Increased COUP-TFII expression in adult hearts induces mitochondrial dysfunction resulting in heart failure

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Mitochondrial dysfunction and metabolic remodelling are pivotal in the development of cardiomyopathy. Here, we show that myocardial COUP-TFII overexpression causes heart failure in mice, suggesting a causal effect of elevated COUP-TFII levels on development of dilated cardiomyopathy. COUP-TFII represses genes critical for mitochondrial electron transport chain enzyme activity, oxidative stress detoxification and mitochondrial dynamics, resulting in increased levels of reactive oxygen species and lower rates of oxygen consumption in mitochondria. COUP-TFII also suppresses the metabolic regulator PGC-1 network and decreases the expression of key glucose and lipid utilization genes, leading to a reduction in both glucose and oleate oxidation in the hearts. These data suggest that COUP-TFII affects mitochondrial function, impairs metabolic remodelling and has a key role in dilated cardiomyopathy. Last, COUP-TFII haploinsufficiency attenuates the progression of cardiac dilation and improves survival in a calcineurin transgenic mouse model, indicating that COUP-TFII may serve as a therapeutic target for the treatment of dilated cardiomyopathy.
Heart failure is the leading cause of death in developed countries, and dilated cardiomyopathy (DCM) is the most common form of heart failure. The aetiology for the majority of non-ischaemic DCM remains unclear. Studies on human specimens and animal models suggest that impaired mitochondrial electron transport chain (ETC) reduces production of high-energy phosphates, leading to energy starvation of the cells. Although the mitochondrial ETC primarily produces ATP, it also generates reactive oxygen species (ROS) as part of a normal respiration process. A defective ETC has been linked to excessive production of ROS, which imposes oxidative stress in failing hearts by damaging mitochondrial DNA and proteins and triggering more ROS formation. In addition, mitochondrial dynamics also contribute to mitochondrial homeostasis in the hearts. Impairment of mitochondrial fusion by Mfn1/Mfn2 double knockout (DKO) results in mitochondrial fragmentation, respiratory dysfunction, leading to a rapid development of DCM.

Metabolic remodelling also emerges as a major player in pathogenesis of heart failure. We have proposed that metabolic remodelling precedes, initiates and sustains functional and structural remodelling. The PGC-1 regulatory network is known as the major network-modulating cardiac metabolism. This network comprises coregulators PGC-1α and PGC-1β that coactivate multiple nuclear receptors, including estrogen-related receptor (ERR)α, ERRy and peroxisome proliferator-activated receptor (PPAR)α, to control expression of genes essential for energy and mitochondrial homeostasis. Loss of key members in this regulatory network produces a range of metabolic defects, including heart failure, defective mitochondrial biogenesis and dynamics and maladaptation to cardiac stress in mice.

COUP-TFII (Nr2f2), a member of the nuclear receptor family, is highly expressed in the embryonic atria, whereas its expression in ventricular cardiomyocytes remains very low from embryo to adult. Under pathological conditions, the expression of COUP-TFII is elevated in the stressed ventricles of non-ischaemic cardiomyopathy patients and a pressure overload mouse model.

In the present study, we generated a mouse model by ectopically expressing COUP-TFII in adult cardiomyocytes to understand the role of COUP-TFII in the development of cardiomyopathy. Increased COUP-TFII levels alter expression of key mitochondrial and metabolic genes, enhance oxidative stress, disturb metabolic homeostasis and lead to DCM. On the other hand, reduced COUP-TFII expression partially mitigates calcineurin-induced cardiac dysfunction and improves survival of calcineurin transgenic mice. Our results reveal the causative role of COUP-TFII in the development of heart failure.

**Results**

**Increased COUP-TFII expression in stressed hearts.** When we reviewed available human DCM data sets, we found a significant increase in COUP-TFII expression levels (3.2-fold) in 13 myocardial tissues of end-stage non-ischaemic DCM (Fig. 1a). In a second cohort of patients, an average of 1.8-fold increase on COUP-TFII levels was also observed in the heart of 86 patients with idiopathic DCM (GSE5406). Results from these two independent cohorts of patients suggest an association between the ventricular COUP-TFII levels and DCM in human.

We found that in response to stress imposed by transaortic constriction (TAC), the expression of ventricular COUP-TFII mRNA was induced in mice (Supplementary Fig. 1a). This result is consistent with previous findings of increased COUP-TFII protein levels in this model. Similarly, ventricles of calcineurin transgenic mice (GrTg), known to develop hypertrophy and subsequent DCM, also exhibited an elevated expression of the COUP-TFII gene (Supplementary Fig. 1b). In addition, COUP-TFII protein levels were increased in isolated cardiomyocytes of CnTg mice (Supplementary Fig. 1c). Together, these results implicate a strong association of increased COUP-TFII expression with cardiomyopathy in mice and in humans.

