, and cell death. In support of an essential role for PINCH in maintaining myocardial cell-matrix and cell-cell interactions in vivo, electron microscopy analysis revealed disruption of costameric structures and intercalated disks in PINCH CDKO myocardium. Consistent with this, PINCH CDKO myocytes in culture failed to attach and spread and exhibited disrupted focal adhesions. Mutant myocytes were markedly smaller and variable in size. Multiple signaling pathways have been implicated in cardiac growth and survival, many of which converge on activation of the PI3K-Akt signaling pathway. Indeed, we observed significantly decreased phosphorylation and activation of Akt and markedly increased cell death in hearts of PINCH CDKO mice. These results revealed a critical role of PINCH in cell attachment and survival and hypertrophic growth of cardiomyocytes, in part at least via ILK activation of the Akt signaling pathway.

Myocardial cell-matrix adhesion is mediated by the integrin-β1 receptor and its associated ILK, Parvin, and PINCH complex. We observed that cardiac deletion of PINCH1 and PINCH2 leads to markedly reduced levels of ILK and Parvin, as seen in PINCH1-null embryos and embryonic stem cells.14,15,34 Reduced ILK might account, at least in part, for reduced Akt phosphorylation and increased cell death.12,14 Expression of integrin-β1 was relatively spared, at least during early postnatal stages. Expression levels of Talin and vinculin, components of another complex linking integrin to actin filament,20,35–37 were unchanged in PINCH CDKO hearts.

A defining feature of postnatal myocardial remodeling and maturation is the redistribution and segregation of distinct cell-cell and cell-matrix complexes. Profound changes in cell-cell adhesion in PINCH CDKO myocardium suggest that PINCH may be required for the expression and distribution of cell adhesion proteins within the intercalated disks. Consis-
tent with this, expression of proteins that function in adherens junctions and gap junctions of the intercalated disks is markedly affected in PINCH CDKO myocardium. In CDKO myocardium, connexin 43/H9251-E-catenin, and ZO-1 were significantly reduced and retained a disperse expression pattern throughout the lateral membrane comparable to that seen at earlier stages of development, rather than being expressed at the intercalated disk as seen in control myocardium. A similar aberrant expression pattern was observed for β-catenin and vinculin, although overall amounts of these 2 proteins were unchanged. These data revealed a critical requirement for PINCH in modulating the stability and polarized distribution of intercalated disk proteins during postnatal myocardial maturation and remodeling.

Previous studies have suggested that PINCH1 and ILK may play an essential role in promoting cardiomyocyte migration and survival after cardiac injury by interacting with thymosin-β4. However, a direct involvement of PINCH in this process has not been determined. In this study, we observed that, although the presence of either PINCH1 or PINCH2 is sufficient to maintain basal myocardial structure and functional integrity, loss of either PINCH1 or PINCH2 leads to exacerbated ischemic damage and deteriorated cardiac function in this setting. The inability of PINCH1 or PINCH2 to compensate for each other in ischemic myocardium may suggest that PINCH1 and PINCH2 may play distinct roles in myocardium or reflect a dose-dependent requirement for PINCH in response to ischemia.

Figure 7. Either cardiac-specific PINCH1 mutants (MUT) or global PINCH2 knockouts show susceptibility to injury after myocardial infarction (MI). A, MI in cardiac-specific PINCH1 mutants (PINCH1 KO) (n=6) and controls (wild type [WT]; n=6) at 20 weeks. a and b, Macroscopic appearance of representative hearts of WT (a) and MUT (b) at 12 days after MI. c and d, Representative trichrome stain of transverse heart sections at comparable levels 12 days after MI in WT (c) and PINCH1 KO (d). e, Echocardiogram of left ventricles 12 days after MI in WT and MUT. Left ventricular fractional shortening (FS) of PINCH1 KO hearts is significantly (P<0.01) decreased vs WT. B, MI global PINCH2 knockouts (PINCH2 KO) (n=6) and WT (n=6) at 20 weeks. f and g, Macroscopic appearance of representative mouse hearts of WT (f) and PINCH2 KO (g). h and i, Representative trichrome stain of transverse heart sections in comparable levels at 12 days after MI in WT (h) and PINCH2 KO (i). j, Echocardiogram of left ventricles 12 days after MI in WT and PINCH1 KO. Left ventricular fractional shortening (LVFS) of PINCH2 KO hearts is significantly decreased vs WT (P<0.01). Arrows indicate the damaged area of the control heart; arrowheads, the damaged area of the PINCH1 or PINCH2 mutant heart.

Taken together, these results demonstrate that PINCH proteins play essential roles in myocardial growth, maturation, remodeling, and function. In addition, the present study further highlights the importance of studying the role of PINCH proteins in human cardiac injury and cardiomyopathy.

Acknowledgments
We thank Dr Banerje for critical reading of the manuscript. Knockout mice were generated at the transgenic core facility at University of California at San Diego.

Source of Funding
The work was supported by National Institutes of Health grants for Drs Chen, Wu, and Evans.

Disclosures
None.

