LRRC10 is required to maintain cardiac function in response to pressure overload

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We previously reported that the cardiomyocyte-specific protein, Leucine-rich repeat containing 10 (LRRC10), has critical functions in the mammalian heart. Here, we tested the role of LRRC10 in the response of the heart to biomechanical stress by performing transverse aortic constriction on \textit{Lrrc10} null (\textit{Lrrc10}^{+/−}) mice. Mild pressure overload induces severe cardiac dysfunction and ventricular dilation in \textit{Lrrc10}^{+/−} mice as compared to controls. In addition to dilation and cardiomyopathy, \textit{Lrrc10}^{+/−} mice showed a pronounced increase in heart weight with pressure overload stimulation and a more dramatic loss of cardiac ventricular performance, collectively suggesting that the absence of LRRC10 renders the heart more disease prone with greater hypertrophy and structural remodeling, although rates of cardiac fibrosis and myocyte drop-out were not different from control. \textit{Lrrc10}^{−/−} cardiomyocytes also exhibit reduced contractility in response to β-adrenergic stimulation, consistent with loss of cardiac ventricular performance after pressure overload. We have previously shown that LRRC10 interacts with actin in the heart. Here, we show that histidine 150 of LRRC10 is required for interaction with actin and this interaction is reduced after pressure overload, suggesting an integral role for LRRC10 in the response of the heart to mechanical stress. Importantly, these studies demonstrate that LRRC10 is required to maintain cardiac performance in response to pressure overload and suggest that dysregulated expression or mutation of \textit{LRRC10} may greatly sensitize human patients to more severe cardiac disease in conditions such as chronic hypertension or aortic stenosis.

Keywords: LRRC10, pressure overload, cardiomyopathy, eccentric hypertrophy
Absence of LRRC10 sensitizes mice to severe cardiac dysfunction and
decompensation after pressure overload without altering myocardial fibrosis or
cardiomyocyte death, suggesting a unique requirement for LRRC10 in maintaining
cardiac functional performance. These results suggest important roles for LRRC10 under
conditions of pressure overload, such as hypertension or aortic stenosis.
INTRODUCTION

Cardiomyocytes hypertrophy in response to extracellular stimuli, such as neurohumoral or growth factor stimulation of membrane-bound receptors (13, 23, 44), or in response to mechanical stretch or strain that is sensed by mechanosensory machinery embedded in the Z-disc, cytoskeleton, and sarcolemma (25, 38, 49). At the whole organ level, the heart undergoes hypertrophy as an adaptive mechanism to maintain cardiac output and reduce ventricular wall stress (20, 23, 38). While initially beneficial, prolonged cardiac hypertrophy becomes maladaptive and progresses to cardiac dilation, decompensation, heart failure, and/or sudden death (20, 23, 38). Cardiac hypertrophy can be induced by a number of cardiovascular diseases, such as myocardial infarction or chronic hypertension (23, 37). Indeed, high blood pressure is a common precursor to heart disease, particularly in the United States, where chronic hypertension is widespread (18, 21, 42). Despite increased awareness and treatment of hypertension, recent epidemiological studies suggest its prevalence continues to rise, affecting approximately 55-65 million adults in the United States alone (18, 21). Therefore, understanding the factors that are required to maintain cardiac function in the setting of pressure overload is critical for developing efficacious therapeutic strategies to treat heart disease.

Cardiac remodeling can be characterized as concentric or eccentric. Concentric hypertrophy occurs in pressure overload, such as from chronic hypertension or aortic valve stenosis, or in inherited hypertrophic cardiomyopathy induced by mutations in genes encoding sarcomeric proteins (22, 37, 38, 46, 49). Concentric hypertrophy results from the addition of sarcomeres in parallel within cardiomyocytes, resulting in greater myocyte cell surface area, disproportionate growth in cardiomyocyte width relative to
length, and thickening of the ventricular wall (26, 52). In contrast, eccentric cardiac hypertrophy is caused by serial addition of sarcomeres, resulting in a disproportionate increase in the length relative to the width of cardiomyocytes that causes ventricular dilation (26, 52). Eccentric cardiac hypertrophy occurs in conditions of volume overload, such as valvular regurgitation or after myocardial infarction, and in inherited cardiomyopathies associated with a wide range of genes, including many cytoskeletal and Z-disc genes (8, 11, 19, 26, 29, 37, 50, 56). Therefore, the heart hypertrophies in response to a variety of pathological stimuli, however, the nature and resulting geometry of cardiac remodeling are stimulus-dependent.

Leucine-rich repeat containing 10 (LRRC10) is a cardiac-specific member of the leucine-rich repeat containing protein (LRRC) superfamily, which have in common leucine-rich repeat (LRR) motifs that function as protein interaction domains (31, 33). LRRC10 has no other known functional domains other than its LRRs (28), suggesting that it mediates its biological functions by interacting with other proteins. Lrrc10 is expressed exclusively in cardiomyocytes and is dramatically upregulated after birth, with robust expression maintained in the adult heart (7, 28), implying important roles in the postnatal heart. LRRC10 interacts with actin and α-actinin in the heart (8), and localizes at the dyad region where the Z-disc comes into close juxtaposition to the T-tubule and sarcoplasmic reticulum (8, 28). This places LRRC10 at a mechanosensitive signaling hub in cardiomyocytes (19).

Lrrc10-null (Lrrc10−/−) mice exhibit perinatal defects in cardiac function and moderate dilated cardiomyopathy in adulthood (8). We have also recently reported that the cardiomyocyte-specific expression of Lrrc10 is regulated by Nkx2-5, GATA4, and
serum response factor (SRF) (7). Importantly, heterozygous missense mutations in $LRRC10$ have been recently identified in human dilated cardiomyopathy patients (43). This strongly supports our working model that $LRRC10$ is a dilated cardiomyopathy candidate gene in humans. However, the roles of LRRC10 in the context of pathological stimulation, such as pressure overload, remain unknown.

