Distinct functions of junD in cardiac hypertrophy and heart failure

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Cardiac hypertrophic stimuli induce both adaptive and maladaptive growth response pathways in heart. Here we show that mice lacking junD develop less adaptive hypertrophy in heart after mechanical pressure overload, while cardiomyocyte-specific expression of junD in mice results in spontaneous ventricular dilation and decreased contractility. In contrast, fra-1 conditional knock-out mice have a normal hypertrophic response, whereas hearts from fra-1 transgenic mice decompensate prematurely. Moreover, fra-1 transgenic mice simultaneously lacking junD reveal a spontaneous dilated cardiomyopathy associated with increased cardiomyocyte apoptosis and a primary mitochondrial defect. These data suggest that junD promotes both adaptive-protective and maladaptive hypertrophy in heart, depending on its expression levels.

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JunD is essential for concentric cardiac hypertrophy upon mechanical pressure overload

We initially examined the expression of the different AP-1 members in the heart after mechanical pressure overload induced by TAC in wild-type mice using RNase protection assay (RPA). Consistent with previous studies [Rockman et al. 1991], we observed an early immediate up-regulation of c-jun, junB, and c-fos 1 h post-TAC followed by down-regulation 4 h after TAC. Likewise, fra-2 was strongly induced after banding and was decreased after 4 h. Basal levels of fra-1 and fosB were very low and only a minor induction of these two genes could be observed [Fig. 1A]. JunD was only slightly induced after...
myocyte cross-sectional areas (MCSA) in −/− and adaptive myocardial hypertrophy in these mice, absent (Supplementary Fig. S1A). Despite the absence of junD heart-to-body weight ratios in reduced compared with control mice (\(n=9\), five males and four females, 4 mo old for sham operation for both genotypes). (*\(p<0.01\)) (Fig. 1D,E). Consistently, myocyte cross-sectional areas were smaller compared with junD+/− mice after TAC (\(p<0.01\)) [Fig. 1C]. Up-regulation of natriuretic peptides characterizes many forms of cardiac hypertrophy (Chien et al. 1993). Decreased hypertrophic response in junD+/− mice was associated with decreased expression of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) after aortic banding of wild-type mice. RNase protection assay showed marked early immediate up-regulation of c-jun, junB, c-fos, and fra-2. junD was expressed at high steady-state levels in hearts without stimulation. (\(A\)) Expression of different AP-1 members and loading control [L32, indicated on the left] after aortic banding of wild-type mice. RNase protection assay showed marked early immediate up-regulation of c-jun, junB, c-fos, and fra-2. junD was expressed at high steady-state levels in native hearts and was slightly increased after banding. Although fra-1 as well as fosB expression was low, a slight induction after aortic banding is visible on a five times higher exposure. (\(B, C\)) Heart-to-body weight ratios \(\text{[AU]}\) Arbitrary units. Hypertrophic response in junD−/− mice after TAC, but was expressed at high steady-state levels in hearts without stimulation.

Next, we asked for the role of junD in cardiac hypertrophy in response to chronic pressure overload and β-adrenergic stimulation by isoproterenol. We subjected junD+/− and control mice to severe and mild TAC. Although a significant increase of heart-to-body weight ratios in junD−/− mice was observed after severe TAC compared with sham-operated knock-out mice \(p<0.05\), the same stimulus led to significantly more hypertrophy in junD+/− control mice \(p<0.01\) [Fig. 1B]. Consistently, myocyte cross-sectional areas were smaller compared with junD+/− mice after TAC \(p<0.01\) [Fig. 1C]. Up-regulation of natriuretic peptides characterizes many forms of cardiac hypertrophy (Chien et al. 1993). Decreased hypertrophic response in junD+/− mice was associated with decreased expression of atrial natriuretic factor [ANF] and brain natriuretic peptide [BNP] after TAC \(p<0.01\) [Fig. 1D,E].

