Circulation

ORIGINAL RESEARCH ARTICLE

Epigenetic Reader BRD4 (Bromodomain-Containing Protein 4) Governs Nucleus-Encoded Mitochondrial Transcriptome to Regulate Cardiac Function

BACKGROUND: BET (bromodomain and extraterminal) epigenetic reader proteins, in particular BRD4 (bromodomain-containing protein 4), have emerged as potential therapeutic targets in a number of pathological conditions, including cancer and cardiovascular disease. Small-molecule BET protein inhibitors such as JQ1 have demonstrated efficacy in reversing cardiac hypertrophy and heart failure in preclinical models. Yet, genetic studies elucidating the biology of BET proteins in the heart have not been conducted to validate pharmacological findings and to unveil potential pharmacological side effects.

METHODS: By engineering a cardiomyocyte-specific BRD4 knockout mouse, we investigated the role of BRD4 in cardiac pathophysiology. We performed functional, transcriptomic, and mitochondrial analyses to evaluate BRD4 function in developing and mature hearts.

RESULTS: Unlike pharmacological inhibition, loss of BRD4 protein triggered progressive declines in myocardial function, culminating in dilated cardiomyopathy. Transcriptome analysis of BRD4 knockout mouse heart tissue identified early and specific disruption of genes essential to mitochondrial energy production and homeostasis. Functional analysis of isolated mitochondria from these hearts confirmed that BRD4 ablation triggered significant changes in mitochondrial electron transport chain protein expression and activity. Computational analysis identified candidate transcription factors participating in the BRD4-regulated transcriptome. In particular, estrogen-related receptor α, a key nuclear receptor in metabolic gene regulation, was enriched in promoters of BRD4-regulated mitochondrial genes.

CONCLUSIONS: In aggregate, we describe a previously unrecognized role for BRD4 in regulating cardiomyocyte mitochondrial homeostasis, observing that its function is indispensable to the maintenance of normal cardiac function.

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Clinical Perspective

What Is New?

- Cardiomyocyte-specific BRD4 (bromodomain-containing protein 4) silencing in developing and adult hearts results in progressive deterioration in cardiac contractile function that culminates in dilated cardiomyopathy.
- BRD4 is essential to maintenance of mitochondrial electron transport chain function via transcriptional regulation of a nuclear mitochondrial gene network.
- BRD4 heterozygous deletion resulted in delayed heart failure and pharmacological BRD4 inhibition with JQ1 induced modest changes in mitochondrial genes, suggesting potential cardiac toxicity in targeting BRD4 at baseline.

What Are the Clinical Implications?

- As more potent and specific inhibitors are developed targeting BRD4 for clinical settings in oncology and other diseases, we must carefully monitor basal cardiac performance for functional and mitochondrial deterioration.

Regulation of chromatin architecture in a condensed (inactive) versus open (active) state is controlled through posttranslational modifications of core histone proteins. Governance of chromatin structure through these modifications has emerged as a key driver of transcriptional responses in diverse cell types.[1] Regulation of chromatin by reversible incorporation of acetyl groups within histone tails is among the best understood. The protein machinery that adds (histone acetyltransferase), removes (histone deacetylase), or recognizes (bromodomain; BRD) the acetyl groups has emerged as a central target of therapeutics for a variety of diseases ranging from cancer to heart disease. In the cardiovascular field, molecules that target histone deacetylases and BET (bromodomain and extraterminal) bromodomain adapters (BRD) have been highlighted for their efficacy in a variety of preclinical models of heart failure and for their existing clinical endorsements in oncology applications.[1]

Histone deacetylase inhibitors have been studied for nearly 3 decades, and the biology of different histone deacetylase classes has been extensively characterized in the heart.[2,3] In contrast, small-molecule BET protein inhibitors are new to the cardiovascular field, emerging with studies reporting that the pan-BET inhibitor JQ1 is effective in experimental heart failure.[4,5] The BET family of proteins includes BRD2, BRD3, BRD4, and BRDT. These proteins bind acetyl-lysine in histone tails via tandem bromodomains (BD1 and BD2) and nonhistone proteins via protein-interaction domains.[6] These proteins function primarily in the nucleus to recruit and modulate transcriptional machinery at acetylated loci; BET inhibitors target the acetyl-lysine binding pockets of BRDs to displace them from chromatin.[7]

Since the initial reports, researchers have corroborated the efficacy of BET protein inhibitors in multiple preclinical disease models, identifying the double bromodomain-containing chromatin adaptor BRD4 as a major participant in pathological cardiac remodeling. Not only did JQ1 blunt the development of pressure overload-induced cardiac hypertrophy in mice, but siRNA knockdown of BRD4 in neonatal cardiac myocytes in culture inhibited the hypertrophic response triggered by extracellular growth cues.[4,5] In addition to these direct effects on cardiomyocytes, recent transcriptomics studies suggest that inhibition of BET proteins affects nonmyocyte pathways such as fibrosis and inflammation in preclinical heart failure models.[8] Despite the confirmed therapeutic potential, the precise biology of BET proteins in the heart, including the role of BRD4 in normal cardiac physiology, is unknown.

To support unremitting contractile activity, cardiomyocytes harbor extensive networks of mitochondria, accounting for more than a third of cell volume[9] and generating 90% of the ATP in the heart.[10] Indeed, cardiomyocytes are uniquely mitochondrion dependent given the requirement of continuous energy supply to support costly myocardial function. Recent studies in the oncology field identified a key role of BET proteins, in particular BRD4, in controlling mitochondrial morphology and function, but with conflicting results: One study suggested BRD4 is a repressor of nucleus-encoded mitochondrial genes,[11] whereas another proposed that BRD4 drives mitochondrial gene expression and fission.[12,13] Considering the importance of mitochondrial energetics in myocardial function, we set out to unveil how BRD proteins regulate mitochondrial function in the cardiomyocyte.

To date, all studies conducted in the heart targeted BRD proteins using pharmacological inhibitors, including pan-BET protein inhibitors; no genetic intervention targeting specific BRD proteins has been reported. In the present report, we directly investigated the role of BRD4, a suspected regulator of adverse cardiac remodeling, in cardiac physiology and pathophysiology by engineering a cardiomyocyte-specific BRD4-deletion model. Our findings provide insight into the normal physiological role of myocardial BRD4 and bring to light potential adverse side effects of BRD targeting in the heart.

METHODS

Detailed methods are described in Materials and Methods in the Data Supplement. Below is a brief summary of the most relevant methods. The authors will make the original data, methods used in analysis, and materials used to conduct the

research available to investigators for purposes of reproducing the results or replicating the procedures.

Animal Studies

The BRD4 exon 5 floxed allele was engineered at the Institute of Model Animal, Wuhan University, by CRISPR-mediated homologous recombination. All animal studies were conducted according to ethics guidelines provided by the Institutional Animal Care and Use Committee at UT Southwestern. The BRD4 floxed line was bred with αMHC-Cre and αMHC-MerCreMer (MCM) lines to generate cardiomyocyte-specific BRD4 knockout models. To induce MCM activity, tamoxifen dissolved in corn oil was administered intraperitoneally (20 mg/kg per day for 5 days). Wild-type (WT) animals used in the αMHC-Cre study were mixed cohorts of BRD4$^{f/f}$ Cre– and WT αMHC-Cre+ littermates. WT animals used in the αMHC-MCM study were mixed cohorts of BRD4$^{f/f}$ Cre– and WT αMHC-MCM+ littermates, all of which were treated with the same tamoxifen regimen as the experimental group. Transverse aortic constriction (TAC) surgery was conducted as previously described.[14] Transthoracic echocardiography was performed on conscious mice and analyzed in blinded fashion using a Vevo2100 system with MS400 transducer.