Here, we performed transverse aortic constriction (TAC) on $Lrrc10^{-/}$ mice to test the hypothesis that LRRC10 is important for the response of the heart to mechanical stress. $Lrrc10^{-/}$ mice exhibit severely deteriorated cardiac function after four weeks of mild pressure overload stimulation, while cardiac function in wildtype (WT) mice is preserved. Interestingly, $Lrrc10^{-/}$ hearts undergo a normal concentric growth response and show similar increases in apoptosis or fibrosis in response to TAC as compared to controls. These data indicate that LRRC10 is uniquely required to maintain cardiac contractile performance but is not necessary for concentric cardiac remodeling in the setting of pressure overload. Additionally, we demonstrate that histidine 150 of LRRC10 mediates its interaction with actin in the heart and the interaction of LRRC10 with actin is reduced after pressure overload, suggesting a potential mechanosensing function for LRRC10.
EXPERIMENTAL PROCEDURES

Animals

Lrrc10-null (Lrrc10-/-) mice have been previously described (8, 39). Experiments utilized Lrrc10-/- mice and littermate or age-matched wildtype (WT) controls maintained on the same C57BL/6 genetic background (8, 39). Mice were euthanized by cervical dislocation to harvest tissue for biochemical and histological analyses. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH) and the University of Wisconsin Research Animal Resource Center policies and approved by a University of Wisconsin-Madison Institutional Animal Care and Use Committee (Protocol #M01461).

Transverse Aortic Constriction (TAC) and Echocardiography

Transverse aortic constriction (TAC) was performed to induce pressure overload hypertrophy as described previously (6, 10). Briefly, three month old mice were anesthetized by face mask administration of 3% isoflurane and then intubated and placed on a ventilator (Harvard Apparatus) with supplemental oxygen and 1.5% isoflurane using a tidal volume of 0.2 mL and respiratory rate of 110 per min. The chest cavity was entered in the second intercostal space at the upper sternal border through a small incision and aortic constriction was performed by tying a 7-0 nylon suture ligature against a 27-gauge needle. The needle was then removed and the pneumothorax was evacuated and extubated. Subcutaneous buprenorphine (0.8 mg/kg) was administered for pain relief and mice were allowed to recover in a heated chamber with 100% oxygen. Pressure gradients across the constriction were estimated from Doppler echocardiography.
using the modified Bernoulli equation. Animals were sacrificed and tissues harvested for
analysis after 28 days of TAC.

Transthoracic echocardiography was performed on mice under 1% isofluorane
gas anesthesia using a Visual Sonics 770 ultrasonograph with a 30-MHz transducer
(RMV 707B) (Visual Sonics, Toronto) as described previously (8, 22). Two-
dimensionally guided M-mode images of the left ventricle (LV) were acquired at the tips
of the papillary muscles. Doppler studies at the level of the aortic constriction were also
acquired. LV mass-to-body weight ratio (LV/BW), LV dimension in diastole (LVIDd),
thickness of the posterior walls in diastole, and isovolumetric relaxation time were
recorded. Parameters were measured over at least three consecutive cycles (8, 10, 58).

Histology and Immunoistochemistry

Hematoxylin and Eosin (H&E) staining was done as described previously (8).
Masson’s trichrome (Sigma) and TUNEL (Millipore) staining of cardiac sections
prepared from paraffin embedded hearts were performed to detect collagen deposition
and apoptosis, respectively, according to the manufacturer’s instructions. Cardiomyocyte
cross-sectional area (CSA) was measured in wheat germ agglutinin (WGA) (conjugated
to Oregon Green 488, 10 μg/mL, Invitrogen) stained cardiac sections costained with
phalloidin (conjugated to Alexa fluor 546, 165 nM, Invitrogen). CSA was evaluated in at
least 300 cardiomyocytes per animal from identical areas of the left ventricle (8).
Fibrosis was quantified as previously described (12) using images taken from the left
ventricular free wall of trichrome stained cardiac sections and percent fibrosis was
averaged from seven 20X images per heart. Imaging of heart sections was done on a
Zeiss Axiovert 200 microscope with Zeiss AxioCam. Morphometric quantitation was performed with NIH Image J software.

For cryosectioning, hearts isolated 4 weeks after TAC or sham surgeries were transferred into 30% sucrose in PBS for 1 hour, then placed in a 1:1 mixture of 30% sucrose in PBS and optimal cutting temperature (OCT) compound for an additional hour. Hearts were then embedded in 100% OCT compound and frozen on dry ice before being sectioned at 10 μm on a Leica CM3050S cryostat. For immunohistochemistry, sections were fixed in acetone for 15 minutes at -20°C and blocked in PBS with 0.1% Tween-20 (PBST), 5% normal goat serum and 2 mg/mL bovine serum albumin for 1 hour. Sections were incubated with LRRC10 (Novus Biologicals, NBP1-81704) and α-sarcomeric actin (Sigma, A-2172), α-actinin (Sigma, A-7811), or ryanodine receptor (Thermo Pierce, MA3-925) primary antibodies at a 1:200 dilution for 1 hour followed by appropriate secondary Alexa-Fluor® antibodies (Molecular Probes) in PBST for 90 minutes. Slides were mounted using Prolong Gold Antifade Reagent with DAPI (Life Technologies). Images were acquired and processed using a fluorescent microscope (ECLIPSE 90i; Nikon), NIS-Elements software suite (Nikon), and Photoshop in Adobe Creative Suite.

