Myocardial-specific ablation of Jarid1 and AT-rich interaction domain–containing 2 (Jarid2) leads to dilated cardiomyopathy in mice

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Cardiomyopathy is a common myocardial disease that can lead to sudden death. However, molecular mechanisms underlying cardiomyopathy remain unclear. Jumonji and AT-rich interaction domain–containing 2 (Jarid2) is necessary for embryonic heart development, but functions of Jarid2 after birth remain to be elucidated. Here, we report that myocardial-

Heart failure is a major cause of mortality affecting 40 million people globally (1). Prolonged exposure to pathological and physiological stresses in the adult myocardium triggers adaptive responses followed by maladaptive responses, including the disruption of transcriptional homeostasis and re-expression of fetal sarcomere and metabolism genes. Persistent volume overload induces axial cardiomyocyte lengthening and chamber dilation, known as eccentric remodeling or dilated cardiomyopathy (DCM), resulting in reduced systolic function (2). In contrast, pressure overload induces radial cardiomyocyte widening and ventricular wall thickening, resulting in concentric growth or hypertrophic cardiomyopathy (HCM) with diastolic dysfunction (3). DCM and HCM are the most common forms of cardiomyopathy that can lead to sudden death (4). Many mutations in sarcomere genes, such as Myosin heavy chain 7 (Myh7, BMHC), Tropomyosin 1 (Tpm1), and Cardiac troponin T (Tnnt2) cause both DCM and HCM. However, molecular and genetic causes of DCM are more heterogeneous than HCM (5–7), which include cytoskeletal, costamere, and nuclear membrane proteins (8, 9). Thus, the molecular mechanisms underlying DCM are not fully understood.

Cardiomyocytes undergo dramatic molecular and developmental changes during the first 2 weeks after birth (herein, referred to as “neonatal” stages), to generate mature functioning cardiomyocytes (10, 11). These changes include the regulation of gene expression and isoform switching from fetal to adult forms, which are involved in contractility, calcium handling, energy utilization, and cell proliferation. In the mouse ventricle, BMHC, the predominant isoform during fetal stages, is replaced by αMHC after birth (10). Expression of metabolic genes for glycolytic processes is decreased, whereas oxidative and fatty acid metabolism genes are induced in the postnatal

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2 The abbreviations used are: DCM, dilated cardiomyopathy; Jarid2, Jumonji A/T-rich interaction domain–containing 2; Jarid2αMHC, αMHC::Cre/+; Jarid2−/−genotype; PDDE, posterior probability of differential expression; DE, differentially expressed; nn, postnatal day n; HCM, hypertrophic cardiomyopathy; Nrg, neuregulin; KO, knockout; qRT-PCR, quantitative real-time PCR; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; H&E, hematoxylin and eosin; CSA, cross-sectional area; WGA, wheat germ agglutinin; LV, left ventricle/ventricular; LVIDd, LV inner diameter at end diastole; LVIDs, LV inner diameter at end systole; EF, ejection fraction; FS, fractional shortening; GO, Gene Ontology; BP, biological process; CC, cellular component; SERCA, sarco-/plasmic/endoplasmic reticulum Ca2+ ATPase; MF, molecular function; PPAR, peroxisome proliferator–activated receptor; TPM, transcript per million; FDR, false discovery rate.
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Heart. Mitochondrial density increases as the metabolic rate increases (12). Cardiomyocyte proliferation in mice ceases during the neonatal period, and physiological hypertrophic growth occurs (13). However, it is poorly understood how these transitions are integrated during neonatal stages and how alterations in these changes contribute to heart failure later in life. Neuregulin (Nrgs) are secreted from the endocardium/endothelial cells and bind to ErbB2 and ErbB4 receptors in cardiomyocytes (14). Nrg1–ErbB signaling has crucial roles in cardiac development and function (15) and activate Akt, Erk, or Jnk pathways required for cell growth and survival (16). Nrg1 improves heart function (16), but this is controversial (17, 18). Thus, it is unclear how Nrg1–ErbB signaling is regulated and what their roles are in the progression of heart failure.

Jarid2 is required for normal embryonic development. Knockout (KO) of Jarid2 in mice causes developmental defects in the heart, liver, or hematopoietic tissues and lethality (19). Endothelial/endocardial deletion of Jarid2 shows cardiac defects recapitulating those in Endothelial/endocardial deletion of Jarid2 in cardiac development and function (p10. At 7 months, exceeding the neonatal period, and physiological hypertrophic growth increases (13). Cardiac alpha-actin increased from p10 to 7 months in the normal heart such as regulating genes in collagen fibril organization and metabolic process pathways. Further, a subset of sarcomeric genes were regulated after birth, but Jarid2 deletion in the differentiated myocardium does not exhibit developmental abnormalities (21). Jarid2 functions as an epigenetic regulator by interacting with methyltransferase enzymes (22). Jarid2 is a component of Polycomb repressive complex 2, a histone H3 lysine 27 (H3K27) methyltransferase. We have demonstrated that Jarid2 and polycomb repressive complex 2 accumulate on the IslI promoter during heart development and repress IslI expression (21). Jarid2 recruits Setdb1 on the Notch1 promoter and represses Notch1 expression via H3K9 methylation in endothelial/endocardial cells of the developing heart (23). Jarid2 can repress transcriptional activity of cardiac transcription factors (24). Interestingly, Jarid2 expression is reduced in heart failure patients (25). In mice, reduced Jarid2 expression is associated with cardiac hypertrophy (26), implying potential roles of Jarid2 in the adult heart. However, roles of Jarid2 in the adult heart remain unclear.

Here, we analyzed mice with a cardiomyocyte-specific deletion of Jarid2 using aMHC::Cre mice. All Jarid2aMHC mice died by 9 months of age with DCM. Gene expression profiling was performed on p10 and 7-month hearts during neonatal stages and DCM, respectively. Our pathway analyses of the neonatal heart from Jarid2aMHC mice indicated that heart development and muscle contraction pathways were significantly dysregulated, despite normal heart morphology at this age. Specifically, immature sarcomere genes, such as Troponin I type 1 (Tnni1) and Smooth muscle alpha-actin 2 (Acta2), and heart failure–associated genes were up-regulated in Jarid2aMHC hearts at p10. At 7 months, Jarid2aMHC hearts were enriched with dysregulated genes in collagen fibril organization and metabolic process pathways. Further, a subset of sarcomeric genes were increased from p10 to 7 months in the normal heart such as Cardiac alpha-actin (Actc1), Myh6, Tnni3, Tnnt2, Tropomyosin (Tpm1), Myosin regulatory light chain (Myl2), and Phospholamban (Pln), but not in Jarid2aMHC hearts. Interestingly, Nrg1, ErbB4, and Ankyrin repeat domain 1 (Ankyd1) expression levels were already dysregulated in Jarid2aMHC hearts prior to DCM. Thus, our data suggest that Jarid2 is required for neonatal myocardial maturation, which impacts on adult cardiac function and homeostasis.