RNA-Sequencing and Analysis

BRD4$^{f/f}$ αMHC-MCM animals were injected with tamoxifen, and left ventricles (LVs) were collected 2 weeks after induction. JQ1 stock was freshly diluted in 10% 2-hydroxypropyl-β-cyclodextrin and injected intraperitoneally daily for 2 weeks at 50 mg/kg; LVs were collected within 8 hours of the last injection. Snap-frozen LVs were processed for RNA extraction. Samples with RNA Integrity Number >8 were submitted for library preparation and next-generation sequencing at Novogene Inc (Santa Clara, CA). Briefly, cDNA libraries were prepared using a NEB library kit with poly-A enrichment. Purified cDNA libraries were sequenced by NovaSeq6000 with >30 million raw reads (150 bp, pair ended) per sample. RNA-sequencing (RNA-seq) data sets were analyzed for differential expression, Gene Ontology, Gene Set Enrichment Analysis, gene modules, and candidate transcription factors (TFs). Data are accessible at National Center for Biotechnology Information, BioProject PRJNA672136.

Mitochondrial Enzyme Activity

Mitochondrial enzyme assays were performed with freshly isolated ventricular mitochondria from mouse ventricles collected at zeitgeber time 2. Subsarcolemmal mitochondria were isolated by ultracentrifugation in ice-cold isolation buffer as previously described.[15] Either intact or sonicated mitochondria were used for enzyme assays at room temperature using a cuvette spectrophotometer in kinetic mode (Agilent). NAD-linked electron transport chain (ETC) complexes were assayed with previously described methods.[15,16] Briefly, mitochondrial NADH oxidase activity (coupled activity of complexes I, III, IV) was measured as the rate of NADH oxidation in intact mitochondria. With sonicated mitochondria, specific activities of complexes I, III, and IV were measured. Specificity of each assay was verified with complex-specific inhibitors. Tricarboxylic acid (TCA) cycle enzyme activities were measured in mitochondria disrupted with 0.01% TritonX-100. Specific activities of Malate Dehydrogenase, Citrate Synthase, Alpha-ketoglutarate Dehydrogenase, Succinate Dehydrogenase were measured. All enzyme activities were substrate dependent and measured in the linear range. All measurements were performed in duplicates and normalized to protein content.

Mitochondrial Respiration Assay

Mitochondrial respiration was measured at 37 °C with freshly isolated mitochondria according to previously described methods.[17,18] Oxygen consumption rate was monitored with a fluorescence-based oxygen sensor (Ocean Optics, Neofox HIOXY oxygen probe). Mitochondria (0.25 mg/mL) were loaded into an airtight chamber in oxidative phosphorylation buffer (210 mmol/L MOPS (4-Morpholinepropanesulfonic acid), 70 mmol/L mannitol, 10 mmol/L sucrose, 5 mmol/L KH_2PO_4, pH 7.4) with the following substrates: (1) malate (1 mmol/L) and pyruvate (0.1 mmol/L) or (2) malate (1 mmol/L) and palmitoyl-L-carnitine (25 µmol/L) in buffer supplemented with 0.05% fatty acid–free BSA. Mitochondrial respiratory state II was measured as the basal oxygen consumption rate; state III was measured as the maximal oxygen consumption rate on ADP supplementation; and state IV was measured as the ADP-independent oxygen consumption rate after ADP exhaustion. Each sample was assayed 3 times with each substrate.

Statistical Analysis

Data are expressed mean±SEM. Statistical difference was assessed by 2-tailed, unpaired Student *t* tests for experiments with 2 groups and 1-way or 2-way ANOVA with Tukey post hoc analysis for multiple comparisons as appropriate in experiments with ≥3 groups. A value of $P<0.05$ was considered statistically significant. Statistical analyses were conducted with GraphPad Prism software 8.0.

RESULTS

Cardiomyocyte-Restricted BRD4 Ablation Results in Contractile Dysfunction and Dilated Cardiomyopathy

To evaluate the role of BRD4 in cardiac physiology and disease, we engineered mice with cardiomyocyte-specific BRD4 deletion by targeting the critical exon 5 (Figure 1A). With a Cre-loxP system, αMHC-Cre–mediated targeting yielded efficient and specific decreases in BRD4 protein levels in the myocardium (Figure 1B and Figure IA and IB in the Data Supplement), specifically from the nucleus of cardiomyocytes (Figure IC and IB in the Data Supplement). Deletion of cardiomyocyte BRD4 during late cardiogenesis (embryonic day 11.5–12.5 by αMHC) did not affect gross cardiac morphology at birth or alter animal growth (Figure ID and IE in the Data Supplement). However, mice with cardiomyocyte BRD4 knockout (cKO) manifested progressive contractile dysfunction with age. Fractional shortening, a measure of ventricular contractility, manifested a slight decrease in cKO mice

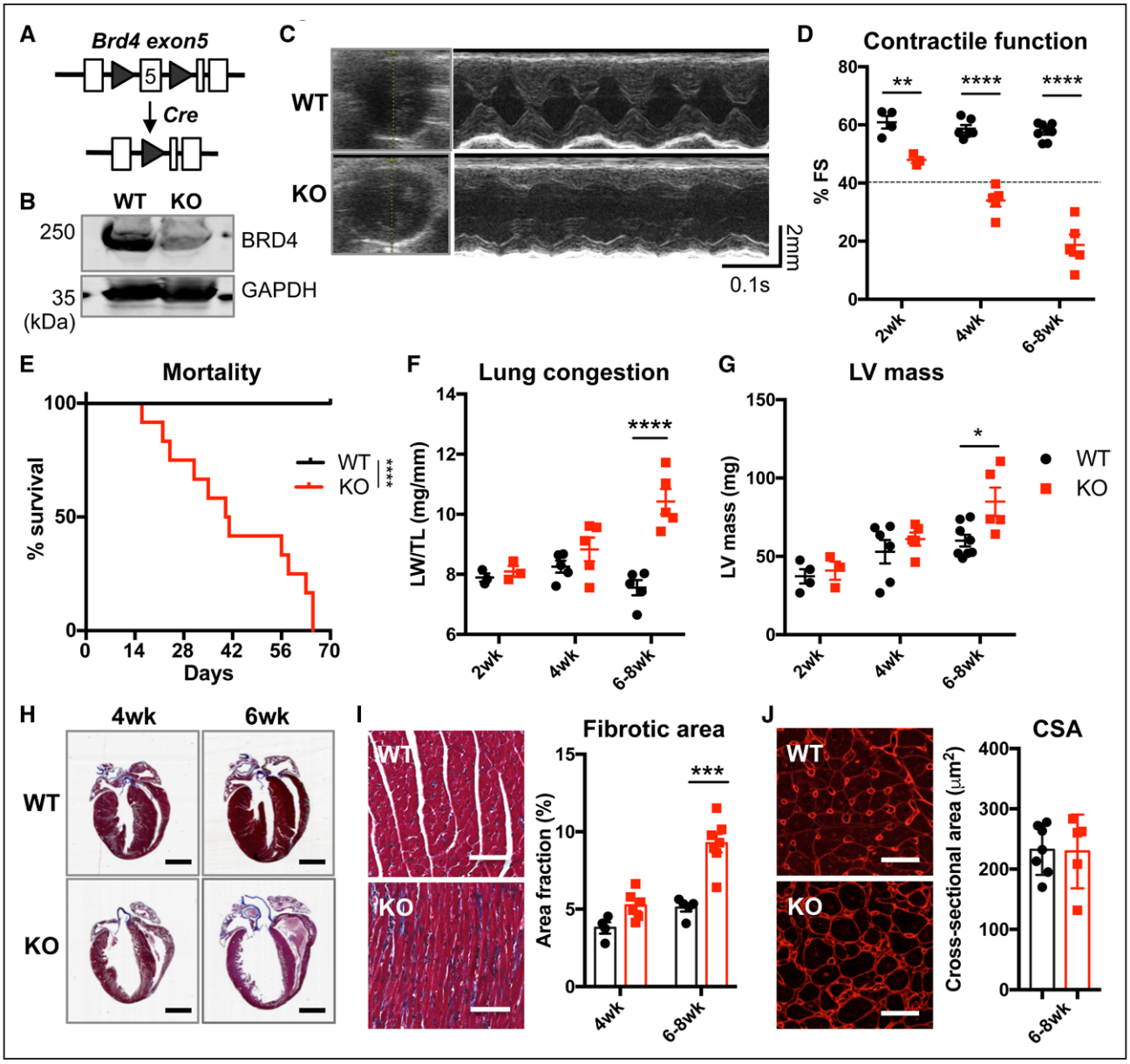

Figure 1. Cardiomyocyte-specific silencing of BRD4 (bromodomain-containing protein 4) in developing heart results in postnatal dilated cardiomyopathy.