Generation of LRRC10 Mutants and Glutathione S-Transferase (GST)-pulldown Assays

Repeat Conservation Mapping (24) was used to identify residues in LRRC10 that are likely to be important for protein:protein interactions. A model of the predicted structure of mouse LRRC10 was created with 3D Jigsaw (4) and visualized with First Glance in Jmol to map the location of putative functionally important amino acids within LRRC10. To produce the GST-LRRC10 fusion protein, mouse Lrrc10 was amplified by
PCR and inserted in frame into the pGEX-2T vector containing an N-terminal GST (GE Healthcare) (8). Mutant GST-LRRC10 fusion proteins were generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) to mutate the wildtype GST-LRRC10 sequence. All new constructs were subjected to diagnostic digestion and confirmed by DNA sequencing. The oligonucleotides used to generate point mutations in LRRC10 are as follows:

LRRC10 Y104A For 5′GAAACAGCTCTGCATCCTCGCCCTGG

LRRC10 W127A For 5′GAACCTACGGACCCTGGCGCTCGAAT

LRRC10 H150A For 5′TCTCCTTTAAAACCCTGGCTGCCGGTTC

GST, GST-LRRC10, or mutant GST-LRRC10 proteins were expressed in E.Coli BL21 (DE3, Merck) and purified with glutathione agarose (Sigma) as described (8, 41). Pulldown assays were performed with purified rabbit skeletal muscle α-actin (Cytoskeleton, AKL95) exactly as described previously (8). For pulldown of heart extracts, adult wild type mouse hearts were homogenized in extraction buffer (50 mM Tris•HCl, pH 7.4, 150 mM NaCl, 1% triton X-100, 0.4% sodium deoxycholate, and protease inhibitors) and lysates were diluted in lysate incubation buffer (20 mM Tris•HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 1% triton-X 100) and incubated with GST, GST-LRRC10, or GST-LRRC10H150A for two hours. Proteins bound to beads were then washed in lysate incubation buffer, resolved by SDS-PAGE, and immunoblotted.

Quantitative Real Time PCR (qRT-PCR), Western Blotting and Immunoprecipitation

qRT-PCR was performed using FastStart SYBR Green Master (Roche) on a BioRad iCycler as described (8). Data were generated using the standard curve method.
and normalized to 18S expression levels. Primers were evaluated by melt curve analysis
to ensure specific amplification of a single, desired amplicon. Primer sequences for 18S,
\textit{ANF}, and \textit{β-MHC} are published (8).

Immunoblotting was performed using standard methods with primary antibodies
for LRRC10 (28) and \textit{α}-sarcomeric actin (Sigma) as previously described (8, 9). For
coimmunoprecipitation, cardiac lysates were made in extraction buffer as described under
GST-pulldown assays from mice 4 weeks after sham or TAC surgery and diluted in
lysate incubation buffer containing rabbit IgG (Santa Cruz) or anti-LRRC10 (1) coupled
to Protein G Dynabeads (Invitrogen) and incubated overnight at 4°C. Protein G beads
were then washed three times with lysate incubation buffer and immunoprecipitated
proteins were eluted, resolved by SDS-PAGE, and immunoblotted as described above (9).

\textbf{Myocyte Fractional Shortening}

Myocyte contractility was measured simultaneously in freshly isolated ventricular
myocytes from WT and \textit{Lrrc10}\textsuperscript{-/-} mice. Isolated cardiomyocytes were incubated with
Tyrodes solution (142 mM NaCl, 5.4 mM KCl, 1MgCl\textsubscript{2}, 1.8 mM CaCl\textsubscript{2}, 5 mM Glu, 5
mM HEPES, 0.33 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.4 with NaOH) in a temperature control chamber
at 37°C. Myocyte length was measured using video-based microscope camera system
(IonOptix, Milton, MA, USA). After allowing cells to settle down for 5 minutes, stable
rod-shaped cardiomyocytes with clear striations were field stimulated for 5 minutes at 0.5
Hz using 2 ms pulses at twice of the threshold voltages applied to two remote platinum
electrodes placed in the perfusing solution by myopacer (IonOptix). Only myocytes with
a resting sarcomere length >1.7 μm that followed the field stimulation protocol 1:1 were
used. Once the steady stimulation was establish, myocytes were stimulated at frequency
of 0.5 Hz, 1 Hz, and 2 Hz separately, for at least 10 continuous contractions at each
stimulation frequency. Then 10 nM isoproterenol (ISO) was applied through the
perfusion solution and recording was repeated under similar pacing stimulation. Myocyte
length was calculated by performing Fast Fourier Transforms of the images and the
resulting plot of the calculated myocyte length was recorded. The indices used to describe
the myocyte contractility were fractional shortening (FS), time to peak (ms), and time to
50% relaxation (RT 50%). All indices were analyzed off-line using IonWizard (IonOptix,
Milton, MA, USA) and were obtained by averaging 10 steady-state transients for each
myocyte.

Statistical Analyses

All results are expressed as mean ± standard error of the mean except Figure 7
where results are expressed as the mean ± standard deviation. All statistical analysis was
performed by a student’s t-test unless otherwise stated. P-value <0.05 was considered
statistically significant. *p<0.05, **p<0.01, ***p<0.001.
RESULTS

Lrrc10−/− mice exhibit increased cardiac growth in response to pressure overload

To test the role of LRRC10 in the response of the heart to biomechanical stress, we performed transverse aortic constriction (TAC) on mice to induce pressure overload hypertrophy. Cardiac structure and function of Lrrc10−/− mice were evaluated after 28 days of pressure overload stimulation as compared to wildtype controls (WT). Lrrc10−/− mice underwent greater cardiac growth and ventricular dilation than WT mice after TAC as observed by histology (Fig 1A). Heart weight-to-body weight ratios (HW/BW) indicate a modest increase in cardiac growth in Lrrc10−/− mice at baseline (Fig 1B), as previously reported (8). However, in response to TAC, Lrrc10−/− mice undergo substantially more cardiac enlargement than WT mice (54% increase in HW/BW compared to 34% in WT) (Fig 1B). Control and Lrrc10−/− mice experienced an equivalent, relatively mild (see Discussion) pressure overload stimulus from the TAC procedure (Fig 1C), indicating that differences in cardiac growth or function after TAC are due to the absence of LRRC10.