Results

Generation of mice with cardiac-specific deletion of Jarid2

Jarid2 is indispensable for normal embryonic development (20, 21, 27). To determine cardiac-specific roles of Jarid2 in the adult heart, we analyzed Jarid2aMHC mice, in which aMHC::Cre specifically inactivates the conditional Jarid2 floxed allele in differentiated cardiomyocytes (28, 29). PCR analyses of genomic DNAs showed that Jarid2 was deleted only in the heart but not in the tail of Jarid2aMHC mice because a Jarid2 floxed-out band (354 bp) was detected only in the heart of Jarid2aMHC but not in the control heart or in the tail (Fig. 1A). qRT-PCR data confirmed a reduction in Jarid2 transcripts in Jarid2aMHC versus control hearts at p10 (Fig. 1B). Given efficient deletions of Jarid2 floxed allele by various Cre (21, 29) and increased noncardiomyocyte populations after birth, the unrecombined Jarid2 f/f allele in the 3-month-old heart (Fig. 1A) or residual mRNA (Fig. 1B) can be attributed to noncardiomyocytes. Immunostaining and Western blotting data also showed a marked reduction of Jarid2 levels in Jarid2aMHC hearts (Fig. 1C and D). To examine Jarid2 levels in the normal heart at different stages, we performed X-gal staining on tissue sections from heterozygous Jarid2 gene trapped mice, in which LacZ expression recapitulates endogenous Jarid2 expression (27). X-gal staining was reduced by 1 month of age (Fig. 1E). Western blotting confirmed that Jarid2 expression was high until p2 but significantly reduced in the adult heart (Fig. 1F). These data indicate that Jarid2 continues to be expressed in the neonatal heart followed by a marked decrease at approximately 1 month of age, implying an important role of Jarid2 during neonatal stages.

Jarid2aMHC mice exhibited dilated cardiomyopathy and premature death

The Jarid2aMHC mice showed expected Mendelian ratios and no gross morphological defects in the embryonic (20) or young adult heart (Fig. S1A and Table S1). However, the mutant mice exhibited 100% mortality by 9 months (Fig. 2A), likely because of heart failure. Thus, Jarid2aMHC hearts at 7 months were subjected to histological examination. The mutant heart was enlarged compared with controls at 7 months (Fig. 2B). Hematoxylin and eosin (H&E) staining images showed similar ventricular wall or septal thickness in the mutant heart (Fig. 2C). Apoptosis levels were not significantly changed in mutant hearts versus controls (Fig. S1B). The cross-sectional area (CSA) of cardiomyocytes was measured by wheat germ agglutinin (WGA) staining to demarcate plasma membrane boundaries in the ventricular myocardium (Fig. 2D). Jarid2aMHC hearts did not show any significant changes in CSA (Fig. 2F) and a total number of cells in the same square area (Fig. S1C). Increased fibrosis was detected in mutant versus control hearts as indicated by PicroSirius red staining (Fig. 2E). Expression of heart failure markers, Nppa, Nppb, Myh7, and Acta1 were normal at 3 months in Jarid2aMHC hearts but significantly

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increased at 7 months compared with controls (Fig. 2G). These data suggest that Jarid2<sup>−/−</sup> MHC mice do not exhibit cardiomyopathy at 3 months but progress to cardiomyopathy, leading to premature death between 6 and 9 months of age.

Next, we investigated cardiac structural and functional parameters by echocardiography at 3 and 7 months of age (Table 1). Jarid2<sup>−/−</sup> MHC mice at 7 months showed increases in left ventricular inner diameter at end diastole (LVID<sub>d</sub>) and end systole (LVID<sub>s</sub>) and LV mass/body weight ratios as compared with controls (Fig. 3, A–C). The dilation was not accompanied by alternation in interventricular septal or posterior wall thickness, whereas LV volumes were significantly increased at end diastole and end systole, indicating the enlarged and dilated LV (Table 1). The left ventricular thickness/radius ratio (LV wall thickness/chamber radius) was significantly reduced in Jarid2<sup>−/−</sup> MHC versus control hearts, indicating ventricular dilation. Decreases in ejection fraction (EF; Fig. 3D) and the percentages of fractional shortening (FS; Fig. 3E) were detected at 7 months, indicating defective cardiac contractility and DCM.

To determine the time-dependent changes in cardiac parameters of Jarid2<sup>−/−</sup> MHC mice, echocardiography was performed at 3 months (Table 1). Interestingly, Jarid2<sup>−/−</sup> MHC mice showed hyperperforming hearts as evidenced by increases in stroke volume, EF, FS, and cardiac output (Fig. 3, D–F). However, LV chamber dimensions remained similar to controls. Histological analyses of Jarid2<sup>−/−</sup> MHC hearts at 3 months did not show morphological defects (Fig. S2). Thus far, our data indicate that Jarid2<sup>−/−</sup> MHC mice exhibit hyperperforming compensating hearts at 3 months followed by decomposition and pathological remodeling by 6–7 months of age. Aortic peak velocity in the mutant heart was not changed compared with the control.
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Figure 2. Jarid2<sup>−/−MHC</sup> mice died at 6–9 months of age. A, Kaplan–Meier survival curves of control (Ctrl) and Jarid2<sup>−/−MHC</sup> mice were assessed by log-rank test, p < 0.001 (n = 10). B, gross morphology of Jarid2<sup>−/−MHC</sup> hearts at 7 months. Scale bar, 1 mm. C–E, the frontal (top panels) or transverse midline (bottom panels) heart sections were stained with H&E (C), and frontal sections for WGA (D), or PicroSirius red (E) staining on control and Jarid2<sup>−/−MHC</sup> hearts at 7 months. Scale bar, 1 mm. F, cardiomyocyte surface CSA in the left ventricular wall of 7-month hearts was measured by WGA staining using ImageJ software (n = 3). G, expression levels of hypertrophy marker genes were measured by qRT-PCR on control and Jarid2<sup>−/−MHC</sup> hearts at 3 or 7 months and normalized to control levels (n = 3–5, m, months).

Table 1
Echocardiographic assessment of cardiac structure and function in Jarid2<sup>−/−MHC</sup> mice