After mild TAC, hypertrophy assessed by measuring heart-to-body weight ratios in junD+/− hearts was almost absent [Supplementary Fig. S1A]. Despite the absence of adaptive myocardial hypertrophy in these mice, junD+/− hearts displayed normal echocardiographical parameters for cardiac function and contractility, since fractional shortening (FS), velocity of circumferential shortening corrected for heart rate [VCFc], and peak aortic velocity corrected for heart rate [PAVc] were not reduced [Supplementary Fig. S1B–D] at least 6 wk after mild TAC. To test whether these findings were specific for pressure overload-induced hypertrophy, we exposed junD−/− mice to β-adrenergic stimulation using isoproterenol infusions at a dosage that does not increase blood pressure. Isoproterenol was administered over a 14-d period. However, the absence of junD did not attenuate cardiac hypertrophy in response to isoproterenol [Supplementary Fig. S1E].

**JunD promotes ventricular dilation when overexpressed specifically in cardiomyocytes**

We next asked whether junD-dependent regulation of hypertrophy is cardiomyocyte specific. For this purpose, we first transfected cultured primary rat cardiomyocytes with a junD expression vector. Indeed, overexpression of junD in these cells led to a marked hypertrophic response compared with nontransfected cardiomyocytes [Supplementary Fig. S2]. We then generated transgenic mice that express junD under the control of the cardiomyocyte-specific α-MHC promoter [α-MHC–junDtg mice]. Southern blot analysis confirmed stable integration of the transgene [Supplementary Fig. S3]. Heart-specific expression of junD was tested by Western blotting [Fig. 2A]. Echocardiography in 8-wk-old α-MHC–junDtg mice showed no significant alteration in anterior and posterior wall thickness [data not shown] as well as in left ventricular mass compared with wild-type controls [Fig. 2B]. However, we found decreased heart contractility since FS was reduced in α-MHC–junDtg mice \(p<0.02\) [Fig. 2C]. Decreased contractility was associated with ventricular dilation since hearts displayed increased left ventricular end systolic [LVESD] [Fig. 2D] and left ventricular end diastolic [LVEDD] diameters [Fig. 2E] \(p<0.01\).

**Fra-1 overexpression causes premature heart failure upon pressure overload and β-adrenergic stimulation**

In order to investigate the function of fra-1 in cardiac hypertrophy, we subjected fra-1 conditional knockout mice [fra-1−/− mice] and fra-1 transgenic mice [fra-1tg mice] to mild TAC. TAC resulted in a comparable hypertrophic response between fra-1−/− mice and wild-type mice, since heart-to-body weight ratios were similar [Supplementary Fig. S4A]. Contractility assessed by echocardiography was preserved 6 wk after surgery in both groups [Supplementary Fig. 5AB–D].

In contrast, although heart-to-body weight ratios were similarly increased [Fig. 3A], cardiac contractility was reduced in fra-1tg mice compared with wild-type mice since FS [Fig. 3B], VCFc [Supplementary Fig. S5A], and PAVc were decreased [Supplementary Fig. S5B] \(p<0.01\). M-mode echocardiography revealed that LVESD and LVEDD in fra-1tg hearts were significantly increased \(p<0.01\) [Fig. 3C,D]. Moreover, mortality was increased in fra-1−/− mice after aortic banding since three out of eight mice died during follow-up. Fra-1tg mice were next subjected to isoproterenol stimulation. The hypertrophic response after infusions of isoproterenol was almost absent in both fra-1 transgenic and wild-type hearts. However, increased mortality associated with
cardiac decompensation could be observed (data not shown).