Expression of the hypertrophy marker genes ANF (atrial natriuretic peptide, Nppa) and β-MHC (β-myosin heavy chain, Myh7) were analyzed by qPCR. As previously reported (8), adult Lrrc10−/− hearts exhibit a mild induction of ANF and robust increase in β-MHC expression without hypertrophic stimulation (Fig 1D, E). In response to pressure overload, Lrrc10−/− mice induce ANF expression to a similar extent as WT mice (Fig 1D). β-MHC expression is elevated in Lrrc10−/− hearts compared to WT at baseline and after TAC (Fig 1E).
Deletion of Lrrc10 greatly exacerbates cardiac dysfunction in response to pressure overload

Next, we tested the hypothesis that LRRC10 is important for cardiac function in response to pressure overload by performing echocardiography on control and Lrrc10−/− mice after TAC or sham surgery. Lrrc10−/− mice undergo significant increases in left ventricular inner diameter (LVID) during systole (Fig 2A) and diastole (Fig 2B) in response to TAC, while left ventricular dimensions are not altered in WT mice (Fig 2A, B). These data indicate that pressure overload greatly exacerbates ventricular dilation in Lrrc10−/− mice as compared to WT. At baseline, Lrrc10−/− mice have moderate dilated cardiomyopathy with increased LVIDs and LVIDd and decreased fractional shortening and ejection fraction compared to WT mice, as we reported previously (8). However, after TAC, cardiac contractility is severely compromised in Lrrc10−/− mice, with drastically reduced fractional shortening (Fig 2C, Table 1) and ejection fraction (Fig 2D, Table 1) compared to shams, while these parameters are not significantly reduced in WT mice in response to TAC (Fig 2C, D).

Comparison of echocardiographic functional parameters before and after 28 days of TAC reveals that Lrrc10−/− hearts undergo much greater reductions in cardiac functional performance due to pressure overload (Fig 2E, F). Isovolumetric relaxation time (IVRT) is significantly increased in Lrrc10−/− hearts after TAC (18.90 ± 1.01 msec in Lrrc10−/− hearts compared to 16.69 ± 0.72 msec in WT, P<0.05), indicating reduced diastolic function in Lrrc10−/− hearts and suggesting poor cardiac performance and functional decompensation after pressure overload. Lrrc10−/− mice have significantly elevated heart rates after TAC (Table 1), resulting in maintenance of cardiac output (data
not shown) despite a marked reduction in cardiac function (Fig 2C-F). Representative M-mode images illustrate modest dilated cardiomyopathy in sham-operated $Lrcc10^{-/-}$ mice versus WT, but greatly exacerbated cardiomyopathy and left ventricular dilation in $Lrcc10^{-/-}$ mice after TAC (Fig 2G). In contrast, cardiac structure and function are largely preserved in WT mice after TAC (Fig 2G, Table 1).

Closer examination of H&E-stained cardiac sections reveals that $Lrcc10^{-/-}$ hearts exhibit overtly normal cardiac morphology in response to pressure overload, without histological abnormalities such as myocyte disarray or alterations in myocyte size between WT and $Lrcc10^{-/-}$ hearts (Fig 3A, top). No differences in fibrosis were detected between WT and $Lrcc10^{-/-}$ hearts after TAC by Masson’s trichrome staining (Fig 3A, bottom, B). Measurement of myocyte cross-sectional area from wheat germ agglutinin (WGA)-stained cardiac sections demonstrates similar increases in WT and $Lrcc10^{-/-}$ mice in response to TAC (Fig 3C, D). This suggests that LRRC10 is dispensable for appropriate concentric left ventricular hypertrophy in response to pressure overload. To further evaluate the nature of cardiac remodeling after pressure overload, the relative wall thickness or H/R ratio (ratio of LV wall and septal thickness to LV chamber radius) was calculated from echocardiography data. Reduced H/R ratios in $Lrcc10^{-/-}$ versus WT mice after sham surgery indicate eccentric cardiac growth and dilation at baseline (Fig 3E), as previously reported (8). $Lrcc10^{-/-}$ mice exhibit a robust concentric hypertrophy response to pressure overload stimulation, as evidenced by a significant increase in H/R ratio after TAC (Fig 3E), consistent with myofiber area measurements (Fig 3D).

Despite severely impaired cardiac function (Fig 2), $Lrcc10^{-/-}$ hearts undergo a similar degree of cardiac fibrosis after pressure overload compared to WT, as detected by
Masson’s trichrome staining (Fig 3A, bottom). Moreover, no difference in the induction of the fibrotic marker gene, Ctgf, was detected between WT and Lrrc10\(^{-/-}\) hearts with or without TAC surgery by qPCR (data not shown), consistent with histological analyses (Fig 3A). There was no difference in cardiomyocyte apoptosis in Lrrc10\(^{-/-}\) hearts compared to WT controls 28 days after TAC or sham surgery (Fig 4A, B), indicating that apoptosis does not exacerbate pressure overload-induced cardiomyopathy in Lrrc10\(^{-/-}\) mice. Therefore, although cardiac functional performance is drastically reduced in Lrrc10\(^{-/-}\) mice after pressure overload, the extent of cardiac fibrosis and apoptosis are unchanged compared to WT mice.