| 3 months | 7 months |
|----------|----------|
|          | Control  | Jarid2<sup>−/−MHC</sup> |
|          | Control  | Jarid2<sup>−/−MHC</sup> |
| n        | 9        | 10        | 9        | 9        |
| LVIDd (mm) | 4.12 ± 0.1 | 4.34 ± 0.1 | 4.23 ± 0.1 | 5.22 ± 0.3<sup>a</sup> |
| LVIDs (mm) | 3.33 ± 0.1 | 3.20 ± 0.2 | 3.02 ± 0.09 | 4.36 ± 0.3<sup>a</sup> |
| LV PW, d (mm) | 0.60 ± 0.03 | 0.65 ± 0.02 | 0.67 ± 0.02 | 0.64 ± 0.02 |
| LV PW, s (mm) | 0.77 ± 0.03 | 0.90 ± 0.03<sup>a</sup> | 0.78 ± 0.02 | 0.77 ± 0.02 |
| LV AW, d (mm) | 0.60 ± 0.03 | 0.66 ± 0.02 | 0.67 ± 0.02 | 0.64 ± 0.02 |
| LV AW, s (mm) | 0.82 ± 0.04 | 0.89 ± 0.03 | 0.77 ± 0.02 | 0.76 ± 0.02 |
| H/R | 0.29 ± 0.01 | 0.30 ± 0.01 | 0.32 ± 0.01 | 0.25 ± 0.01<sup>b</sup> |
| LV volume, d (μL) | 79.04 ± 4.5 | 86.39 ± 6.9 | 80.83 ± 5.2 | 135.34 ± 19.1<sup>a</sup> |
| LV volume, s (μL) | 45.54 ± 3.6 | 42.49 ± 5.5 | 35.96 ± 2.6 | 93.06 ± 20.8<sup>b</sup> |
| Stroke volume (μL) | 33.50 ± 1.9 | 43.90 ± 2.3<sup>a</sup> | 44.87 ± 3.1 | 42.28 ± 3.7 |
| EF (%) | 42.71 ± 2.1 | 51.95 ± 2.7<sup>a</sup> | 55.37 ± 1.5 | 35.21 ± 4.3<sup>a</sup> |
| FS (%) | 20.87 ± 1.1 | 26.64 ± 1.7<sup>a</sup> | 28.65 ± 1.0 | 17.25 ± 2.3<sup>a</sup> |
| Heart rate (bpm) | 424.44 ± 6.3 | 419.20 ± 11.9 | 485.67 ± 13.1 | 448.11 ± 18.1 |
| Cardiac output (ml/m) | 14.17 ± 0.7 | 18.47 ± 1.2<sup>a</sup> | 21.75 ± 1.5 | 19.10 ± 2.0 |
| IVRT (ms) | 23.75 ± 1.5 | 18.46 ± 1.1<sup>a</sup> | 19.52 ± 1.7 | 22.22 ± 1.7 |
| MV E/A | 1.65 ± 0.1 | 1.32 ± 0.1<sup>b</sup> | 1.37 ± 0.1 | 1.25 ± 0.2 |
| LV mass (mg) | 90.27 ± 8.9 | 106.29 ± 9.7 | 101.87 ± 5.2 | 142.50 ± 14.1<sup>b</sup> |
| Body weight (g) | 27.67 ± 2.0 | 26.70 ± 1.5 | 33.78 ± 1.9 | 33.22 ± 1.7 |
| LV mass/BW (mg/g) | 3.24 ± 0.1 | 3.98 ± 0.3<sup>a</sup> | 3.06 ± 0.2 | 4.37 ± 0.5<sup>b</sup> |

<sup>a</sup>p < 0.05 compared with controls.
<sup>b</sup>p < 0.05 compared with controls.

Aortic peak velocity (mm/s) values at 3 months were 1332.11 ± 120.1 and 2068.50 ± 381.4 for controls and mutants, respectively (p = 0.01, statistically not significant). At 7 months, it was 1817.04 ± 120.7 for controls versus 1497.00 ± 190.2 for mutants (p = 0.17, statistically not significant). Altogether, we demonstrated for the first time that Jarid2 within the myocardium is required for maintaining normal cardiac function in the adult heart.
Gene expression profiling in the Jarid2^MHC heart at neonatal stages

The heart undergoes crucial maturation processes during the first 2 weeks after birth to achieve normal adult cardiac morphology and function (12). Jarid2 expression was significantly reduced by 1 month of age (Fig. 1, E and F), but Jarid2^MHC mice did not show DCM until much later. Thus, we hypothesized that early defects during neonatal stages in the mutant heart play critical roles in the initiation and progression to DCM later in life. We set out to determine molecular changes in Jarid2^MHC hearts at p10, which will provide crucial information on the function of Jarid2 in the postnatal hearts, as well as defective molecular pathways that underlie DCM pathogenesis. The Jarid2^MHC heart exhibited normal morphology (Fig. 4, A and B), and hypertrophic marker genes were not altered in Jarid2^MHC versus control hearts (Fig. 4D). CSA (Fig. 4C) and cell proliferation examined by immunostaining of Ki67 or phospho-histone H3 (Fig. S1D) was not altered in the Jarid2^MHC heart. Together, Jarid2^MHC hearts present grossly normal phenotypes at p10.

Thus, Jarid2^MHC hearts at p10 would provide an excellent opportunity to investigate the molecular function of Jarid2 in the postnatal heart before the onset of secondary compensatory processes or pathological remodeling of the heart. We analyzed gene expression profiling in Jarid2^MHC versus control hearts by performing RNA-seq at p10 (Fig. 5). We employed two different analysis methods, EBSeq (Fig. 5A) and DESeq2 (Fig. S3A), to determine differentially expressed (DE) genes between Jarid2^MHC and control hearts (30). As shown in Fig. 5B, 61 DE genes were identified by EBSeq analysis, whereas 20 DE genes were identified by DESeq2 analysis. First, we analyzed all 72 DE genes identified by either DESeq2 or EBSeq analysis (Table S2). The majority of DE genes (54 genes, 75%) were up-regulated in the absence of Jarid2 (Fig. 5C and Table S2), likely reflecting the transcriptional repression function of Jarid2. Gene ontology (GO) term analysis on biological process (BP) showed that organ morphogenesis, ion transmembrane transport, heart development, and muscle contraction were significantly dysregulated (Fig. 5, C and D). The genes enriched in organ morphogenesis were involved mainly in noncardiac organ development such as lung (Irx1 and Irx2), neuron (Ntm1, Cdh2 and Gli1), epidermal stem cell and intestinal stem cell (Lrig1), and...
These results suggest that other organ developmental genes are repressed by Jarid2 in the heart at neonatal stages. Among the 54 up-regulated genes, 5 genes (Ankrd1, Abcc9, Actc1, Pln, and Gyg) have been shown to be mutated in human cardiomyopathy (2). GO term analysis on cellular component (CC) showed up-regulation of myofibril, contractile fiber, sarcomere, and T-tubule gene pathways in Jarid2/H9251 MHC hearts at p10. These included increased contractility related genes (Actc1, Slc8a1, Abcc9, Atp2a1, Ankrd1, Myl12a, Myl1, and Tmod1) prior to hyperperforming hearts at 3 months in Jarid2/H9251 MHC.

To determine potential direct targets of Jarid2, we overlapped 72 DE genes with our Jarid2 ChIP-chip data (23), yielding 15 genes dysregulated in Jarid2/H9251 MHC hearts whose genomic loci were occupied by Jarid2 (Table S3). Among these, the promoter region of Gli1, Tll1, and Prph were co-occupied by Jarid2 and H3K27 trimethylation. Gli1, a zinc finger transcription factor, is a modulator and target of hedgehog signaling during embryo development (31). Gli1 has been studied in the generation of vascular smooth muscle cells and regulation of fibrosis (32). Prph is a type III intermediate filament protein present in neurons of the mammalian peripheral nervous system and neuroblastoma cells (33). Tll1 is a member of the tubulin tyrosine ligase superfamily and important for interaction and axoneme motility (34). Other genes occupied by Jarid2 included Tmem100. Because Tmem100 is expressed in endothelial cells and involved in endothelial differentiation (35), it may not be a direct target of Jarid2 in cardiomyocytes. Tmem100 KO mice are embryonic lethal with cardiovascular failure (36). The sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) is a major component of Ca\(^{2+}\) cycling, and reduction of SERCA expression and activity have been linked to diastolic dysfunction in failing hearts (37). Although SERCA2a is the major cardiac-specific isoform, SERCA1a overexpression in the heart reveals faster Ca\(^{2+}\) transport kinetics by reduction in endogenous SERCA2a levels (37). Thus, increased Atp2a1 (SERCA1a) levels in Jarid2/H9251 MHC may impact on SERCA2a activity and thus Ca\(^{2+}\) uptake and cardiac contractility.