Fra-1<sup>−/−</sup> junD<sup>−/−</sup> mice develop dilative cardiomyopathy

We next asked whether junD-dependent compensatory cardiac hypertrophy might protect fra-1<sup>−/−</sup> mice from heart failure. To answer this question we generated fra-1 transgenic mice, which simultaneously lack junD [fra-1<sup>−/−</sup> junD<sup>−/−</sup> mice]. Mice with all possible genotypes were recovered at Mendelian frequency. Approximately 60% of fra-1<sup>−/−</sup> junD<sup>−/−</sup> mice died within the first 2 wk after birth (Fig. 4A). At necropsy, fra-1<sup>−/−</sup> junD<sup>−/−</sup> pups displayed enlarged hearts (Fig. 3B). Histological examination showed marked dilation of all cardiac chambers of fra-1<sup>−/−</sup> junD<sup>−/−</sup> mice [Supplementary Fig. S6A]. No developmental cardiac abnormalities and no defects in other organs could be detected. Dilation of the heart in fra-1<sup>−/−</sup> junD<sup>−/−</sup> mice could not be found immediately before birth (data not shown). Thus, the observed phenotype reflects a rapid progressive process starting after birth together with the switch from the low-pressure fetal to a high-pressure post-natal circulation.

fra-1<sup>−/−</sup> junD<sup>−/−</sup> mice surviving the first month after birth developed peripheral edema and ascites, became dyspneic and lethargic, and died [Fig. 4C]. Histopathological analyses revealed signs of advanced chronic hepatic and pulmonary congestion (data not shown). The heart size of adult fra-1<sup>−/−</sup> junD<sup>−/−</sup> mice was markedly increased (Fig. 4D), which was confirmed by the increased heart-to-body weight ratios in fra-1<sup>−/−</sup> junD<sup>−/−</sup> mice compared with mice with all other genotypes [p < 0.01] [Supplementary Fig. S6B]. Histological analyses showed cardiomyocyte hypertrophy and disarray [Supplementary Fig. S6C,D]. In addition, we found extensive focal interstitial fibrosis in fra-1<sup>−/−</sup> junD<sup>−/−</sup> ventricles [Supplementary Fig. S6E]. Overall, the cardiac phenotype in adult fra-1<sup>−/−</sup> junD<sup>−/−</sup> mice mimics human heart failure resulting from dilated cardiomyopathy [Hunter and Chien 1999].

Subcellular and molecular analyses of heart phenotype in fra-1<sup>−/−</sup> junD<sup>−/−</sup> mice

We next investigated whether overexpression of fra-1 and the lack of junD in the heart affected transcriptional expression of other AP-1 members using RPA. In the fra-1<sup>−/−</sup> junD<sup>−/−</sup> hearts the fra-1 transgene was efficiently expressed and junD expression was completely absent. No change in mRNA levels of any other AP-1 members was detected in fra-1<sup>−/−</sup> junD<sup>−/−</sup> hearts [Supplementary Fig. S7A]. Reactivation of embryonic genes as well as up-regulation of natriuretic peptides and contractile proteins characterize many forms of cardiac hypertrophy [Chien et al. 1993]. Indeed, mRNA levels of β-myosin heavy chain were markedly elevated in fra-1<sup>−/−</sup> junD<sup>−/−</sup>-hearts [Fig. 4E]. ANF was consistently increased [Fig. 4E]. Likewise, α-skeletal actin was elevated. Expression of SERCA2a, a Ca<sup>2+</sup>-ATPase of the sarcoplasmic reticulum representing an important regulator of cardiac contraction and relaxation, is often reduced in failing hearts [Muller et al. 2003]. In fra-1<sup>−/−</sup> junD<sup>−/−</sup> hearts SERCA2a was slightly down-regulated [Fig. 4E].

fra-1<sup>−/−</sup> junD<sup>−/−</sup> mice upon TAC. (A) Heart-to-body weight [Heart/BW] ratios in fra-1<sup>−/−</sup> and wild-type [wt] control mice 6 wk after mild banding [black bars] and sham operation [open bars]. No differences in both genotypes could be observed. (B) Longitudinal M-mode studies in fra-1<sup>−/−</sup> and wild-type [wt] mice 6 wk after mild TAC [black bars] and sham operation [open bars]. (C) Representative M-mode image of wild-type [wt] and fra-1<sup>−/−</sup> mice showed increased left ventricular end systolic and diastolic diameters (LVESD and LVEDD) compared with wild-type [wt] controls, indicating eccentric hypertrophy. (D) Quantification of different measurements of LVESD showed an increase of this parameter in fra-1<sup>−/−</sup> mice after mild TAC, indicating ventricular dilation [p = 0.05, three males and two females, 3 mo old for TAC and n = 5, three males and two females, 3 mo old for sham operation for both genotypes]. (*) p < 0.01 compared with respective sham group (student unpaired t-test); (**) p < 0.01 compared with all other groups (ANOVA with post hoc student Neuman Keuls multiple comparison test).
The roles of junD and fra-1 in heart failure