A, Schematic of BRD4 conditional allele. **B**, Representative immunoblot of BRD4 in wild-type (WT) vs knockout (KO) left ventricular (LV) lysates at 4 weeks of age. **C**, Representative echo image at 4 weeks of age, B-mode and M-mode. **D**, Echocardiographic analysis of WT and BRD4 cardiomyocyte-specific KO (cKO) at 2, 4, and 6 to 8 weeks of age (n=3–5 per group). ***P*<0.01, ****P*<0.005, *****P*<0.001 vs age-matched WT. **E**, Survival curve of BRD4 cardiomyocyte deletion mouse (n=11–12 per group). *****P*<0.001 vs WT. **F**, Lung congestion measured by wet lung weight normalized to tibia length over at 2, 4, and 6 to 8 weeks of age (n=3–5 per group). *****P*<0.001 vs WT. **G**, LV mass measured by echo at 2, 4, and 6 to 8 weeks (n=3–5 per group). **P*<0.05 vs WT. **H**, Representative 4-chamber cross section at 4 and 6 weeks of age, trichrome stained. Scale bar=20 mm. **I** and **J**, Representative images and quantification of cardiac fibrosis by trichrome staining at 4 to 8 weeks of age (n=5–8 per group); scale bar, 100 μm. FS indicates fractional shortening. ****P*<0.005 vs WT and cardiac fiber size by wheat germ agglutinin staining at 6 to 8 weeks of age (n=5–8 per group); scale bar, 25 μm.

at the earliest time points tested (2 weeks), with progressive declines over time (Figure 1C and 1D and Table I in the Data Supplement). These mice also manifested pulmonary edema, a sign of heart failure, and increased mortality: 50% mortality at 6 weeks and nearly 100% mortality by 10 weeks of age (Figure 1E and 1F). Histological analysis of LV tissue revealed significant thinning of the ventricles and interstitial fibrosis (Figure 1H and 1I). Whereas measurements of LV mass and heart weight/tibia length revealed significant increases at late time points (6–8 weeks) compared with age-matched WT mice (Figure 1G and Table I in the Data Supplement), measurements of cross-sectional area did not reveal evidence of an increase, suggesting a lack of concentric hypertrophic growth (Figure 1J). The cKO hearts exhibited eccentric remodeling (elevated LV mass and relative wall thickness <0.42; Table I in the Data Supplement). In summary, genetic disruption of BRD4 selectively in

cardiomyocytes triggered an unexpected and progressive pathological remodeling response, culminating in dilated cardiomyopathy (DCM) and early mortality.

BRD4 Deletion in Adult Heart Provokes Contractile Dysfunction and Heart Failure

Next, we tested the role of BRD4 in mature adult hearts by engineering an inducible cKO model using an αMHC-MCM driver. The adult cKO cohorts were injected with tamoxifen at 8 weeks of age, and a variety of cardiac parameters were monitored over time (Figure IF in the Data Supplement). The inducible cKO line manifested tamoxifen- and Cre-specific deletion of BRD4 protein from the adult myocardium by 2 weeks after tamoxifen injection (Figure 2A and Figure IG and IH in the Data Supplement). Adult BRD4-null hearts maintained normal contractile function until 3 weeks after injection and then developed progressive systolic dysfunction and chamber dilation, coupled with signs of heart failure and increased mortality, similar to αMHC-Cre cKO mice (Figure 2B–2E and Table II in the Data Supplement). In contrast to the αMHC-Cre cKO, adult-onset BRD4-null hearts did not develop significant fibrosis but did manifest tissue hypertrophy as evidenced by elevated cross-sectional area of LV fibers and LV mass (Figure 2F and 2G and Table II in the Data Supplement). Appropriate control groups for tamoxifen injections and αMHC-MCM transgene were tested (Figure II in the Data Supplement).

Together, findings from the 2 BRD4 cKO models indicate that BRD4 is required to maintain contractile

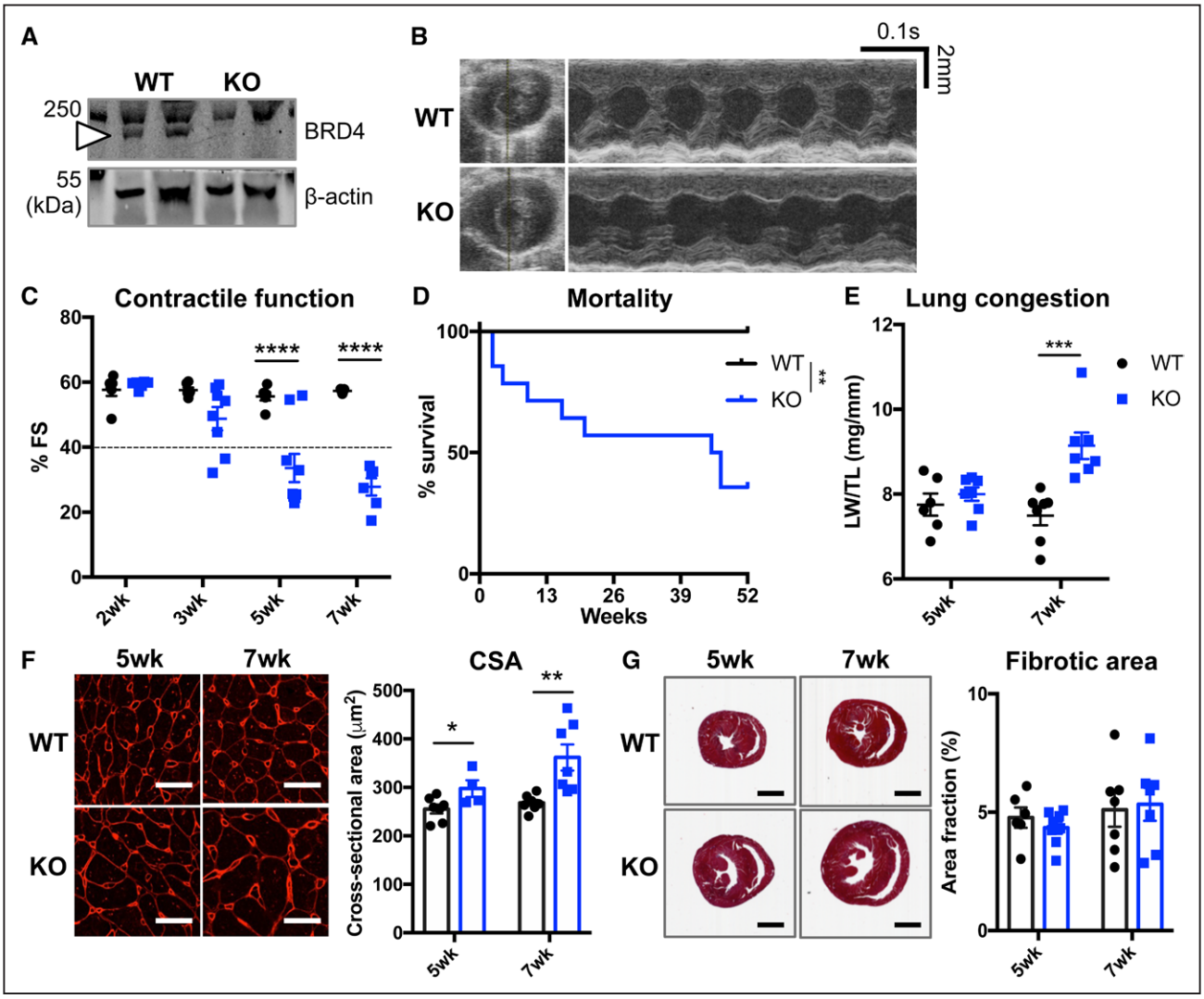

Figure 2. Cardiomyocyte (CM)-specific deletion of BRD4 (bromodomain-containing protein 4) in adult heart results in heart failure.
A, Representative immunoblot for BRD4 in wild-type (WT) vs knockout (KO) left ventricular (LV) lysates at 2 weeks after tamoxifen (Tam) injection (arrow indicates the BRD4-FL band). **B**, Representative echo image at 5 weeks after Tam, B-mode and M-mode. **C**, Echocardiographic analysis of WT and BRD4 CM-specific KO (cKO) at 2, 3, 5, and 7 weeks after Tam (n=3–7 per group). ****P<0.001 vs WT. **D**, Survival curve of BRD4 CM-deletion mouse (n=10–14 per group). **P<0.01 vs WT. **E**, Lung congestion measured by wet lung weight (LW) normalized to tibia length (TL) at 5 and 7 weeks after Tam (n=6–8 per group). ***P<0.005 vs WT. **F**, Representative images and quantification of cardiac fiber size by wheat germ agglutinin stain (n=6–8 per group); scale bar, 25 μm. *P<0.05, **P<0.01 vs WT. **G**, Representative 2-chamber cross section, trichrome-stained at 5 and 7 weeks after Tam, and quantifications of tissue fibrosis (n=6–8 per group).