There were seven DE genes identified by both EBSeq and DESeq2 analyses (Table 2). ADP-ribosylhydrolase–like 1

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**Figure 5. Analyses of gene expression profiling in Jarid2\(^{\text{MHC}}\) versus control hearts at p10.** A, volcano plots showed differentially expressed genes by EBSeq analyses. 61 genes were dysregulated at a statistically significant level (PPDE > 0.95), which was magnified in the right panel. Gray lines indicate ±1-fold change. Each gene is indicated by a dot. B, Venn diagram demonstrates numbers of DE genes by EBSeq and DESeq2. C, GO term analysis was performed with all 72 DE genes to determine significant BP and CC. The x axis indicates log-transformed FDR adjusted (adj) p value, and dotted lines indicate adjusted p value of <0.05. Colors reflect z scores. D, heat map indicates genes involved in organ morphogenesis, ion transmembrane transport, heart development, and muscle contraction. Increased genes are indicated in red, intermediate genes are in black, and decreased genes are in green.
Table 2
List of seven DE genes identified by both EBSeq and DESeq2 analyses at p10

| Gene          | Fold change | PPDE | Name                                           |
|---------------|-------------|------|------------------------------------------------|
| Ankrd1        | 1.31        | 1.00 | Ankyrin repeat domain 1 (cardiac muscle)       |
| Ndrg4         | 0.65        | 1.00 | N-myc downstream regulated gene 4              |
| Sulf2         | 0.61        | 1.00 | Sulfatase 2                                    |
| Slc8a1        | 0.58        | 1.00 | Solute carrier family 8 (sodium/calcium         |
|               |             |      | exchanger), member 1                          |
| Abcc9         | 0.55        | 1.00 | ATP-binding cassette, subfamily C              |
|               |             |      | (CFTR/MRP), member 9                          |
| Adprhl1       | 0.53        | 1.00 | ADP-ribosylhydrolase like 1                   |
| Gyg           | 0.53        | 1.00 | Glycogenin                                    |

(Adprhl1) is a member of the ADP-ribosylhydrolase family (38). Although Adprhl1 is enzymatically inactive, it is important for heart chamber outgrowth and myofibril assembly in Xenopus embryos (39). The function of Adprhl1 in the mouse heart has not been studied, but elevated Adprhl1 expression levels may lead to abnormal orientation of myofibrils or contractile defects in Jarid2αMHC hearts. Glycogen is a primary form of energy storage in eukaryotes. Glycogenin (Gyg) is a glycosyltransferase that catalyzes the addition of glucose as a primer for glycogen synthesis (40). GYGI deficiency has been associated with cardiomyopathies because of an abnormal storage material, polyglucosan in the heart (41). The elevated Gyg and Glycogen branching enzyme 1 (Gbe1) in Jarid2αMHC hearts suggest that glucose metabolism may be altered. Sodium-calcium exchanger 1 (Slc8a1, NCX1) is an antiporter membrane protein that maintains cytosolic Ca<sup>2+</sup> homeostasis (42). Dysregulation of NCX1 in humans is observed in end-stage heart failure, and expression and function of NCX1 are increased during heart failure (42). Therefore, increased Slc8a1 expression in Jarid2αMHC hearts may cause a disruption in Ca<sup>2+</sup> homeostasis. Abcc9 is an ABC family member and encodes a membrane-associated receptor, SUR2, in the mitochondria and cell membrane. ABCC9 mutation causes DCM in humans, and KO mice die in the neonatal period with progressive cardiac dysfunction and a failure to transition from fetal to mature myocardial metabolism (43). Abcc9 expression is increased by low oxygen stress mediated by ERK or AKT signaling as a protective effect (44). In Jarid2αMHC hearts, elevated Abcc9 levels imply cardiac dysfunction or defective maturation. Sulfatase 2 (Sulf2) is an extracellular endosulfatase and removes the 6-O-sulfate from heparan sulfate. Heparan sulfate is a dynamic molecule in the extracellular matrix and involved in signaling by interacting with growth factors (45). N-myc downstream regulated gene 4 (Ndrg4) is a cytoplasmic protein highly expressed in the heart and brain. Down-regulation of Ndrg4 leads to hypoplastic hearts in zebrafish, and Ndrg4 expression is down-regulated by Tbx2 in mice (46, 47). In our RNA-seq data, Tbx2 expression was down-regulated, suggesting that an increase in Ndrg4 expression may be mediated by reduced Tbx2 expression. Ankrd1 is a part of the muscle ankyrin repeat protein family and a cardiac-specific stress-response protein. Ankrd1 functions as a transcription co-factor in the nucleus and maintains sarcomere assembly by interacting with titin in the heart (48). Although Ankrd1 KO mice are viable and display normal heart function, Ankrd1 has been proposed as a potential biomarker for heart failure. Ankrd1 transcription is directly activated by Nkx2.5 and GATA4, and their transcriptional activities can be repressed by Jarid2 (24). Thus, increased Nkx2.5 and GATA4 activities in Jarid2αMHC hearts may activate Ankrd1 expression. These seven up-regulated genes in Jarid2αMHC hearts indicate alterations in expression of critical genes at p10. Interestingly, among the seven genes, four genes (Gyg, Slc8a1, Abcc9, and Ankrd1) are heart failure–associated genes.

Gene expression profiling in the Jarid2αMHC heart during DCM/heart failure

To determine molecular changes in DCM hearts, we performed RNA-seq on 7-month hearts (Fig. 6). A total of 2375 genes were significantly dysregulated in Jarid2αMHC hearts by either EBSeq (Fig. 6A) or DESeq2 analysis (Fig. S3B). Among those, 1005 genes were common DE genes identified by both analyses (Fig. 6B). Major gene ontology of the 1005 genes by BP analysis indicated dysregulation of collagen fibril organization and fatty acid metabolism gene pathways, which consist of mostly up- and down-regulated genes, respectively (Fig. 6C). Likewise, the extracellular region and mitochondrion in CC pathways were dysregulated, which consisted of mostly up- and down-regulated genes in Jarid2αMHC hearts, respectively. Similarly, fibrinectin binding and oxidoreductase activity were identified by molecular function (MF), consisting of up- and down-regulated genes, respectively. Specifically, aerobic metabolism, including fatty acid oxidation and oxidation-reduction process by BP were enriched with down-regulated genes in Jarid2αMHC hearts with DCM.