His150 of LRRC10 mediates binding to actin

Some actin binding proteins are critical for the response of the heart to biomechanical stress (5, 17, 34, 55), suggesting that localization to the Z-disc or actin cytoskeleton within the cardiomyocyte provides an optimal environment for sensing or transducing signals in response to mechanical stretch or strain. We have shown that LRRC10 interacts with \(\alpha\)-actin in the heart and all actin isoforms in vitro (8), suggesting that LRRC10 serves as a pan-actin binding protein. LRRCs physically interact with other proteins via hydrophobic residues on the interior concave face of the solenoid-shaped structure imparted by the LRR domains (31-33). To probe further into mechanisms mediating interaction of LRRC10 with actin, we utilized Repeat Conservation Mapping, a computational method to predict functional sites in LRR domains (24), to predict protein:protein interaction sites in LRRC10. Residues Y104, W127, and H150 were identified as putative functional sites in LRRC10, which map to the interior concave face
of the predicted protein structure of LRRC10 (Fig 5A). To determine if these amino acids are critical for the interaction of LRRC10 with actin, Y104, W127, or H150 were mutated to alanine, and in vitro pulldown assays were performed with GST or a GST-LRRC10 fusion protein and purified α-actin (Fig 5B). As expected, a GST fusion protein containing wildtype (WT) LRRC10 interacts with actin, while GST alone does not. Mutation of Y104 or W127 does not affect the interaction of LRRC10 with actin. In contrast, mutation of H150 of LRRC10 abrogates its interaction with actin (Fig 5B), indicating that H150 of LRRC10 is essential for actin binding. To test if H150 is important for the interaction of LRRC10 with actin in the heart, mouse heart extracts were incubated and pulled down with GST, GST-LRRC10, or GST-LRRC10\textsuperscript{H150A}, and then immunoblotted for α-actin (Fig 5C). WT LRRC10 interacts with α-actin in the heart, as previously reported (8), but mutation of H150 abolishes the interaction of LRRC10 with actin (Fig 5C), indicating that H150 of LRRC10 is a functional actin binding site. Predictive modeling of the structures of WT and mutant H150A LRRC10 illustrates a similar protein conformation (Fig 5D), suggesting mutation of this histidine to alanine does not change the overall conformation of LRRC10 but specifically alters the actin binding motif within LRRC10.

We next determined if the interaction of LRRC10 with actin in the heart is altered in response to biomechanical stress. We performed immunoprecipitation with an LRRC10 antibody on cardiac lysates from sham or TAC-operated mice and immunoblotted for actin. Results demonstrate a reduced interaction of LRRC10 with actin in TAC hearts (Fig 6A), indicating that binding of LRRC10 to actin is reduced during pressure overload and that association of LRRC10 with the actin thin filaments is
dynamically regulated in response to mechanical stress. To determine if LRRC10 undergoes a substantial change in subcellular localization after pressure overload, immunohistochemistry was performed on cardiac sections from sham or TAC mice. Intracellular localization of LRRC10 appears normal in TAC hearts as compared to sham-operated hearts (Fig 6B). Despite reduced interaction of LRRC10 and α-actin, LRRC10 properly colocalizes in a striated pattern with α-actin (Fig 6B), as well as the Z-disc protein, α-actinin, and the ryanodine receptor, a sarcoplasmic reticulum protein, in both control and pressure overloaded hearts (data not shown). These data indicate that LRRC10 remains at or near the dyad region under pressure overload, maintaining its close proximity to the Z-disc, sarcoplasmic reticulum and T-tubule.

Myocyte contractility

To determine if the absence of LRRC10 alters cardiomyocyte contractile function, single cell contractility was evaluated in myocytes isolated from WT and Lrrc10−/− hearts. The impact of loss of LRRC10 on myocyte contractile function was determined by measuring myocyte shortening (fractional shortening, FS) under field stimulation. The baseline FS was not significantly different between myocytes from WT and Lrrc10−/− mice at all stimulation frequencies (0.5, 1 and 2 Hz) (Fig 7A, B). However, following perfusion with 10 nM isoproterenol (ISO), the FS was significantly increased at all stimulation frequencies in WT myocytes, while the FS in ventricular myocytes from Lrrc10−/− hearts did not increase in response to ISO (Fig 7A, B), demonstrating that Lrrc10−/− myocytes exhibit a defective contractile response to β-adrenergic stimulation. Furthermore, we analyzed the ratio of FS after and before ISO treatment (FS after ISO /FS before ISO), and found that this ratio was significantly reduced in Lrrc10−/− myocytes
compared to WT at 1 Hz and 2 Hz (Fig 7C), further highlighting the impaired contractile response of Lrrc10-/- myocytes in response to ISO. Time to peak contraction and the time of 50% relaxation were not altered in the Lrrc10-/- and WT myocytes at basal conditions and with ISO perfusion (Fig 7D, E).

DISCUSSION

Pressure overload occurs in response to cardiovascular diseases, including hypertension and left ventricular outflow tract obstruction, and results in concentric cardiac hypertrophy that when prolonged becomes maladaptive and can progress to congestive heart failure (14, 27, 36, 37, 42). Pressure overload activates mechanosensitive signals that increase myocardial mass via addition of sarcomeres in parallel, resulting in radial growth of cardiomyocytes and increased left ventricular wall thickness (26, 27, 37, 49). Here, we tested the hypothesis that LRRC10 is important for the response of the heart to mechanical stress and pressure overload hypertrophy.