function of the myocardium in postnatal stages and in adulthood. These genetic BRD4 disruption studies are in marked contrast to pharmacological inhibition studies that reported stable baseline function (up to 4 weeks reported).[4] In addition, we observed that haploinsufficiency of BRD4 ($BRD4^{f/-}$ αMHC-Cre line) resulted in a decrease in BRD4 protein levels and development of heart failure by 6 months of age, followed by premature mortality by 12 months (Figure IIIA–IIID in the Data Supplement). In aggregate, these findings strongly suggest the existence of a dose-dependent requirement for BRD4 protein to maintain myocardial functional homeostasis.

Transcriptomic Profiling Reveals Shared and Distinct Profiles

To identify mechanisms leading to myocardial dysfunction after BRD4 silencing in cardiomyocytes, we conducted a comprehensive RNA-seq analysis of early transcriptomic changes in BRD4-null hearts. To minimize the contribution of secondary changes occurring as a result of a heart failure phenotype, we studied adult inducible BRD4 cKO animals before the emergence of an overt cardiac phenotype. To query mechanisms underlying the phenotypic differences between genetic BRD4 ablation and pharmacological BRD4 inhibition, we studied JQ1-treated hearts as a comparison group (Figure 3A). At the time of tissue collection, all groups exhibited normal contractile function with unaltered hypertrophy markers except for a slight elevation of ANP (atrial natriuretic peptide) in the cKO group (Figure IVA–IVC in the Data Supplement). BET protein inhibitor response genes were altered in JQ1-treated hearts as previously reported.[4]

The poly A–enriched transcriptome was evaluated from whole LV of WT, cKO, and JQ1-treated mice. The resulting data set manifested distinct segregation across the 3 groups (Figure IVD in the Data Supplement), with >3000 differentially expressed genes (Figure 3B). Comparative analysis of cKO and JQ1 groups revealed shared and disparate gene sets (Figure 3C). A quarter of the JQ1-induced transcriptome changes overlapped with the cKO gene set, suggesting cardiomyocyte BRD4-specific targets. The JQ1-unique gene set, which represents BRD4-independent changes in cardiomyocytes plus BET inhibition-regulated transcripts in noncardiomyocytes, was enriched in cell adhesion, cell growth, and autophagy pathways (Figure 3D).

Whereas the JQ1-unique group involved a larger number of altered genes, the enriched pathways displayed relatively higher adjusted *P* values, suggesting overall broad and scattered alterations. In contrast, the cardiomyocyte BRD4-specific gene sets, both shared and cKO unique, were enriched in mitochondrial metabolism and ETC pathways with significantly smaller adjusted *P* values (Figure 3D). Half of the genes altered in cKO were not shared with JQ1; a cKO-unique gene set suggests existence of bromodomain-independent transcriptional control by BRD4 or a large efficacy gap between JQ1-mediated inhibition and the complete silencing of BRD4.

We dissected differential gene expression further by conducting module analysis to identify gene subsets (Figure 3E and 3F). A total of 16 gene modules were identified with the top 5 showing significant enrichment scores. The first ranked module (M1), encompassing genes uniquely downregulated by JQ1, was enriched in cell growth and mobility pathways, whereas modules uniquely downregulated in the cKO data set (M2) were enriched in mitochondrial protein complex IV genes. Genes downregulated in both the cKO and JQ1 data sets (but with greater enrichment in cKO) strongly conformed to mitochondrial energetic pathways (M3). Genes downregulated in both (but to a greater degree in JQ1; M4) or uniquely upregulated in JQ1 (M5) are involved in fatty acid and lipid metabolism. For each module, gene hub analysis was performed to identify potential signaling nodes by assessing known interactions and coexpression patterns between genes (Figure V and Table III in the Data Supplement). Many of the identified gene hubs in top modules included highly muscle-enriched molecules, whereas gene hubs in lower-ranking modules were ubiquitously expressed.

Altogether, the RNA-seq findings provide a detailed transcriptomic comparison between pharmacological BET protein inhibition and genetic ablation of BRD4. BET protein inhibition results in broad changes in cardiac transcript abundance, whereas BRD4-specific disruption, both deletion and inhibition, results in early and significant changes in nucleus-encoded mitochondrial genes.

Cardiomyocyte BRD4 Regulates Nucleus-Encoded Mitochondrial Gene Expression

To further dissect BRD4-dependent regulation of mitochondria, we isolated cKO-specific gene sets for in depth analysis, finding that there are 2-fold more downregulated genes than upregulated genes in cKO compared with WT (Figure 4A). As in the previous analysis, the most significant pathways altered in cKO were mitochondrial ETC genes regulating ATP synthesis and oxidative phosphorylation; in addition, we identified significant downregulation of calcium handling/excitation-contraction coupling pathways and upregulation of myocardial remodeling genes (Figure 4B and 4C). RNA-seq–identified transcript changes were validated by quantitative polymerase chain reaction (qPCR) in both LV and isolated adult mouse ventricular myocyte samples, at both early and late stages, and in both Cre lines (Figure 4E and Figure IVE in the Data Supplement). Furthermore, the validated mitochondrial genes were