Because highly dysregulated genes in the mutant heart may play important roles in mediating DCM at 7 months, we analyzed 319 dysregulated genes that showed more than 2-fold changes (Fig. 6D). Among the 319 DE genes, 128 were down-regulated and 191 were up-regulated. GO term analyses on the down-regulated genes showed action potential, single-organism metabolic process, and regulation of transmembrane transport by BP analysis. The CC analysis indicated sarcolemma and T-tubule, and MF analysis showed voltage-gated ion channel activities. The up-regulated genes were mainly involved in extracellular region (84 of 191). Interestingly, PI3K-Akt signaling pathway was identified by KEGG analyses, which included many extracellular matrix genes (Col1a1, Col1a2, Col3a1, Col5a2, Col6a3, Col11a1, Cdkn1a, Gng8, Itga11, Lpar3, Myc, Spp1, Tnc, and Thbs4). Additionally, heart failure–associated genes or extracellular matrix genes were up-regulated, including Tissue inhibitor of metalloprotease-1 (Timp1), A disintegrin and metalloproteinase with thrombospondin repeats-1 (Adams1), and Small proline-rich repeat protein 1a (Sprrr1a). Timp1 is a collagenase inhibitor (49). Adams1 mutation causes geleophysic dysplasia with cardiac defects, including ventricular septal defect and thickened and nonstenotic aortic valve (50). Sprrr1a is overexpressed in myocytes after mechanical stress and may prevent the myocytes against permanent damages (51). Therefore, Jarid2αMHC hearts exhibited increases in stress-response genes and fibrosis related genes.
Jarid2 is required for myocardial maturation

Next, we determined genes that are continuously dysregulated at both p10 and 7 months in the mutant heart to increase mechanistic insights into the Jarid2 function and DCM progression. Our RNA-seq data indicated that only 12 genes were significantly dysregulated in \( \text{Jarid2}^{+/H9251} \text{MHC} \) hearts at both p10 and 7 months (Fig. 7A). Most of these genes (Lpar3, Sulf2, Gsg1l, Irx2, Pam, Ankrd1, Gyg, Lnx1, and Adprhl1) continued to be up-regulated in \( \text{Jarid2}^{+/H9251} \text{MHC} \) hearts at p10 and 7 months compared with controls, implying that Jarid2 may function in suppressing these genes in normal hearts. Lpar3 is a receptor for lysophosphatidic acid belonging to the G protein–coupled receptor family and is expressed in the myocardium during postnatal maturation stages (52). Pam is a membrane protein containing peptidylglycine \( \alpha \)-hydroxylating monoxygenase and \( \alpha \)-amidating enzymes (53). Pam has been studied in the atrium for \( \alpha \)-amidation and pro-ANP packaging in secretory granules (54). Lnx1 is an E3 ubiquitin ligase that mediates the ubiquitination and degradation of Numb and also ubiquitinates ErbB2 receptors in Schwann cells for the maturation (55). Therefore, increased Lnx1 might regulate Nrg1–ErbB signaling pathways in \( \text{Jarid2}^{+/H9251} \text{MHC} \) hearts. Gsg1l is a transmembrane auxiliary subunit of \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, which are ionotropic glutamate receptors in the central nervous system (56). 

Figure 6. Analyses of gene expression profiling in \( \text{Jarid2}^{+/H9251} \text{MHC} \) versus control hearts at 7 months. A, volcano plots showed DE genes by EBSeq. 1044 genes were statistically significant (PPDE > 0.95), indicated by dotted line, and magnified in the right panel. All genes are indicated by dots. B, Venn diagram demonstrates numbers of differentially expressed genes by EBSeq and DESeq2. 1005 genes were common DE genes in EBSeq and DESeq2. C, GO term analysis was performed with 1005 genes. The top ten BPs and top five CCs and MFs are indicated (FDR adjusted \( \text{adj} p \) value of <0.05). D, GO term analysis was performed with 319 DE genes that showed greater than 2-fold changes. All significant categories were shown for down-regulated genes, and the top three significant categories were shown for up-regulated genes (FDR adjusted \( p \) value < 0.05). KEGG, Kyoto Encyclopedia of Genes and Genomes.
expressed higher in control hearts at p10 and 7 months compared with mutants. *Gli1* was down-regulated at p10 but up-regulated at 7 months in mutant hearts.

Because the heart undergoes postnatal development, we reasoned that comparing normal age-dependent changes in gene expression patterns from p10 to 7 months in control versus mutant hearts may provide important information to identify developmental defects in the mutant heart (Fig. 7B). There were 16 genes showing significantly different time-dependent patterns between control and *Jarid2<sup>ΔH9251</sup>MHC* hearts. In *Jarid2<sup>ΔH9251</sup>MHC* hearts, *Eukaryotic elongation factor 1* (*Eef1a1*), *Nppa*, *Nppb*, and *Ankrd1* were significantly increased compared with controls, which are linked to heart failure. In contrast, metabolic process–related genes, such as *Cytochrome c oxidase subunit 7a1* (*Cox7a1*) and *Enoyl CoA hydratase 1* (*Ech1*), were down-regulated in mutant hearts compared with controls. *Cox7a1* is a complex of the mitochondrial respiratory chain and a heart and skeletal muscle-specific enzyme. *Cox7a1* KO mice develop DCM with reduced Cox activities in the muscle (57). *Ech1* is the second enzyme of mitochondrial fatty acid β-oxidase pathways and associated with cell growth and apoptosis (58).

Interestingly, *Ankrd1* and *Nppb* expression levels were slightly up-regulated in control hearts from p10 to 7 months (1.6- and 1.4-fold, respectively), even though these genes were highly up-regulated in *Jarid2<sup>ΔH9251</sup>MHC* hearts (2.5- and 3.2-fold, respectively). Contractile gene expression (*Tpm1, Myl2, Myh6, Actc1, Pln, Tnni2, and Tnni3*) was increased in control hearts from p10 to 7 months, whereas expression of these genes was not increased as much in *Jarid2<sup>ΔH9251</sup>MHC* hearts. Indeed, expression levels of *Tpm1, Myl2, Myh6, Actc1, Pln*, and *Tnni2* were reduced from p10 to 7 months in *Jarid2<sup>ΔH9251</sup>MHC* hearts. These results suggest that Jarid2 is important to increase or maintain the expression of contractile genes for mature cardiomyocytes.

To analyze sarcomere gene expression, we examined expression levels of fetal isoforms such as *Myl7, Tnni1*, and *Acta2* by qRT-PCR on p10 and 7-month hearts (Fig. 7C). In general, these fetal genes are switched to the adult forms during myocardial maturation (59). *Tnni1* and *Acta2* were significantly increased in the mutant neonatal heart. *Ankrd1* is expressed at a higher level in the embryonic heart than the adult heart (48). *Ankrd1* expression was continuously increased in p10 and 7-month *Jarid2<sup>ΔH9251</sup>MHC* hearts. *Myh7*, a fetal isoform, was only...
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increased at 7 months, which is a hallmark for heart failure, namely “fetal gene re-expression.” Tpm1, Myl2, and Pln are important contractile genes that are increased as the heart matures, and dysregulations of these genes are linked to DCM (60). However, these genes were down-regulated in 7-month Jarid2\textsuperscript{−/−} hearts, although Myl2 and Pln expression levels were not altered at p10 (Fig. 7C). These data suggest that Jarid2 is necessary to repress expression of fetal genes and maintain mature genes in the adult heart.