Dilation of hearts was shown to be associated with cardiomyocyte apoptosis (Wencker et al. 2003). Since very few apoptotic cells in control and fra-1

hearts could be observed by TUNEL (data not shown), we performed a more sensitive ligation-mediated PCR (LM-PCR) DNA fragmentation assay. Indeed, DNA fragmentation was increased in fra-1

hearts (Fig. 4F). Although a chronic low rate cardiomyocyte death may lead to ventricular dilation and dilative cardiomyopathy, these results do not prove whether apoptosis is causally involved in the observed heart phenotype. Many key events in the apoptotic machinery are linked to mitochondria (Green and Reed 1998). Therefore, we next analyzed whether the cardiomyopathy in fra-1

mice could be associated with a primary mitochondrial defect, which leads to mitochondrial-associated cell death. Transmission electron microscopy revealed that mitochondria were randomly dispersed in clusters and appeared swollen in fra-1

cardiomyocytes [Fig. 4G]. To assess the mitochondrial content, the relative level of mitochondrial DNA was examined by Southern blot analysis of cytochrome b, a mitochondrial-encoded gene, as previously described [Naya et al. 2002]. The mitochondrial DNA copy number was equal in wild-type and fra-1

hearts, suggesting that the total cellular mitochondrial content in fra-1

mice is not increased [Supplementary Fig. S8A]. To address whether there is a primary mitochondrial defect in these mice, we investigated mitochondrial function in mouse embryonic fibroblasts (MEFs). Mitochondrial transmembrane potential (ΔΨm) was monitored by tetramethylrhodamine methyl ester (TMRM), a fluorescent probe that accumulates in polarized mitochondria and is released upon mechanical pressure overload, leading to premature heart failure. The data in fra-1

hearts (Fig. 4F) suggest that mitochondrial dysfunction might contribute to the observed cardiac phenotype in fra-1

mice.

Recent studies in mice and humans provide evidence that specific forms of cardiac hypertrophy triggered by specific pathways seem to be associated with cardiac dilation and heart failure and do not represent a normal adaptive growth response [Levy et al. 1990; Wettstein et al. 1997; Banki-Harrington and Rockman 2003]. Although dispensable in cardiac hypertrophy, overexpression of fra-1 resulted in an overshooting response upon mechanical pressure overload, leading to premature heart failure. The data in junD

mice after trans-aortic constriction suggest that junD is required for adaptive concentric hypertrophy in heart and therefore might be protective against transition from concentric/compensated to eccentric/decompensated hypertrophy. We also show that these protective effects prevented spontaneous heart failure in fra-1

mice since fra-1

mice developed a dilative cardiomyopathy. Moreover, we provide evidence that a primary mitochondrial dysfunction might contribute to the observed cardiac phenotype in fra-1

mice.

Many forms of cardiomyopathies show cytoskeletal and extracellular matrix defects in cardiomyocytes [Chien 2000]. Immunofluorescence (IF) analysis of neonatal hearts with antibodies against α2-laminin and sarcomeric α-actinin as well as desmin, vimentin, muscle LIM protein (MLP), β-dystroglycan, and α-sarcoglycan, revealed no differences between control and fra-1

hearts [Supplementary Fig. S7B; data not shown].