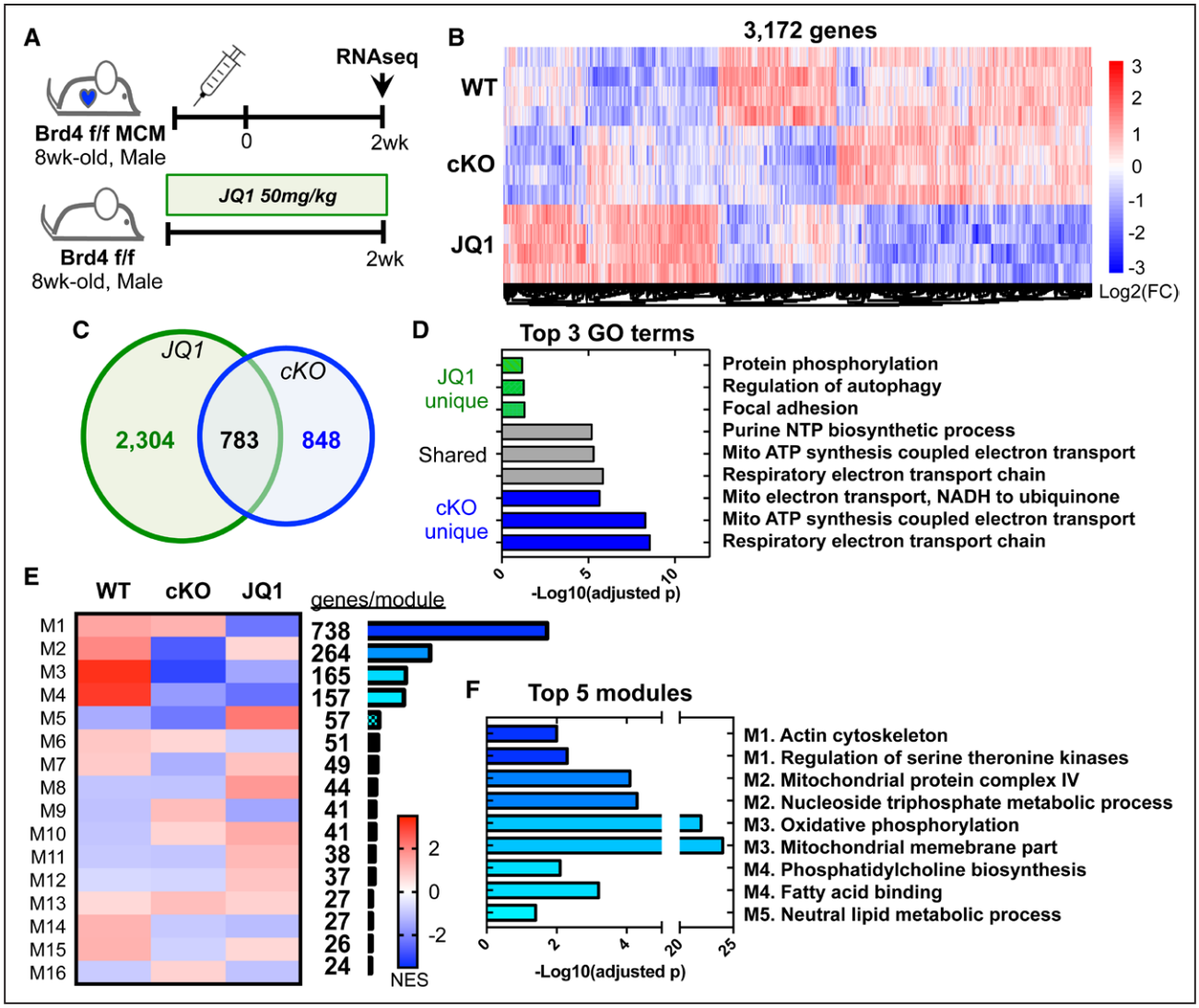

Figure 3. BRD4 (bromodomain-containing protein 4) deletion vs inhibition reveals shared and unique transcriptomic profiles.
A, Schematic for RNA-sequencing sample collection with 3 groups: wild-type (WT), cardiomyocyte-specific knockout (cKO) at 2 weeks after induction, and JQ1 after 2 weeks of treatment (n=4 per group). **B**, Heat map of differentially expressed genes between all groups (adjusted *P*<0.05). **C** and **D**, Venn diagram between cKO vs WT and JQ1 vs WT groups and top 3 Gene Ontology (GO) terms for each compartment (adjusted *P*<0.05). **E**, Heat map of the module analysis for differentially expressed genes between all groups (fold change>1.5, *P*<0.05) with total number of genes per module. **F**, Significant GO terms for top 5 gene modules.

not altered in conventional pressure overload–induced heart failure samples (Figure IVF in the Data Supplement), suggesting BRD4-specific regulation rather than heart failure–driven changes. Metabolite network analysis of downregulated genes in cKO tightly conformed to NADH, oxygen, and redox-sensitive metabolites (Figure 4D), corroborating the finding that cardiomyocyte BRD4 is specifically involved in driving genes involved in the mitochondrial ETC.

BRD4 Controls Mitochondrial Homeostasis in the Myocardium by Driving ETC Protein Expression

To test whether BRD4-driven changes in transcription result in functional changes in cardiac ETC capacity, we conducted enzyme activity assays for each mitochondrial ETC complex. On the basis of RNA-seq trends, complexes I, III, and IV, as well as TCA cycle enzymes, were expected to be altered (Figure 5A). Mitochondria were isolated from WT and cKO ventricles at the onset of cardiac dysfunction (Figure 5B); sham and TAC hearts with comparable myocardial function were used as controls to distinguish BRD4-driven changes from heart failure–driven changes (Figure 5C and Figure VIA and VIB in the Data Supplement).

Mitochondria from BRD4 KO myocardium displayed marked deterioration in ETC activity per mitochondrion. Overall proton pump capacity (complexes I, III, IV), measured by maximal NADH oxidation rate, was significantly decreased in both the constitutive and inducible cKOs (Figure 5D and Figure VIC in the Data Supplement). Each complex measured in isolation (complexes I, III, IV) corroborated reduced ETC activity (Figure 5E). In

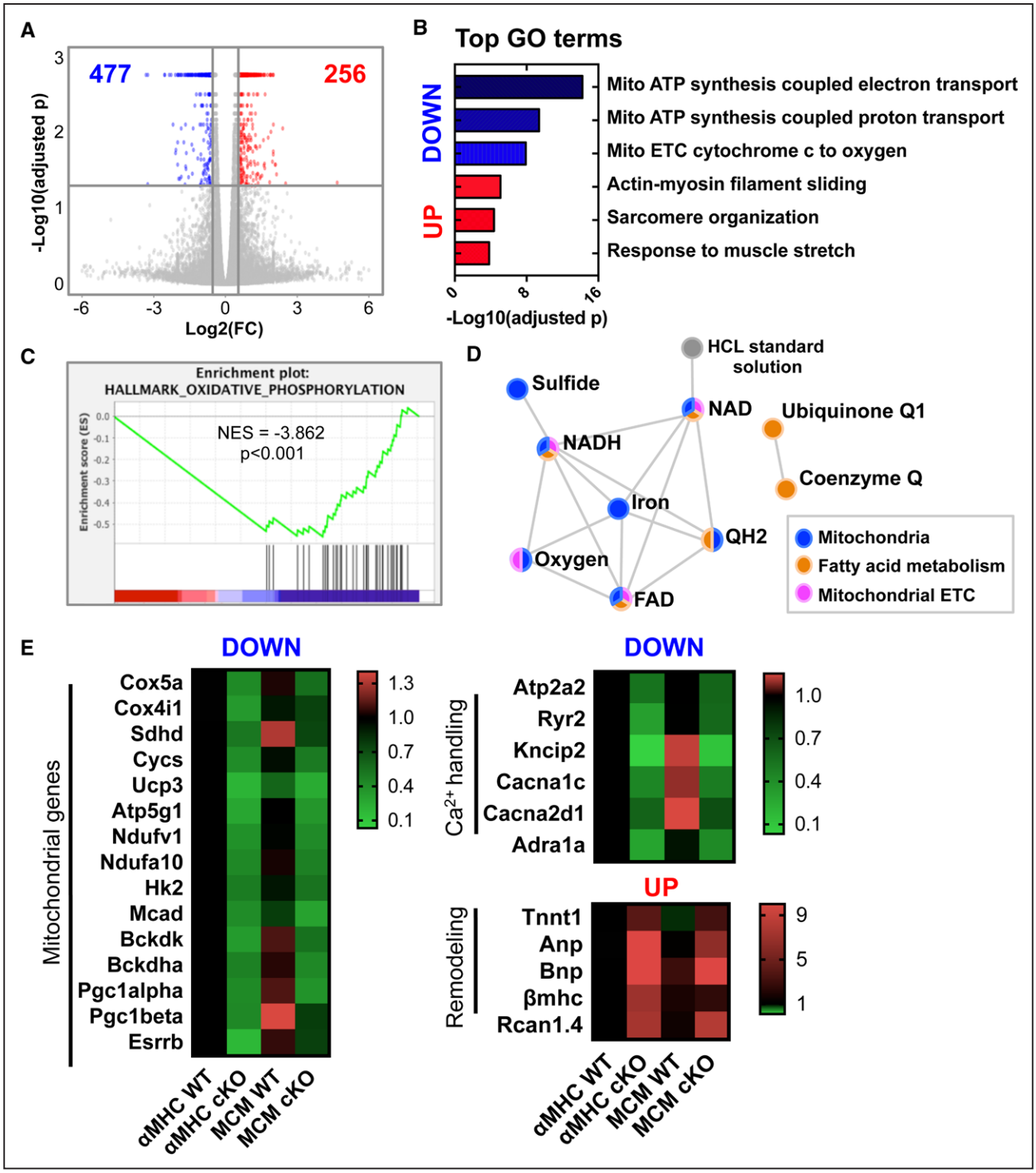

Figure 4. Transcriptome analysis of BRD4 (bromodomain-containing protein 4)–null heart reveals early and significant changes in nucleus-encoded mitochondrial gene network involved in the electron transport chain (ETC).