Dysregulation of Nrg1–ErbB4 signaling pathway in Jarid2-deficient hearts

We have previously shown that Notch1–Nrg1–ErbB signaling pathways were increased in Jarid2 KO embryonic hearts (20). Myocardial-specific induction of ErbB2 causes cardiac hypertrophy and dedifferentiation of the cardiomyocytes mediated by ERK and AKT signaling pathways (15). In our RNA-seq data, Nrg1 was highly up-regulated, whereas ErbB4 was down-regulated in Jarid2\textsuperscript{−/−} hearts at 7 months (Fig. 6A). Therefore, we examined whether the altered immature gene expression is correlated with Nrg1–ErbB signaling. First, we determined the expression levels of Nrg1 and ErbB4 by qRT-PCR (Fig. 8, A and B). Interestingly, Nrg1 expression was continuously increased in Jarid2\textsuperscript{−/−} hearts from 1 to 7 months (DCM). In contrast, ErbB4 expression in the myocardium was temporarily increased at p10 but down-regulated by 7 months in Jarid2\textsuperscript{−/−} hearts. Because Nrg1–ErbB signaling activates AKT and ERK pathways (15), we examined ErbB4 protein levels and downstream signals (Fig. 8, C and D). ErbB4 protein expression was increased in Jarid2\textsuperscript{−/−} hearts compared with controls at p10, correlating with mRNA levels. Phospho-AKT and phospho-ERK1/2 expression levels were significantly elevated, whereas total AKT and ERK1/2 expression levels were not changed. To determine whether increased ErbB4 signaling pathways directly regulate fetal gene expression, isolated cardiomyocytes from control and Jarid2\textsuperscript{−/−} hearts were incubated in ErbB inhibitors. Under the vehicle-treated control condition, Tnni1 and Acta2 expression levels were significantly higher in Jarid2\textsuperscript{−/−} versus control hearts (Fig. 8E). However, these increases were normalized to control levels by the treatment with an ErbB receptor inhibitor (Fig. 8F). These results suggest that Jarid2 may mediate transition from fetal to adult gene expression via ErbB4 signaling pathways during neonatal stages. Further experiments are required to determine whether Jarid2 is a direct regulator of ErbB4 signaling and to examine in vivo effects of blocking ErbB signaling on gene regulation in the mutant heart.

Discussion

In the present study, the molecular and developmental functions of Jarid2 were determined in the postnatal heart. Jarid2\textsuperscript{−/−} mice exhibited DCM with contractility defects, leading to premature death at approximately 6–9 months of age. Because Jarid2 expression was maintained in neonatal hearts but significantly decreased thereafter, we reasoned that Jarid2 functions critically in neonatal hearts. These studies will increase our understanding on the molecular etiology of DCM. Thus, we analyzed gene expression profiling at p10 (neonatal) and 7 months (DCM) hearts. At p10, dysregulated genes in Jarid2\textsuperscript{−/−} hearts represented mainly heart development and muscle contraction pathways, and heart failure-related genes were significantly elevated, although the mutant heart morphology appears normal. At 7 months, the metabolic process and ion channel activity pathways were enriched with down-regulated genes, and extracellular matrix component genes were up-regulated in Jarid2\textsuperscript{−/−} hearts. These data indicate that Jarid2 is important for regulating genes for neonatal heart development and muscle contraction at p10, even when cardiac morphology and function seem normal (Fig. 9). These early changes in gene expression may contribute to hyperfunctioning hearts, and lethal DCM at 7 months. To determine whether Jarid2 plays important roles in maturation of cardiomyocytes postnatally, we analyzed gene expression patterns from p10 to 7 months. In normal hearts, sarcomere- and contractile-associated genes were up-regulated from p10 to 7 months in an age-dependent manner, whereas these genes were not sufficiently increased with age in Jarid2\textsuperscript{−/−} hearts. These data suggest that Jarid2 is essential in cardiomyocyte maturation and normal sarcomere development.
Jarid2 deletion mice by αMHC::Cre survived to adulthood and showed DCM, whereas those by Nkx2.5::Cre died with cardiac malformations within 1 day after birth (21). Although both Cre lines are cardiac-specific, Nkx2.5::Cre expresses Cre earlier than αMHC::Cre and is considered as cardiac progenitor-specific (28), whereas αMHC::Cre as differentiated cardiomyocyte-specific. Accordingly, Nkx2.5::Cre expression domains include noncardiomyocytes such as endocardial cells. However, it has been reported that Nkx2.5::Cre did not delete certain genes in the endocardium (61). Jarid2 deletion mice by Nkx2.5::Cre also showed that Jarid2 was expressed and functional in the endocardium (21), supporting the critical function of Jarid2 in the cardiomyocyte progenitors during early development. αMHC::Cre begins expressing Cre in embryonic cardiomyocytes. Thus, one cannot exclude a possibility that some cardiac defects in Jarid2αMHC mice may be embryonic origin. The adult function of Jarid2 can be determined by time-dependent cardiac-specific deletion of Jarid2, such as tamoxifen-induced deletion after birth. If an adult conditional deletion of Jarid2 produces no phenotype, heart failure in Jarid2αMHC mice may have congenital origins. However, given that the neonatal heart of Jarid2αMHC mice is morphologically normal and shows a small number of dysregulated genes at p10 and that the αMHC promoter becomes stronger after birth, embryonic defects may not be a major contributor in DCM progression.

Jarid2 KO mice develop congenital cardiac defects such as thin myocardium, hypertrabeculation, and ventricular septal defects during embryonic stages (27). Although Jarid2 is an enzymatically inactive member of the JMJ histone demethylase family, Jarid2 can function as an epigenetic regulator by recruiting histone methyltransferases in the developing heart (22). However, the function of Jarid2 in the adult heart is not fully understood. The RNA-seq data at p10 were overlapped with our Jarid2 ChIP-chip data from the developing heart (23) to determine potential targets of Jarid2 (Table S3). Among the 72 DE genes, Jarid2 occupies the promoters of the 15 genes, which are potential direct targets of Jarid2. Contractile-related genes (Ttll1 and Atp2a1) were up-regulated in mutant hearts, correlating with the transcriptional repression function of Jarid2. Cell proliferation and differentiation-related genes (Tmem100, Gli1, Tgfb1, and Egfl7) were down-regulated in mutant hearts, implying potential transcriptional activation functions of Jarid2. However, because ChIP-chip assays were performed using embryonic hearts, this overlapping has limitations caused by age and environmental differences. Further investigation into Jarid2 occupancy in the postnatal heart would be important to determine direct targets of Jarid2. In Jarid2αMHC mice at p10, cardiac morphology is overtly normal, yet altered expression of genes involved in ion transmembrane transport and muscle contraction has already occurred. This suggests that dysregulation of these genes is not sufficient to develop morphological and functional defects at p10 but may be causal to hyperfunctioning hearts at 3 months and maladaptation to DCM at 7 months. Although Jarid2 expression is significantly reduced within 1 month of birth, DCM was not manifested until much later in the mutant heart. It is plausible that dysregulation of gene expression during neonatal stages may be permanent, leading to DCM later in life. Alternatively or concomitantly, compensatory mechanisms may contribute to hyperperforming heart in the mutant, followed by pathological remodeling caused by cumulative compensation and chronic stimuli. Given that Jarid2 expression level is not significant in the adult heart, it would be interesting to determine whether epigenetic changes on genomic loci of the DE genes at p10 are maintained later in the adult heart by performing ChIP assays. Jarid2αMHC mice exhibited DCM and increased gene expression involved in collagen fibril organization and extracellular...
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matrix remodeling, indicating increased fibrosis, a common feature of pathological remodeling in the failing heart. Cardiac fibrosis can cause disruption of myocardial architecture and impairment of systolic and diastolic function (62, 63). Jarid2<sub>MHC</sub> hearts showed dysregulated collagen synthesis (Col6a3, Col1a2, Col5a2, Col3a1, Col1a1, Col14a1, Col11a1, and Col9a2). In addition, genes involved in extracellular matrix degradation were dysregulated such as metalloproteases (Mmp19, Mmp2, Mmp14, Mmp23, Adamts7, Adamts3, Adamts2, Adamts12, Adam22, and Adam 11) and metalloprotease inhibitors (Timp1). Secreted factors regulating fibrosis (Fgf11, Fgf9, Ptn, and Fstl3) were also dysregulated in Jarid2<sub>MHC</sub> hearts. Interestingly, Serpin isoforms (Serpinf1, Serpina3n, Serpine1, and Serpinb1c) were highly increased in Jarid2<sub>MHC</sub> hearts at 7 months. Serpins are serine protease inhibitors, which regulate anti-fibrinolysis. Serpine1 (PAI1) inhibits the Mmp activity involved in fibrosis (64). Together, these findings suggest that increased fibrosis in Jarid2<sub>MHC</sub> hearts is mediated by dysregulation of collagen, Mmp, and Serpin expression.