Figure 4. Early post-natal mortality and dilative cardiomyopathy in fra-1

mice. (A) Survival curve of offspring from intercrosses of males fra-1

and females fra-1

or fra-1

. Within 3 mo >90% of fra-1

mice died suddenly or developed a congestive heart failure. Numbers of mice of each genotype were as follows: fra-1

(n = 41, red); fra-1

(n = 62, black); all other genotypes, fra-1

(n = 181, black). (B) The sizes of/MyHC was found to be markedly up-regulated in

−

junD played severe edema as compared with

−

junD. Surviving fra-1

newborn hearts, confirming a myocardial hypertrophic change. (C) Immunofluorescence (IF) analysis of neonatal hearts was performed with antibodies against MyHC and corresponding actin. (D) Western blot analysis of cytochrome b, a mitochondrial-encoded gene, as previously described [Naya et al. 2002]. The mitochondrial DNA copy number was equal in wild-type and fra-1

hearts, suggesting that the total cellular mitochondrial content in fra-1

mice is not increased [Supplementary Fig. S8A]. To address whether there is a primary mitochondrial defect in these mice, we investigated mitochondrial function in mouse embryonic fibroblasts (MEFs). Mitochondrial transmembrane potential (ΔΨm) was monitored by tetramethylrhodamine methyl ester (TMRM), a fluorescent probe that accumulates in polarized mitochondria and is released upon mechanical pressure overload, leading to premature heart failure. The data in fra-1

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mice.
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On the other hand, we show that ectopic expression of \textit{junD} specifically in heart leads to a spontaneous heart dilation, and cardiomyocytes overexpressing \textit{junD} exhibit pronounced hypertrophy. These experiments indicate that \textit{junD} also seems to promote maladaptive hypertrophy when expressed at unphysiological levels.

We conclude that a well-balanced expression of \textit{junD} is crucial for heart integrity as well as function, which is supported by the fact that \textit{junD} was only slightly induced after TAC, but was expressed at high steady-state levels in hearts without stimulation. Thus, \textit{junD} and \textit{fra-1} might represent potential therapeutic targets in heart failure patients in future.

Materials and methods

\textit{Generation of fra-1\textsuperscript{−/−}, junD\textsuperscript{−/−}, and fra-1\textsuperscript{Δ28} mice}

\textit{Fra-1}\textsuperscript{−/−} (C57/BL6/129sv) and \textit{junD}\textsuperscript{−/−} deficient mice (C57/BL6/129sv) have been described previously [Joehum et al. 2000; Theop et al. 2000]. These mice have been intercrossed to obtain \textit{fra-1\textsuperscript{−/−} junD\textsuperscript{−/−}} mice. F2 generations have been used for all experiments. \textit{Fra-1} conditional knock-out mice (C57/BL6/129oala) have been generated by crossing \textit{fra-1} floxed mice with the MORE-Cre mice [Eerle et al. 2004].

\textit{Generation of α-MHC-junD\textsuperscript{−/−} mice}

The α-MHC-junD\textsuperscript{−/−} transgene construct consists of the entire \textit{junD} cDNA cloned into the Sall-digested pMHC, poly A vector (kind gift of Dr. J. Robbins, Division of Molecular Cardiovascular Biology, Department of Pediatrics, The Cincinnati Children’s Hospital Medical Center, Cincinnati, OH). The construct was injected into the male pronucleus of fertilized single-cell embryos to produce α-MHC-junD\textsuperscript{−/−} mice. Transgenic founders were identified by Southern blot analysis of tail DNA according to standard procedures. Only offspring from founder mouse number 9 were used for analyses.

\textit{Transverse aortic banding}

Transverse aortic banding or sham operation was performed in age- and weight-matched mice as previously described [Tarnavskiy et al. 2004].

\textit{Administration of isoproterenol}

Isoproterenol was administered chronically to mice using a surgically implanted mini-osmotic pump (ALZET) that released isoproterenol in 0.9% NaCl at a rate of 30 mg/kg/d. Control mice carried pumps containing 0.9% NaCl solution alone.