A, Volcano plot of differentially expressed coding and noncoding genes between wild-type (WT) and cardiomyocyte-specific knockout (cKO) groups (fold change [FC]>1.5, adjusted *P*<0.05). **B**, Top 3 Gene Ontology (GO) terms, upregulated or downregulated in cKO vs WT (FC>1.5, adjusted *P*<0.05). **C**, Top enriched gene set from Gene Set Enrichment Analysis for cKO vs WT against Hallmark gene sets. Adjusted *P*<0.05 represents statistical significance. **D**, Metabolic network analysis of the altered genes in cKO vs WT (FC>1.5, adjusted *P*<0.05). Nodes labeled by top ontology terms from the Human Metabolome Database. **E**, Quantitative polymerase chain reaction validation of RNA-sequencing–identified cKO-specific downregulated and upregulated genes in αMHC-Cre lines at 4 weeks of age and αMHC-MerCreMer at 5 weeks after tamoxifen Tam (n=4–5 per group); all shown genes are significantly altered compared with WT (*P*<0.05 by 2-way ANOVA). NES indicates Normalized Enrichment Score.

comparison, mitochondria isolated from TAC-induced failing hearts exhibited intact ETC function per mitochondrion, suggesting that the diminished ETC activity in cKO is a heart failure–independent phenotype. In addition to ETC complexes, TCA cycle enzymes downregulated in cKO (MDH, CS, SDH) manifested decreased

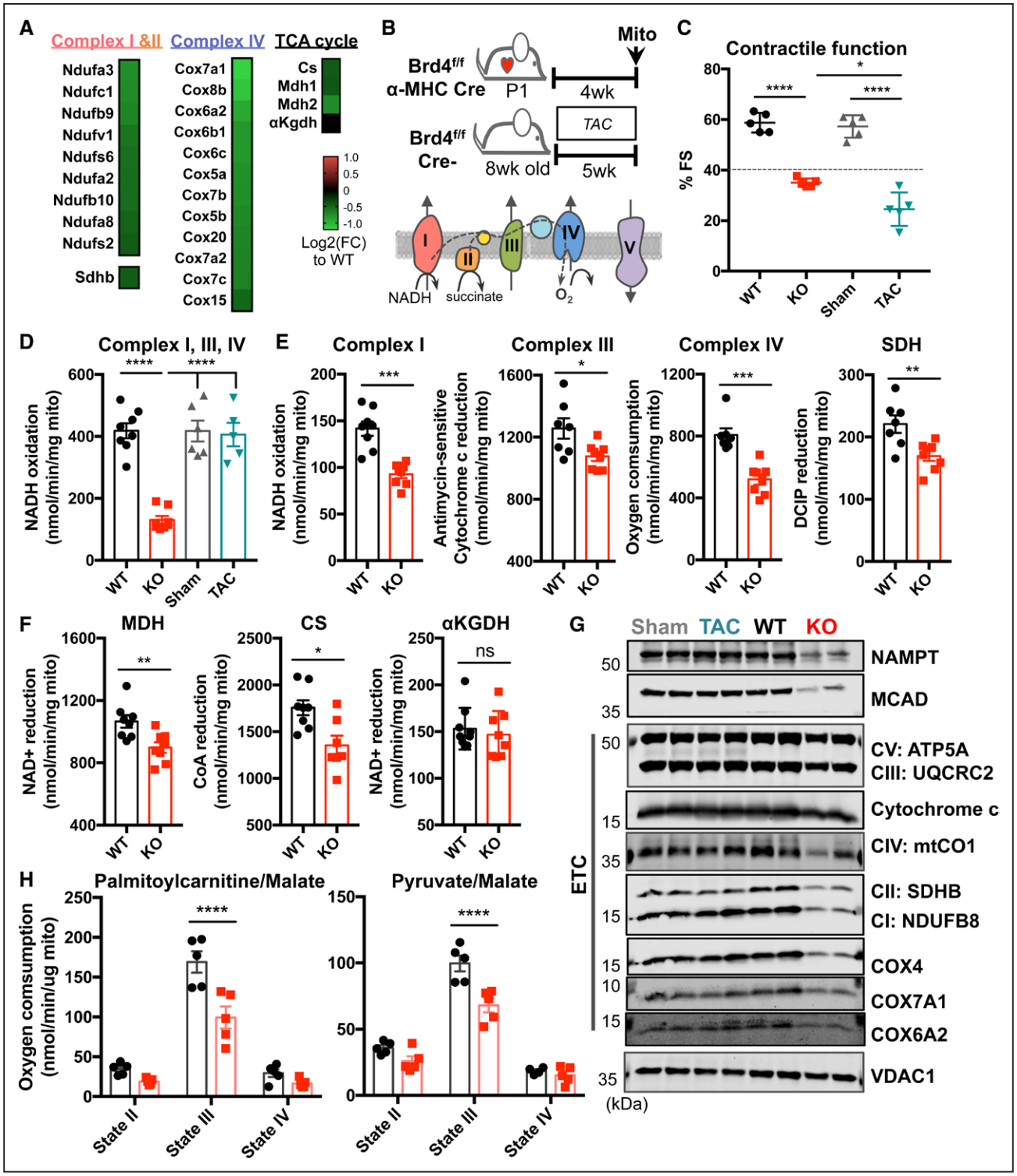

Figure 5. BRD4 (bromodomain-containing protein 4) deletion in cardiomyocytes leads to mitochondrial electron transport chain (ETC) dysfunction. **A**, Significant RNA-sequencing transcript changes of ETC and tricarboxylic acid (TCA) cycle genes in cardiomyocyte-specific knockout (cKO) vs wild-type (WT). **B**, Schematic of mitochondrial ETC and animal models for ventricular mitochondria isolation: WT and αMHC cKO at 4 weeks of age and sham and TAC 5 weeks after surgery. **C**, Contractile function by echo of samples for mitochondrial enzyme activity assays, αMHC cKO, and TAC sets (n=5–8 per group). **P*<0.05, ****P*<0.005, *****P*<0.001 vs WT or sham. **D**, Maximal NADH oxidase activity measured by NADH consumption rate of isolated ventricular mitochondria at room temperature (RT; n=5–8 per group). ****P*<0.005, *****P*<0.001 vs WT or BRD4 KO. **E**, Individual ETC complex maximal activity measurement of isolated ventricular mitochondria at RT (n=8 per group). **P*<0.05, ***P*<0.01, ****P*<0.005 vs WT. **F**, TCA cycle enzyme maximal activity of isolated mitochondria at RT (n=8 per group). **P*<0.05, ***P*<0.01 vs WT. **G**, Immunoblot of mitochondrial proteins and enzymes in isolated ventricular mitochondria from αMHC cKO and TAC sets. **H**, Oxygen consumption measurement of isolated mitochondria at 37 °C using different substrates, palmitoyl-carnitine and pyruvate (n=5 per group). *****P*<0.001 vs WT.