The adult heart produces 90% of its ATP using fatty acid oxidation. However, this energy metabolism is changed to glycolysis in the failing heart (65). Jarid2<sub>MHC</sub> hearts at 7 months displayed a reduction of fatty acid metabolic process. The peroxisome proliferator-activated receptor (PPAR) pathway is a key regulator in heart metabolism, which is inhibited in DCM mouse models (66). PPAR downstream signaling was significantly reduced in Jarid2<sub>MHC</sub> hearts at 7 months (Slc27a1, Acatd, Esrrb, and Ppard1a). However, this pathway was not altered in p10 hearts, suggesting that reduced fatty acid metabolism is a result of DCM pathogenesis. PPARγ coactivator 1α (PGC1α, Pparc1a) regulates mitochondrial biogenesis and oxidative phosphorylation in the heart (66), implying that mitochondrial function is perturbed in Jarid2<sub>MHC</sub> hearts (Fig. 6C). Glucose metabolism process was not identified as a significantly dysregulated pathway in Jarid2<sub>MHC</sub> hearts at 7 months. However, RNA-seq data showed decreases in glycolysis- and gluconeogenesis-related genes (Ldhb, Galm, Acs11, Aldob, Fbp2, and Aldh9a1) and increases in glucose synthesis enzymes (Ptgesc3, Prkg3, Gck, and Gyg), implying a shift from aerobic metabolism to nonoxidative glucose metabolism in Jarid2<sub>MHC</sub> hearts at 7 months.

In mouse models of heart failure, Nrg1 expression is higher, whereas ErbB2 and ErbB4 expression levels are decreased during the transition from compensatory hypertrophy to heart failure (16). Nrg1 improves heart function and prevents cardiac fibrosis in animal models and heart failure patients (16). Increases in Nrg1 and ErbB4 expression correlate with increases in contractility in animal models of heart failure (17). It is interesting that in our study, Nrg1 expression was increased from 1 month, which was much earlier than the onset of DCM. Thus, it is plausible that early increases in Nrg1 lead to the hyperperforming heart at 3 months. Elevated levels of Nrg1 and decreased ErbB4 in 7 months Jarid2<sub>MHC</sub> hearts correlate with a general heart failure phenotype. Induction of constitutively active ErbB2 in neonatal hearts causes re-entry of cardiomyocytes into the cell cycle mediated by Nrg1 (15), and activation of Nrg1 and ErbB4 induces proliferation of differentiated cardiomyocytes (67). Although ErbB4 expression was altered in Jarid2<sub>MHC</sub> hearts at p10, the cell proliferation was not changed, implying that an increase in ErbB4 expression is not sufficient to induce cell proliferation. Because Nrg1 expression was up-regulated in Jarid2<sub>MHC</sub> hearts from 1 month, increased Nrg1 may serve as an early indicator of cardiomyopathy.

Ankrd1 was highly increased in neonatal as well as failing hearts of Jarid2<sub>MHC</sub> mice. Transgenic mice with Ankrd1 overexpression display normal cardiac morphology (48), and the function of increased Ankrd1 in the adult heart is controversial. Ankrd1 can mediate adaptive and protective responses against pathological damages (68, 69). In contrast, Ankrd1 can accelerate the progression to hypertrophy (70–72). It is possible that increased Ankrd1 at p10 plays a protective role to maintain normal heart morphology and functions, which may lead to the hyperfunctioning heart at 3 months. It is unclear whether chronic overexpression of Ankrd1 is protective or accelerates the progression of DCM in Jarid2<sub>MHC</sub> mice at 7 months. Because Ankrd1 expression is already increased at p10, which is much earlier than the onset of DCM, it may serve as an early sensitive marker of cardiomyopathy. It would be interesting to determine the role of early increases in Nrg1 and Ankrd1 in DCM pathogenesis in Jarid2<sub>MHC</sub> mice.

In our RNA-seq data, only 72 genes were differentially expressed in p10 mutant hearts compared with controls, whereas 2375 genes were dysregulated at 7 months, suggesting that Jarid2<sub>MHC</sub> hearts are not severely perturbed in neonatal stages. However, of the 72 genes, 12 genes were also differentially expressed at 7 months, of which 2 genes were maintained down-regulation and 9 genes were maintained up-regulation in Jarid2<sub>MHC</sub> hearts versus controls, implying that Jarid2 function is critical in regulating expression of certain genes from the neonatal to adult stages. Moreover, in control hearts, sarcomere and contractile-associated genes were increased in an age-dependent manner (Fig. 7, B and C), whereas these genes were not increased in Jarid2<sub>MHC</sub> hearts.

Time-dependent gene expression profiling data have been reported in another DCM mouse model with a phospholamban (PlnR9C) mutation (66). By comparing the published dysregulated genes in pre-DCM mutant hearts with our data at p10, we identified the dysregulated genes common to both hearts. These include Tll1, Myl1, Gyg, and Ankrd1 (in cardiomyocytes) and Amdp3, Atp2a1, Kctd17, Sulf2, and Tgfβ1 (in non-cardiomyocytes). Interestingly, a group of genes that are decreased in PlnR9C mice at the DCM stage (Abcc9, Ank, Esrrg, Irx1, Mdh1, Pln, Sucg2, and Tll1) was up-regulated in Jarid2<sub>MHC</sub> hearts at p10 prior to the development of DCM. Some genes increased in PlnR9C mice at the DCM stage were increased in p10 Jarid2<sub>MHC</sub> hearts, such as Gbe1, Ndr4g, Gyg, and Ankrd1. Thus, Jarid2 controls the gene expression at neonatal stages, which may contribute to DCM development in adulthood. Further studies are necessary to determine whether up-regulation of these DCM-related genes at p10 leads to DCM in mutant hearts. Further investigation into the ability of Jarid2<sub>MHC</sub> mice respond to pathological stresses would be interesting. Together, we demonstrate for the first time that Jarid2 plays crucial roles in the development of postnatal hearts, which will provide an important framework for understanding the molecular and genetic basis of cardiomyopathy in humans.
Experimental procedures

Animal husbandry and echocardiography

All studies using animals were conducted in accordance with the University of Wisconsin Research Animal Resource Center policies and the National Institutes of Health Guide for Care and Use of Laboratory Animals. All animal research was approved by an Institutional Animal Care and Use Committee (protocol M005971). All mice were littermate or age-matched controls and mutants. The studies were not blinded. Herein, Jarid2 conditional deletion mice using aMHC::Cre mice (28), aMHC::Cre/+; Jarid2f/f, were designated as Jarid2mMHC. To generate Jarid2mMHC mice, females homozygous for the floxed Jarid2 allele (Jarid2f/f) (29) were mated with aMHC::Cre/+; Jarid2f/+ males. aMHC::Cre/−; Jarid2f/f mice were used as the control throughout this study. All mice employed in this study were bred to a mixed 129/SvJ and C57BL/6 genetic background, and genotyping was performed by PCR as described (29).