\textit{Transfection experiments}

Neonatal rat hearts were dissected, digested with collagenase [Worthington Biochemical] as well as pancreatic (GIBCO Laboratories), and plated as previously described [Auerbach et al. 1997]. Two hours prior to transfection, plating medium was exchanged to transfection medium (20% M199, 73% DBSS-K [6.8 g/L NaCl, 0.14 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.2 mM CaCl\textsubscript{2}, 0.2 mM MgSO\textsubscript{4}, 1 mM dextrose, 2.7 mM NaHCO\textsubscript{3}], 4% horse serum, and 2% glutamine 200 mM). Cells have been transfected with Flag-tagged \textit{junD} cDNA (generous gift from Dr. Latifa Bakiri) under the control of the cytomegalovirus promoter using Escort III (Sigma) according to the manufacturer’s protocol. After 4 h, medium has been replaced by maintenance medium [Auerbach et al. 1997]. After 72 h, cells have been fixed, stained with a mouse monoclonal anti-Flag antibody (Chemicon) and phalloidin (Molecular Probes), mounted, and analyzed by fluorescence microscopy as previously described. Cell size has been measured using ImageJ image processing software. Experiments have been performed twice.

\textit{Histology, IF, and electron microscopy (EM)}

For histology, hearts were fixed with 4% buffered formalin and embedded in paraffin. Interstitial fibrosis was analyzed by using the Elastin van Gieson staining. IF was performed on nonfixed cryosections [5 min] and stainings were performed according to manufacturer’s recommendations. IF antibodies including α-actinin (sarcomeric) and α2-laminin were purchased from Sigma. EM was performed with hearts fixed in 2.5% glutaraldehyde diluted in phosphate buffer (0.1 M at pH 7.4) according to standard procedures. Myocyte cross-sectional area was determined by tracing the outlines of cardiomyocytes in triochrome-stained sections from the left ventricular region using a video imaging program [Northern Eclipse 5.0] as previously described [Oudit et al. 2003].

\textit{RPA}

Newborn mice were sacrificed and total RNA was isolated with the Ready-To-Go You-Prime First-Strand beats (Amersham Biosciences). Semiquantitative PCR was performed with three different dilutions of cDNA and repeated three times. RNA expression levels for ANF and BNP after banding experiments in the left ventricle was quantified with Real-Time TaqMan RT–PCR using ABI Prism 7700 sequence detection system. Briefly, 1 μg of total RNA was reverse transcribed using random hexamers. Taqman reactions were carried out in 96 well plates using 0.5% cDNA, 12.5 μL of 2× Taqman universal PCR mastermix, 100 μM probe, and 200 μM of each primer and water to a final volume of 25 μL. 18S rRNA was used as an endogenous control and every sample was analyzed in duplicate.

\textit{Blunt-end LM–PCR}

Genomic DNA was isolated from wild-type and \textit{fra-1}\textsuperscript{−/−} \textit{junD}\textsuperscript{−/−} hearts and analyzed by LM–PCR as previously described [Blaschke et al. 1996].

\textit{Assessment of mitochondrial content by Southern blotting}

Southern blotting was performed according to standard procedures and as previously described [Naya et al. 2002].

\textit{Mitochondrial membrane potential assay}

MEFs with different genotypes were plated onto glass coverslips and cultured in DMEM containing 10% FCS 24 h before starting the experiment. We employed the fluorescence dye TMRE (Sigma) to monitor the ΔΨm, as described [Irwin et al. 2003]. Briefly, fibroblasts were loaded with TMRE for 20 min at 37°C. Cells were then placed on the stage of Olympus MT20 inverted fluorescence microscope and Oligomycin [Sigma, 5 μM final concentration] was added. Temperature was maintained at 37°C. Emission was monitored at 580 nm. Sequential images were collected in 60-s intervals for 40 min. The time course of ΔΨm (measured as arbitrary fluorescence units) was performed using Olympus CellR software.

\textit{Statistical analysis}

Data are presented as mean ± SEM. Statistical significance of differences was calculated using an ANOVA with post hoc student Neuman Keuls multiple comparison test and student unpaired t-test, respectively. Significance was accepted at the level of \textit{p} < 0.05.

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