LRRC10 is a cardiomyocyte-specific factor required for proper cardiac function (1, 7, 8, 28), and mutations in the LRRC10 gene have been recently linked to human dilated cardiomyopathy (43). We demonstrate that Lrre10-/- mice exhibit severe cardiac dysfunction in response to TAC and Lrrc10-/- myocytes have reduced single-cell contractility in response to adrenergic stimulation, indicating that LRRC10 is indispensable for the response of the mammalian heart to biomechanical stress. We have previously shown that LRRC10 interacts with actin and α-actinin (8), positioning it in an ideal location to sense or transduce signals in response to biomechanical stress. Embryonic hearts of Lrrc10-/- mice upregulate a number of cytoskeletal genes critical for
the response of the heart to mechanical stress (3, 8, 35, 47), suggesting that the absence of
LRRC10 may render the heart sensitive to stretch or strain. Here, we identify histidine
150 of LRRC10 as necessary for its interaction with actin and show that this interaction is
reduced after pressure overload. Mechanistically how the interaction of LRRC10 with
actin is reduced after TAC is unclear. However, the maintenance of a similar
immunolocalization pattern that colocalizes with α-actin, α-actinin and the sarcoplasmic
reticulum indicates that LRRC10 remains within the dyad region in close proximity to
actin. It is possible that post-translational modifications or biomechanical signals in
response to pressure overload result in displacement of LRRC10 from the actin thin
filaments and/or association with other interacting factors or protein complexes at the Z-
disc, costamere, sarcoplasmic reticulum, or T-tubule. Indeed, the close proximity of
structures within the dyad region (45) makes it so that localization of LRRC10 at the Z-
disc, T-tubule, or dyadic cleft is indistinguishable by immunohistochemistry. Histidine
150 of LRRC10 is located on its interior concave face (Fig 5A). LRRCs commonly bind
other interacting factors on this interior face of the α/β horseshoe fold imparted by the
LRRs (31, 32) so post-translational modifications and/or binding of other factors to
LRRC10 near histidine 150 may be critical for its regulation. Future studies will
investigate molecular defects in the LRRC10 His150Ala mutant as well as LRRC10
mutations recently identified in human dilated cardiomyopathy (43).

 Nonetheless, LRRC10 may serve as a cardiomyocyte-specific scaffolding protein
to tether important structural or signaling molecules to mediate appropriate responses to
mechanical stress. Z-disc proteins such as muscle LIM protein (2, 29), Cypher (56, 57),
and telethonin (29, 30) are thought to compose a mechanosensory signalosome that
allows the cardiomyocyte to internally sense and respond to mechanical stretch or strain. Moreover, the striated muscle-specific LRRC, LRRC39, acts as a mechanosensor at the cardiomyocyte M-line by regulating SRF-dependent transcription (51).

Importantly, \( Lrrc10^{+/} \) mice develop severe dilated cardiomyopathy in response to four weeks of mild pressure overload stimulation. In our hands, performance of TAC to surgically induce pressure overload resulted in a pressure gradient across the aortic constriction of less than 50 mmHg, which caused concentric hypertrophy with preserved cardiac function in control mice that did not progress to decompensation or present clinical signs of heart failure. This is a relatively mild stimulus compared to other TAC models that achieve a greater aortic pressure gradient and induce a robust hypertrophy response and decompensation of ventricular performance in control mice within four weeks of pressure overload stimulation (15, 40, 48). Therefore, our model of mild pressure overload serves as a good pathophysiological model of chronic hypertension, a condition that occurs in nearly one third of Americans (18, 21).

\( Lrrc10^{+/} \) mice exhibit severe cardiomyopathy and decompensated ventricular performance in response to mild pressure overload stimulation, yet are still capable of undergoing appropriate concentric cardiac hypertrophy. This suggests that LRRC10 is uniquely required to maintain functional performance but is not necessary for concentric cardiomyocyte growth in response to pressure overload. \( Lrrc10^{+/} \) hearts become very dilated after TAC, indicative of eccentric growth, but also undergo pressure overload-induced concentric left ventricular remodeling, with increased myocyte cross-sectional area and increased H/R ratios. Therefore, \( Lrrc10^{+/} \) hearts likely undergo a combination of eccentric and concentric remodeling in response to TAC. We have previously reported
that unstimulated adult \( Lrsc10^{-/-} \) cardiomyocytes are longer than WT (8), suggesting eccentric growth by addition of sarcomeres in series, consistent with ventricular dilation. Thus, pressure overload worsens cardiac function in the absence of LRRC10, which may further increase neuroendocrine drive, providing a positive feedback loop that further enhances cardiac growth and exacerbates cardiac dysfunction.

The fact that \( Lrsc10^{-/-} \) mice exhibit greatly diminished cardiac function but a normal concentric hypertrophy response to TAC suggests that cardiac contractility is specifically compromised in the absence of LRRC10. Here, we demonstrate that \( Lrsc10^{-/-} \) mice display reduced cardiac function after pressure overload while isolated \( Lrsc10^{-/-} \) myocytes exhibit normal contractility at baseline but a defective contractile response to \( \beta \)-adrenergic stimulation. Thus, LRRC10 is required for baseline cardiomyocyte contractile function in the loaded intact heart, however, in unloaded isolated myocytes LRRC10 is not necessary for normal myofilament contraction. This data is in agreement with a potential role for LRRC10 as a mechanosensor in that only in the intact heart where myocytes can sense preload and mechanical stretch does the absence of LRRC10 manifest in reduced baseline contractile performance. We have shown that LRRC10 is not required for normal kinetics of myofilament contraction or for myofilament calcium sensitivity in skinned trabeculae (8), indicating that LRRC10 does not directly regulate cardiomyocyte contraction at the level of the myofilament but rather may play fundamental roles in upstream signals required for proper excitation-contraction coupling. Unloaded isolated \( Lrsc10^{-/-} \) myocytes are unable to mount an appropriate contractile response to \( \beta \)-adrenergic stimulation, which is consistent with cardiomyopathy in \( Lrsc10^{-/-} \) mice but could alternatively be due to some direct role for LRRC10 in coupling \( \beta \)-
adrenergic simulation and excitation-contraction coupling. Indeed, in other tissues, LRRCs have been shown to regulate ion channel function (16, 53, 54). Therefore, we speculate that LRRC10 may play critical roles in cardiomyocyte electrophysiology and/or calcium handling, which is essential for regulating normal excitation-contraction coupling. These studies do not identify the precise mechanism whereby Lrrc10−/− mice exhibit reduced contractile function and increased susceptibility to cardiomyopathy but provide insight into the role of LRRC10 in human cardiac disease and pave the way for future studies investigating molecular mechanisms underlying cardiomyopathy when LRRC10 is absent or mutated.