Transthoracic echocardiography was performed on mice under 1% isoflurane gas anesthesia using a VisualSonics 770 ultrasonograph with a 30 or 40-MHz transducer (RMV 707B) as described previously (9). Two-dimensionally guided M-mode images of the LV and Doppler studies were acquired at the tip of the papillary muscles. All cardiac parameters were measured over at least three consecutive cycles and analyzed as shown in Table 1. Fractional shortening was calculated as (LVIDd − LVIDs)/LVIDd × 100.

Western blotting, cardiomyocyte culture, and qRT-PCR

To determine the protein levels, Western blotting was performed using heart extracts as described previously (20). The primary antibodies used were anti-Jarid2 peptide antibodies (20), anti-ErbB4 (Santa Cruz, sc283), anti-phospho-AKT (CST, 3787), anti-AKT (CST, 4685), anti-phospho-ERK1/2 (CST, 9106), anti-ERK1/2 (CST, 9102), anti-Cleaved-Caspase3 (CST, 9664), and anti-GAPDH (Millipore, MAB3744) followed by horseradish peroxidase–conjugated secondary antibodies (Santa Cruz, sc2005 and sc2004). Protein bands were detected by chemiluminescence (Termo Scientific) and quantitated with National Institutes of Health ImageJ software. Cultured primary cardiomyocytes were prepared from newborn (p1) mouse hearts as we described (73), yielding ~70% cardiomyocytes under our culture condition. The cells were plated in Dulbecco’s modified Eagle’s medium with 10% horse serum and 5% fetal bovine serum. ErbB inhibitor, AG1478 (5 μM, Sigma), or vehicle (1:1 ratio of methanol and DMSO) as control was added to serum-free medium for 2 days for qRT-PCR experiments.

qRT-PCR was performed as we described (20). Briefly, mRNAs extracted from mouse ventricles were reverse transcribed to cDNA using cDNA synthesis kit (Thermo Fisher) followed by qRT-PCR using FastStart SYBR Green Master (Roche) on a Bio-Rad iCycler. The appropriate primers for each gene are listed in Table S4. All primers were thoroughly evaluated by melt curve analysis to ensure the amplification of a single, desired amplicon. All samples were assayed in duplicate with nearly identical replicate values. The data were generated using the standard curve method and normalized to 18S expression. qRT-PCR data were analyzed by the RQ analysis algorithm (Bio-Rad).

Histology, Lac Z staining, and immunohistochemistry

H&E staining (27), and immunohistochemistry were performed on paraffin-embedded sections as described (20). Briefly, tissue sections were incubated with primary antibodies, anti-Jarid2, anti-MF-20 (DSHB, MF20), anti-Ki-67 (Abcam, ab15580), and anti-phospho-H3 (Millipore, 06-750). Alexa dye-conjugated secondary antibodies (Invitrogen) or DAB substrate kit (Vector Laboratories, sk-4100) was used for visualization. LacZ staining was performed on hearts of heterozygous mice containing a gene trap insertion of LacZ into the Jarid2 locus as described previously (27). Briefly, the hearts were isolated and fixed with 0.5% glutaraldehyde at different ages. The cryosections were incubated in X-gal staining solution at 37 °C for 2 h. Cardiomyocyte CSA was measured in WGA (Invitrogen) stained cardiac sections as described (9). CSA was evaluated in at least 200 cardiomyocytes per animal from identical areas of the left ventricular wall. Cardiomyocytes only showing the nucleus in the middle of octagonal cell shape were included in the measurements. Hoechst dye was used for counterstaining of nuclei. The images were taken using a Zeiss Axiovert 200 microscope and an AxioCam HRc camera. Picosirius staining for collagen were performed as described (9, 27).

Analyses of RNA-seq data

RNA-seq experiments were performed at the University of Wisconsin Biotechnology Center (74). Briefly, total RNA was extracted from control or Jarid2mMHC hearts at p10 or 7 months. For each group, three biological replicates were prepared. Total RNA samples were synthesized into double-stranded cDNA using SuperScript II reverse transcriptase (Invitrogen) and random primers. The cDNA products were ligated to Illumina adapters and amplified. Quality and quantity of the finished libraries were assessed using an Agilent HS DNA or DNA1000 chip (Agilent Technologies) and Qubit® dsDNA HS assay kit (Invitrogen), respectively. Single-end 100-bp sequencing was performed on an Illumina HiSeq2500 sequencer and analyzed using the standard Illumina Pipeline, version 1.8.2. Following quality assessment, the sequencing reads were aligned to reference sequences of transcriptome from NCBI (GRCm38.p6) using Bowtie v 1.1.2 allowing two mismatches (75). Based on the aligned reads, countable values, expected counts, and transcript per million (TPM) for each transcript were estimated by RSEM v 1.3.0 (76). The statistical programming language R (version 3.4.4) was used for further data processing.

RNA-seq data analyses were performed as described (30). DESeq2 was used to identify DE genes with high precision and accuracy. Genes that have false discovery rate (FDR) adjusted p value < 0.05 were considered as DE genes with statistical significance. DE genes were displayed in an MA plot. Another DE analytic tool, EBSeq, was used to identify DE genes based on the Bayesian empirical approach. Genes with value of posterior probability of differential expression (PPDE) of >0.95 were considered as significantly DE genes that were plotted on a volcano plot. GO term enrichment on DE genes was assessed.
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using the Database for Visualization and Integrative Discovery (DAVID) functional analysis software (https://david.ncifcrf.gov) (77, 78). GO terms were obtained from three categories: BP, CC, and MF. Significance of enrichment for each GO term was determined by FDR adjusted \( p \) value < 0.05 based on EASE scores (79) and modified Fisher’s exact test. Significant GO terms in enrichment were visualized using R packages. The RNA-seq data have been submitted to the Gene Expression Omnibus under accession no. GSE118945.

Time-dependent differential gene expression analysis

We applied a simple and novel technique to rank DE genes by directly comparing the degree of changes of their TPM value under different conditions in terms of geometric calculation over time. For each gene, we compared changes of its TPM values from p10 to 7 months between control and \( \text{Jarid2}^{ \text{ΔMHC}} \) hearts. To quantify the degree of changes, we calculated an angle between two 2D vectors: one vector for \( \text{Jarid2}^{ \text{ΔMHC}} \) and the other vector for the control. The \( y \) value of the starting point in the vectors was obtained from the TPM value at p10, and the \( y \) value of the ending point is from the TPM value at 7 months. The difference between the \( x \) values of the two points in a vector was adopted from the maximum difference of \( y \) values. We calculated an outer product of the two vectors to obtain the angle, which indicates the degree of change. To detect significant genes, we fit the angle data into Gaussian distribution and filter angles with \( p \) values < 0.05. The significant genes were visualized by heat map using TPM values.

Statistical analysis

The data represent the averages of three to five replicates and S.E. The replicate numbers are indicated in the text. Significance was determined by Student’s \( t \) test, and differences are considered significant with \( p \) value of ≤ 0.05 (*) and very significant with a \( p \) value of \( \leq 0.01 \) (**).

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