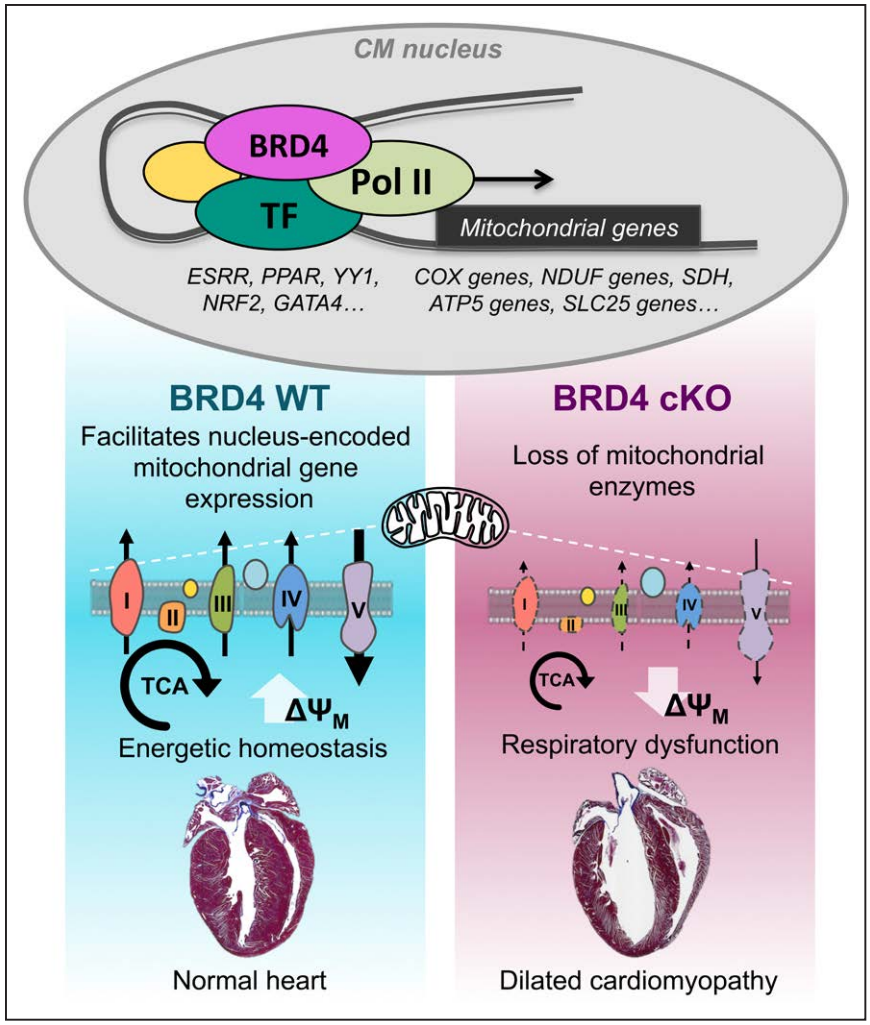

Figure 6. Summary figure.
Cardiomyocyte BRD4 (bromodomain-containing protein 4) plays an indispensable role in driving cardiac gene expression, in particular, nucleus-encoded mitochondrial respiratory enzymes. Without BRD4, cardiomyocyte electron transport chain function deteriorates owing to depletion of key enzymes, resulting in depressed respiration and mitochondrial membrane potential ($\Delta\Psi_M$). BRD4 cardiomyocyte (CM)-specific knockout (cKO) hearts progress to severe contractile dysfunction and heart failure. TCA indicates tricarboxylic acid; TF, transcription factor; and WT, wild-type.

activity per mitochondrion. In contrast, the activity of αKGDH, the transcript of which was not altered by BRD4 ablation, remained intact (Figure 5F). Immunoblot analysis of mitochondrial protein lysate loaded in equal amounts revealed clear reductions in numerous mitochondrial enzymes, including those in the ETC, as well as in other metabolic processes, consistent with alterations in their transcripts (Figure 5G).

To test whether changes in enzyme activities affect mitochondrial oxidative phosphorylation capacity, we measured mitochondrial O_2 consumption in the presence of different metabolic substrates (Figure 5H). Mitochondria from BRD4-null myocardium exhibited depressed capacity to utilize both fatty acid and pyruvate for energy production. To corroborate studies conducted in isolated mitochondria, we imaged intact mitochondria in isolated neonatal rat ventricular myocytes subjected to transient, siRNA-driven BRD4 silencing (Figure VID–VIF in the Data Supplement). BRD4 silencing diminished mitochondrial membrane potential as measured by Mitotracker Red CMXRos staining.

In summary, these findings led us to conclude that cardiomyocyte BRD4 transcriptionally regulates expression of key mitochondrial ETC and TCA cycle enzymes to maintain myocardial energetic homeostasis.

BRD4 Links Key TFs Involved in Cellular Energetics With Mitochondrial Gene Transcription

To unveil a link between the epigenetic adapter BRD4 and transcriptional regulation of mitochondrial gene networks, we explored candidate TFs. To identify putative TFs involved in BRD4-mediated cardiomyocyte gene regulation, the cKO-regulated gene list was queried against multiple TF databases based on chromatin interaction or TF perturbation studies. Converging "top hits" from 4 databases yielded a total of 13 TF groups that appeared more than once across databases; only 1 TF family, estrogen-related receptors (ESRRs), appeared across all databases (Figure VIIA and Table IV in the Data Supplement). Individual TFs identified from the analysis were then scored and ranked by a combined enrichment score in each database and the number of appearances across databases (Figure VIIB in the Data Supplement). Top-ranking TF candidates with known

expression in the heart included ESRRα, PPARα, NFE2L2 (or NRF2), and YY1; all 4 TFs have been shown to regulate cellular energetics in many organs, including the heart. Of note, the top 4 candidate TFs along with 3 additional TFs with significant presence—GATA4, ESRRβ, and ESRRγ—covered >60% of BRD4 cKO downregulated genes (Table V in the Data Supplement).

We tested the top-ranking TF, ESRRα, for involvement in BRD4-mediated mitochondrial gene regulation. Indeed, we located ESRRα-binding motifs in promoter regions of many cKO downregulated mitochondrial genes, including *Mcad* and *Sdhb,* both of which were confirmed with a decrease in message and protein levels (Figure 5G). Chromatin immunoprecipitation (ChIP)–qPCR analysis of *Mcad* and *Sdhb* promoters using cardiomyocyte chromatin was performed on 2 sites, region A, a negative control region without estrogen related receptor element (ERRE), and region B, an ERRE-containing region within 1kb of the transcription start site, against BRD4, ESRRα, and RNA Pol-II (Figure VIIC and VIID in the Data Supplement). ChIP-qPCR results revealed that (1) BRD4 binds regions harboring an ERRE, with clear displacement in BRD4 cKO, and (2) ESRRα is displaced from the ERRE-containing regions of each promoter in the absence of BRD4 (Figure VIIC and VIID in the Data Supplement).

DISCUSSION

In this study, we uncovered an indispensable role for BRD4 protein in preserving myocardial contractile function and mitochondrial homeostasis. Using a cardiomyocyte-specific BRD4-deletion model, we found that BRD4 regulates mitochondrial gene expression in the myocardium. Loss of BRD4 protein leads to dramatic downregulation of expression of key nucleus-encoded mitochondrial genes, as well as genes involved in excitation-contraction coupling. These changes lead to cardiomyocytes depleted of critical enzymes involved in the mitochondrial proton gradient (ETC) and metabolite cycling (TCA cycle and lipid oxidation); the consequence is progressive declines in ventricular contractile performance, ventricular dilatation, and cardiomyopathy. In the end, our findings demonstrate a physiological role for BRD4 in maintaining normal cardiomyocyte biology, in contrast to previous reports suggesting a role for BRD4 in promoting pathological cardiac remodeling.

BRD4 and Transcriptional Control

BRD4 is known to play an integral role in the formation of superenhancers, clusters of enhancers with dense enrichment of transcriptional machinery and activity. A comprehensive characterization of superenhancers within multiple tissue types[19] revealed that many superenhancers are located at genomic loci harboring "cell identity" genes, genes that are highly expressed to enable specialized tissue function. Considering this, our findings that BRD4 is specifically enriched at mitochondrial and calcium handling gene loci, both of which are highly expressed and crucially important in cardiac muscle, are perfectly in line with the known biology. Surprisingly, whereas mitochondrial and excitation-contraction coupling genes were disrupted in BRD4 KO hearts, sarcomere genes, another set of highly expressed, myocyte-specific genes suggested to be regulated by superenhancers, were largely unaltered in BRD4 cKO mice even at late stages after deletion (data not shown). As expected in these failing hearts lacking BRD4, some remodeling-associated cytoskeletal genes were altered. Further analysis using chromatin-surveying techniques such as assay for transposase-accessible chromatin–sequencing and Hi-C seq is required to assess the impact of BRD4 disruption on chromatin topology and accessibility of these genomic regions.