Lrrc10−/− hearts do not exhibit increases in cardiac apoptosis or fibrosis compared to WT in response to TAC, indicating that cardiomyocyte apoptotic cell death and fibrosis do not contribute to the advanced cardiomyopathy observed in Lrrc10−/− mice after mild pressure overload, supporting a specific defect in cardiac functional performance. It is possible that systolic dysfunction in the embryonic Lrrc10−/− heart sensitizes the adult Lrrc10−/− heart to severe cardiac dysfunction after pressure overload. However, defects in cardiac functional performance in Lrrc10−/− mice after TAC are far greater than the additive effects of basal dilated cardiomyopathy observed in unstimulated adult Lrrc10−/− mice (8) combined with the cardiac phenotype of TAC-operated control mice, suggesting an inability of LRRC10-deficient hearts to cope with pressure overload. Therefore, Lrrc10−/− mice represent a unique model of hypersensitivity to biomechanical stress that exhibit hypertensive cardiomyopathy after pressure overload without increased fibrotic remodeling or cardiomyocyte cell death. Our data suggest that human mutations in LRRC10 may increase susceptibility to more severe heart disease or
heart failure in the setting of pressure overload, such as in patients with high blood pressure or aortic stenosis.
**FIGURE LEGENDS**

**Figure 1.** Response of *Lrcc10<sup>-/-</sup>* hearts to pressure overload stimulation. Control (WT) and *Lrcc10<sup>-/-</sup>* mice at three months of age were subjected to 28 days of transverse aortic constriction (TAC) or sham surgery to test the response to biomechanical stress. (A) H&E staining and whole images of WT and *Lrcc10<sup>-/-</sup>* hearts in response to TAC (scale bar = 1 mm). (B) Wet heart weight-to-body weight ratios in sham- and TAC-operated WT and *Lrcc10<sup>-/-</sup>* mice (n=8-12). (C) Left ventricular pressure gradient demonstrates similar pressure overload stimulus in WT and *Lrcc10<sup>-/-</sup>* mice. (D-E) Expression of the hypertrophy marker genes (D) ANF (*Nppa*) and (E) β-MHC (*Myh7*) was evaluated by qPCR (n=5-9). Results are normalized to *18S* expression.

**Figure 2.** *Lrcc10<sup>-/-</sup>* mice have greatly exacerbated cardiomyopathy in response to pressure overload stimulation. (A-F) Cardiac function in control (WT) and *Lrcc10<sup>-/-</sup>* mice subjected to transverse aortic constriction (TAC) was evaluated by echocardiography. Measurements of left ventricular inner diameter (LVID) during (A) systole and (B) diastole, and (C) percent (%) fractional shortening and (D) % ejection fraction 28 days after TAC or sham surgery. (E) % change in fractional shortening (F.S.) and (F) ejection fraction (E.F.) after 28 days of TAC compared to preoperative values. (n=7-11). (G) Representative M-mode images.
Figure 3. Analysis of cardiac growth characteristics and fibrosis in $Lrrc10^{+/−}$ mice in response to pressure overload. (A) Histological examination of WT and $Lrrc10^{+/−}$ heart sections by H&E, and trichrome staining. Scale bar = 50 μm. (B) Quantification of fibrosis from trichrome staining in A. Values indicate average of n=3-4 animals. (C, D) Myocyte cell surface area (CSA) evaluated in WGA-stained cardiac sections revealed similar increases in WT and $Lrrc10^{−/−}$ hearts in response to TAC, indicating that $Lrrc10^{−/−}$ mice undergo normal concentric hypertrophy in response to pressure overload. Values indicate average of n=3-4 animals. At least 300 myocytes evaluated per heart. Scale bar = 10 μm. (E) H/R ratios or relative wall thickness (the ratio of LV wall and septal thickness to LV chamber) was calculated from echocardiography data. Decreased H/R ratios in sham $Lrrc10^{−/−}$ hearts indicate dilation or eccentric cardiac growth and increased H/R ratios after TAC indicate a normal concentric growth in response to TAC. (n=7-11).