References
Cardiomyocytes communicate with their surrounding microenvironment through costameres. In both humans and animal models, dysfunction of costamere adhesion leads to cardiomyopathy and heart failure. Adhesion between cardiomyocytes and the extracellular matrix at the costamere is mediated by integrin-β1 receptor and its associated complex. Engagement of integrin with extracellular matrix leads to recruitment and formation of a cytoplasmic focal adhesion complex that links integrins to the actin cytoskeleton and other intracellular pathways. This complex is composed of integrin-linked kinase, Parvin, and PINCH proteins. Two PINCH proteins, PINCH1 and PINCH2, have been described in mammals and share high homology. Both PINCH1 and PINCH2 are expressed in most tissues and organs, including myocardium. However, whether PINCH is required in normal myocardium or for postinjury healing of myocardium has not been addressed. To explore the functional role of PINCH in cardiomyocytes, we generated several mouse lines in which PINCH1 and PINCH2 are singly or doubly ablated in myocytes. Analysis of these mouse models has demonstrated essential roles for PINCHs in myocardial dysfunction, heart failure, and cardiac remodeling and cardiomyopathy.

CLINICAL PERSPECTIVE
Cardiomyocytes communicate with their surrounding microenvironment through costameres. In both humans and animal models, dysfunction of costamere adhesion leads to cardiomyopathy and heart failure. Adhesion between cardiomyocytes and the extracellular matrix at the costamere is mediated by integrin-β1 receptor and its associated complex. Engagement of integrin with extracellular matrix leads to recruitment and formation of a cytoplasmic focal adhesion complex that links integrins to the actin cytoskeleton and other intracellular pathways. This complex is composed of integrin-linked kinase, Parvin, and PINCH proteins. Two PINCH proteins, PINCH1 and PINCH2, have been described in mammals and share high homology. Both PINCH1 and PINCH2 are expressed in most tissues and organs, including myocardium. However, whether PINCH is required in normal myocardium or for postinjury healing of myocardium has not been addressed. To explore the functional role of PINCH in cardiomyocytes, we generated several mouse lines in which PINCH1 and PINCH2 are singly or doubly ablated in myocytes. Analysis of these mouse models has demonstrated essential roles for PINCHs in myocardial growth and maturation, as well as remodeling in response to injury, and highlights the importance of studying the role of PINCHs in human cardiac remodeling and cardiomyopathy.
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_Circulation._ 2009;120:568-576; originally published online August 3, 2009;
doi: 10.1161/CIRCULATIONAHA.109.864686

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/120/7/568

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2011/02/03/CIRCULATIONAHA.109.864686.DC1

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Expanded Method

Generation of PINCH mutant mouse lines:

Floxed PINCH1 mice were generated as described \(^1\). Cardiac specific PINCH1 knockouts were generated using CTNT-Cre mice. The targeting vector for generating a floxed PINCH2 mouse line was shown in Supplementary Fig. 1A. We generated global PINCH2 null mice using protamine-Cre mice as previously described \(^1\). Genotyping was performed as described \(^1\).

Expanded Results

Generation of conditional PINCH1 and PINCH2 knockout mouse lines

To better understand the role of PINCH2, we generated a floxed PINCH2 mouse line (Supplementary Fig. 1A) using the same approach as previously described \(^1\). We deleted PINCH2 in germ cells using Protamine-Cre mice \(^1\). PINCH2 null mice were viable and fertile. Detailed analyses of several organs including the heart did not reveal any morphological and functional abnormalities (Supplementary Fig. 2 A-G). To determine the role of PINCH1 in cardiomyocytes, we generated a cardiac specific PINCH1 mutant by crossing the cTNT-Cre mouse line \(^2\), with floxed PINCH1 mice \(^1\). The cardiomyocyte specific PINCH1 knockouts (cTNT-Cre;PINCH1\(^{ff}\)) were viable and fertile and showed no overt phenotype. PINCH1 mutants exhibited normal cardiac morphology and function compared to that of controls (Supplementary Fig. 3-G). These data were consistent with those of MLC2v-Cre;PINCH1 cardiac specific knockouts previously characterized \(^1\).
References


Expanded Figure Legends

Supplementary Fig. 1: **Generation of knockout mouse lines.** A: Targeting strategy for generation of the PINCH2 floxed allele. B: Strategy to generate cardiac specific PINCH1 and 2 double knockouts (CDKC) with the cTNT-Cre;PINCH1 cardiac specific knockout and global PINCH2 knockout. C: Western Blotting shows absence of PINCH1 in the cTNT-Cre;PINCH1 cardiac specific knockout myocytes (lane 2) and CDKC (lane 3) compared with that of wild type myocytes (lane 1).

Supplementary Fig. 2. **Analyses of global PINCH2 null hearts.** A and B: Normal spreading and focal adhesion attachment of cardiomyocyte of the new born mutant (B) compared with that of the control (A). C-F: Morphological and histological analyses reveal no pathological changes in the heart of the mutants (D, F) compared with that of wild type controls (C, E). G: Echocardiographic measurements shows normal physiological function of the heart from the mutants compared with the controls. All data are presented as means ±.BW, body weight; HR, heart rate; LVEDD, end-diastolic left ventricular dimension; LVESD, end-systolic left ventricular dimension; PWth, left ventricular posterior wall thickness; IVSth, interventricular wall thickness; LV%FS, left ventricular percent fractional shortening; Vcf, velocity of circumferential fiber shortening. bpm, beats per minute; circ, circumference.

Supplementary Fig. 3. **Analyses of the cardiac specific PINCH1 mutant.** A and B: Normal spreading and focal adhesion attachment of cardiomyocyte of the new born mutant (B) compared with that of the control (A). C-F: Morphological and histological analyses reveal no pathological changes in the heart of the mutants (D, F) compared
with that of wild type controls (C, E). G: Echocardiographic measurements shows normal physiological function of the heart from the mutants compared with the controls. All data are presented as means ± SEM.
Supplementary Fig. 1

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Supplementary Fig. 2

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### Supplementary Fig. 3

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