Gene Defects in DCM

Many genetic defects have been linked to nonischemic DCM. Genetic disruptions of the sarcomere, excitation-contraction coupling, mitochondria, nuclear integrity, and cardiac TFs are major factors contributing to DCM pathogenesis.[20] The present study reveals the epigenetic linker BRD4 as a previously unrecognized, potential element in DCM. Mouse models targeting proteins known to interact with BRD4 on chromatin—Mediator components (eg, CDK8, MED1, MED12) and key cardiac TFs (eg, GATA4, ESRRα/β)—exhibit a phenotype similar to BRD4 cKO: development of systolic dysfunction at a young age that progresses to DCM-like pathology.[21–25] The role of BRD4 in bridging TFs to transcriptional machinery such as the Mediator complex on chromatin, coupled with the converging phenotypes of these mouse models, suggests that BRD4, Mediator, and key cardiac TFs function together to maintain cardiac muscle homeostasis, all taking place in addition to the previously reported role of BRD4 in cardiac remodeling.[4,5,8,26] A unique aspect for BRD4 among its transcriptional partners is that it is a druggable target, with BET BRD inhibitors being actively tested in both preclinical and clinical studies in various applications, including cancer. However, our study, revealing severe phenotypic consequences of BRD4 disruption in the heart, even in the setting of haploinsufficiency, raises concern about potential cardiotoxic side effects of long-term BET inhibitor exposure.

BRD4 and Mitochondria

Genetic disruption of cardiomyocyte BRD4 either in the developing heart (αMHC-Cre) or in the mature

adult heart (inducible Cre) triggered systolic dysfunction. However, the developmental and inducible deletion models differed in phenotype severity: Although both resulted in severe systolic dysfunction, αMHC-Cre cKO displayed DCM-like pathology with dilated ventricles, whereas the adult cKO displayed a heart failure phenotype with myocyte hypertrophy. In the early postnatal phase of development, cardiomyocyte mitochondrial biomass increases as myocytes grow, and metabolism shifts toward fatty acid oxidation. This burst of mitochondrial synthesis may account for the increased sensitivity to loss of BRD4 observed in αMHC-Cre cKO hearts. In contrast, loss of BRD4 in mature adult heart manifests a phenotype later, potentially as the natural turnover of mitochondria continues. In both models, BRD4 ablation led to marked downregulation of nucleus-encoded mitochondrial and metabolic gene transcription before any overt phenotypic manifestation. By the time a cardiac phenotype manifests, mitochondrial ETC and TCA cycle enzyme activities are impaired. In contrast, pressure overload–induced cardiac remodeling, a classic model of systolic HF, did not provoke changes in the mitochondrial transcriptome or mitochondrial function. The unique mitochondrial and metabolic signature of BRD4 KO hearts suggests that BRD4 KO–driven systolic dysfunction arises from inadequate energy provision.

The role of BRD4 in cell cycle control and differentiation has made it an attractive target for therapeutics in the treatment of cancer. For example, many hematopoietic cancers depend on high levels of BRD4 activity for expression of Myc.[27] A recent study[13] suggested that BRD4 may also govern mitochondrial fission in malignant cells to promote metabolism, supporting growth. In fact, many tumor cells are marked by elevated metabolic rates to meet, and even exceed, the bioenergetic and biosynthetic demands of continuous cell growth. Whereas cardiomyocytes are postmitotic, they naturally have high bioenergetic demand to support mechanical contraction. This common feature of high metabolic demand in tumors and the heart may be a shared mechanism of susceptibility to BRD4 targeting. On the other hand, a conflicting report suggested that, in human hybrid cells harboring a specific mitochondrial DNA mutation, BRD4 functioned as a negative regulator of nuclear mitochondrial transcription.[11] Our data suggest that BRD4 acts as a driver of mitochondrial dynamics and function, which may be linked to cardiomyocyte-specific superenhancer activity. A recent study[28] reported that potent BET inhibition with a second-generation BET inhibitor (I-BET-151) induced adverse cardiac side effects, including disrupted mitochondria. This report is in line with our findings that BRD4 activity is essential in driving mitochondrial homeostasis in the heart.

Comparisons With Pharmacological Suppression

Genetic targeting of BRD4 in cardiomyocytes resulted in an outcome distinct from effects observed with JQ1 inhibition. In previous studies, JQ1 administration in animals (once daily for 4 weeks) did not provoke baseline cardiac dysfunction and was protective in the context of pathological hypertrophy.[4] In our study, we also did not observe any effect of JQ1 on baseline cardiac function in the period we monitored (once daily injection for 2 weeks), yet we did observe changes in nucleus-encoded mitochondrial gene transcription in JQ1-treated hearts (Figure 3). In particular, BRD4-specific changes (shared gene set between JQ1 and cKO) were enriched in mitochondrial respiration genes, even though decreases observed in JQ1-treated hearts were blunted compared with cKO hearts. This may be a reflection of the nature of JQ1 as a competitive inhibitor that blocks the acetylation binding pocket in BRD4. It is possible that the multivalent contacts that occur among transcriptional components in superenhancer regions may afford some level of protection to this disruption. In addition, animals with BRD4 heterozygous deletion developed delayed heart failure (Figure III in the Data Supplement), suggesting that a decreased level of BRD4 protein is also toxic to the heart over an extended period of time. Considering the suboptimal pharmacokinetic properties of JQ1 in vivo, second-generation BET inhibitors with improved efficacy and pharmacokinetics will need to be monitored for changes in these gene products. Such studies will help us determine whether enhanced efficacy of BRD4 inhibition has the potential to trigger deleterious effects or point to a new possibility that BRD4 harbors critical bromodomain-independent functions in the heart.

Mammalian BRD4 is known to have splice variants. The long variant is the most extensively studied isoform with a unique C-terminal domain that enables interactions with transcriptional machinery, including pTEFB and Mediator.[29,30] The short variants lack this C-terminal domain; they are less well characterized but have been highlighted recently for their unique biology.[31] In the heart, nothing is known about BRD4 isoforms, yet JQ1 targets a common feature of all BRD4 isoforms. In addition to BRD4 isoforms, we must consider other BRD proteins, BRD2 and BRD3, when interpreting pharmacological effects because these proteins are also inhibited by JQ1 and other BET inhibitors. In fact, our RNA-seq data reveal that cardiomyocyte BRD4-specific changes account for only a quarter of the JQ1-altered transcriptome. Other BRD proteins have been identified with different epigenetic interactomes in various tissue contexts, but no work has been conducted to date in the heart. The present report marks a step toward delineating the precise roles of these epigenetic readers in cardiac biology.

TF Interactions

In addition to its role in enhancer regulation, BRD4 recruits and modifies transcriptional machinery at gene promoters, for example, the RNA Pol-II and pTEFb complexes for transcriptional pause-release and elongation.[32] In the present study, we uncovered candidate TFs that may work with BRD4 in controlling mitochondrial and calcium-handling gene transcription in the myocardium. Most of the top candidates identified—ESRR and PPAR nuclear receptors, NRF2 redox regulator, YY1, and GATA TFs—are known already to have important roles in cardiac development and physiology.[33–36] In validation of the top candidate ESRRα, we located TF-binding motifs in the promoters of cKO downregulated genes and showed with ChIP-qPCR that BRD4 does indeed bind the ESRR motif-containing regions on the target loci, and the loss of BRD4 leads to loss of ESRRα and Pol-II on the ERRE-containing promoter region. These data suggest that BRD4 directly affects TF binding, in addition to the previously described control of transcriptional pause-release.[4]

Conclusions and Study Limitations

Work reported here demonstrates that (1) BRD4 is a novel epigenetic regulator of myocardial energetics and DCM, (2) BRD4 governs nucleus-encoded mitochondrial transcription in cardiomyocytes, and (3) caution is warranted with BET inhibitor–based therapeutics, highlighting the importance of monitoring cardiac function in oncology trials using BRD4 inhibitors.

Our study has limitations. (1) Transcriptomic analysis and ChIP-qPCR experiments do not delineate changes in chromatin landscape. Chromatin landscaping techniques such as assay for transposase-accessible chromatin–sequencing or H3K27ac ChIP-Seq are required to test for alterations in promoter and enhancer occupancy and to probe for potential transcriptional partners of BRD4 in the heart. (2) Enzyme assays that we used measure maximum capacity of each complex rather than activity under physiological conditions. Metabolite analysis or radioactive pulse-chase studies would complement our data to assess metabolic changes in intact heart.

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Disclosures

None.

Supplemental Materials

Supplemental Methods
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