Figure 4. Apoptosis is unaltered in $Lrrc10^{−/−}$ hearts in response to pressure overload. (A) TUNEL staining of WT and $Lrrc10^{−/−}$ heart sections after TAC (TUNEL green, phalloidin red, nuclei blue). Scale bar = 50 μm. (B) Quantification of TUNEL positive cardiomyocytes in sham and TAC-operated WT and $Lrrc10^{−/−}$ hearts.
Figure 5. Histidine 150 of LRRC10 is critical for its interaction with actin. (A) Model of the predicted structure of mouse LRRC10 created in 3D Jigsaw and visualized with First Glance in Jmol. Depicted in yellow are the residues Tyr104, Trp127, and His150 that were predicted to be functional sites with Repeat Conservation Mapping. (B) GST-pulldown assays indicate that residue His150 of LRRC10 is critical for its physical interaction with actin. Purified α-actin was incubated with GST or GST fusion proteins containing LRRC10 or a mutant LRRC10 containing Tyr104, Trp127, or His150 mutated to Ala followed by immunoblotting for α-actin. Shown below is a coomassie stained gel demonstrating equal loading conditions. (C) His 150 of LRRC10 is required for interaction with actin in the heart. Mouse heart extract was incubated with GST, GST-LRRC10, or GST-LRRC10$^{H150A}$ and then immunoblotted for α-actin, indicating that GST-LRRC10 but not GST or the GST-LRRC10$^{H150A}$ mutant interacts with actin in cardiac lysates. (D) Models of the predicted structures of WT and H150A mouse LRRC10.
Figure 6. Reduced interaction of LRRC10 with actin after pressure overload. (A) Heart extracts from TAC or sham operated mice were immunoprecipitated with an LRRC10 antibody and immunoblotted for α-actin, revealing reduced association of LRRC10 and α-actin after pressure overload. IP, immunoprecipitation. * indicates IgG light chain. (B) Immunohistochemistry for LRRC10 (green) and α-actin was performed on cardiac sections of mice subjected to sham or TAC surgery, indicating normal colocalization of LRRC10 with α-actin near the Z-disc after pressure overload. DAPI was used as a nuclear stain in the overlays (blue). Scale bar = 10 μm.

Figure 7. Reduced contractility in Lrrc10-/− myocytes following β-adrenergic stimulation. (A) Representative myocyte shortening traces of WT and Lrrc10-/− myocytes paced at 0.5 Hz, 1 Hz and 2 Hz frequencies. (B) Quantification of myocyte fractional shortening in WT and Lrrc10-/− myocytes at baseline and in response to 10 nM isoproterenol (ISO) treatment. (C) Ratio of fractional shortening after ISO treatment relative to before ISO treatment. ISO/Baseline ratios were calculated from the FS values measured from individual cardiomyocytes after and before ISO perfusion, respectively. (D) Time to peak contraction and (E) time of 50% relaxation in Lrrc10-/− and WT myocytes at basal conditions and with ISO perfusion. n= 18 myocytes from 5 mice for WT and 15 myocytes from 4 mice for Lrrc10-/−. *P<0.05. Data are presented as mean ± standard deviation.
Table 1. Echocardiographic assessment of cardiac structure and function in \textit{Lrrc10}^{-/-} mice after pressure overload.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sham WT (n=7)</th>
<th>\textit{Lrrc10}^{-/-} WT (n=8)</th>
<th>TAC WT (n=11)</th>
<th>\textit{Lrrc10}^{-/-} TAC (n=11)</th>
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<tr>
<td>LVID; d (mm)</td>
<td>4.32±0.15</td>
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<td>4.34±0.11</td>
<td>5.09±0.16**##</td>
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<tr>
<td>LVPW; d (mm)</td>
<td>0.72±0.03</td>
<td>0.59±0.03**</td>
<td>0.77±0.04</td>
<td>0.72±0.03##</td>
</tr>
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<td>LVID; s (mm)</td>
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<td>4.46±0.25**##</td>
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<td>0.91±0.04**</td>
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<td>0.77±0.05</td>
<td>0.72±0.02##</td>
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<td>51.34±2.83</td>
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<tr>
<td>% Fractional Shortening</td>
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<td>26.38±1.69</td>
<td>13.09±2.35***##</td>
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<td>127.96±8.47</td>
<td>156.17±12.70**##</td>
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<td>493±19</td>
<td>542±16*##</td>
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</table>

Evaluation of cardiac structural and functional parameters by echocardiography in \textit{Lrrc10}^{-/-} and control (WT) mice at various ages. \textit{LV}, left ventricle; \textit{ID}, inner diameter; \textit{PW}, posterior wall; \textit{AW}, anterior wall; \textit{d}, diastole; \textit{s}, systole; \textit{E.F.}, ejection fraction; \textit{F.S.}, fractional shortening. Values are represented as the mean ± standard error of the mean.

*P<0.05, **P<0.01, ***P<0.001 compared to WT of the same treatment; ##P<0.05, ###P<0.01 compared to sham of the same genotype.
ACKNOWLEDGEMENTS

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DISCLOSURES

None.
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Figure 2. *Lrrc10*−/− mice have greatly exacerbated cardiomyopathy in response to pressure overload stimulation. Cardiac function in control (WT) and *Lrrc10*−/− mice subjected to transverse aortic constriction (TAC) was evaluated by echocardiography. (A-F) Measurements of left ventricular inner diameter (LVID) during (A) systole and (B) diastole, (C) % fractional shortening, and (D) % ejection fraction 28 days after TAC or sham surgery. (E) % change in fractional shortening (F.S.) and (F) ejection fraction (E.F.) after 28 days of TAC compared to preoperative values. (n=7-11). (G) Representative M-mode images.
A

WT  Lrrc10<sup>−/−</sup>

H&E

trichrome

B

% Fibrosis

WT  Lrrc10<sup>−/−</sup>

C

WT  Lrrc10<sup>−/−</sup>

Sham

TAC

D

CSA (μm<sup>2</sup>)

Sham  TAC

E

H/R ratio

Sham  TAC
**A**

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<tr>
<th>Input</th>
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<th>IP LRRC10</th>
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<tr>
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<td>Sham TAC</td>
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α-actin

LRRC10

**B**

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<tr>
<td></td>
<td>WT</td>
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