**COUP-TFII induces DCM.** The potential link to cardiomyopathy prompted us to investigate whether increased COUP-TFII expression in mice might impact the development of contractile dysfunction. For this purpose, we crossed a previously established COUP-TFII overexpression allele CAG-S-COUP-TFII with a cre driver Myh6-MerCreMer (Myh6-MCM) line to overexpress COUP-TFII specifically in cardiomyocytes after heart development is complete, by administration of tamoxifen (Fig. 1b). Functionally, overexpressing COUP-TFII (OE, Myh6-MCM; CAG-S-COUP-TFII) in 2-month-old mice resulted in a greatly enlarged heart in comparison with the CTRL (Myh6-MCM) mice (Fig. 1c) 16 days after COUP-TFII transgene induction (D16). Echocardiography further revealed that OE mice exhibited characteristics of DCM, including increased left ventricular inner dimension (Fig. 1d) and relative wall thickness (RWT; Supplementary Fig. 1f, right panel). The progressive compromise of cardiac function resulted in increased mortality of OE mice after activation of COUP-TFII expression (Fig. 1f). Notably, day 16 OE hearts also had a 5.3-fold increase of COUP-TFII mRNA levels over CTRL (Supplementary Fig. 1g). By this time, the OE hearts exhibited severe dilation and contractile dysfunction analogous to end-stage DCM in human patients. The overexpressed COUP-TFII mRNA levels between human specimens (3.2X, Fig. 1a) and OE hearts (5.3X, Supplementary Fig. 1g) are not too far apart, suggesting that the mouse model is relevant. Most importantly, the activated COUP-TFII transgene increases COUP-TFII protein levels in cardiomyocytes (Supplementary Fig. 1d) to a similar extent as GrTg does (Supplementary Fig. 1c), which gives us confidence to use this model for dissecting the role of COUP-TFII in cardiomyopathy. In summary, our animal model shows that increased COUP-TFII expression in the heart is sufficient to induce DCM with a high mortality rate.

At the molecular level, OE ventricles exhibited decreased zMHC, increased βMHC and reduced SERCA2a gene expression (Supplementary Fig. 1h), similar to the molecular changes observed in human DCM patients and other mouse models. To gain insights on COUP-TFII’s effect in hearts, we identified the molecular profile of day 16 OE hearts through microarray analysis using ventricular compartments. Unbiased Gene Ontology analysis of the OE profile showed significant enrichment in the category of ‘DCM’ in the Kyoto Encyclopedia of Genes and Genomes (P = 2.2 × 10−8), further supporting the pivotal role of COUP-TFII in DCM. Moreover, using the OE profile as a transcriptomic signature of COUP-TFII activities, we found that the COUP-TFII signature is considerably higher in the DCM patients than the CTRL group (Fig. 1g). This finding suggests a stronger presence of COUP-TFII activities in DCM hearts as a result of higher levels of COUP-TFII expression (Fig. 1a). Collectively, our data reveal that the COUP-TFII signalling also exists in the human hearts and is positively correlated with a pathological profile of DCM.

Mechanistically, the Ingenuity Pathway analysis reveals ‘mitochondrial dysfunction’, ‘fatty acid β-oxidation’ and ‘glycolysis’ as the most prominent pathways affected by COUP-TFII overexpression in cardiomyocytes (Fig. 1h). This result suggests that COUP-TFII alters cardiac energy homeostasis via modulating expression of genes important for energy production, fuel utilization and mitochondrial function.
Impaired cardiac fuel utilization. The OE profile shows a significantly reduced expression of many genes that controls fuel utilization. Quantitative reverse transcription–PCR (qRT–PCR) analysis revealed that genes for uptake, binding, trafficking into the mitochondria and oxidation of fatty acids all exhibited a reduced expression in OE hearts as early as day 4 (Fig. 2a). These findings suggest that fatty acid utilization may be limited in the OE hearts. Indeed, the OE hearts exhibited a lower oleate oxidation rate in ex vivo (Fig. 2d), suggesting a defect in oxidative phosphorylation for energy production.

The PGC-1 network is critical in regulating genes essential for cardiac metabolism\(^{10,13}\). Because the expression of PGC-1\(\alpha\) is upregulated in the adipose tissue of COUP-TFII heterozygous mutant mice\(^{20}\), it is likely that PGC-1\(\alpha\) expression is also altered when COUP-TFII is overexpressed in the hearts. Indeed, PGC-1\(\alpha\) expression was reduced when COUP-TFII is overexpressed (Fig. 2a). Moreover, levels of other members of the PGC-1 network, including PGC-1\(\beta\), ERR\(\alpha\), ERR\(\gamma\) and PPAR\(\alpha\) are all reduced in cardiac tissues in the OE hearts (Fig. 2a). The notion that COUP-TFII suppresses the PGC-1 network is supported by the inverse relationship of COUP-TFII and PGC-1\(\alpha/\beta\)\(^{11}\), ERR\(\alpha\)\(^{21}\) and PPAR\(\alpha\)\(^{22}\) signatures (Supplementary Fig. 2). These results indicate that COUP-TFII is a major metabolic regulator that is central to control cardiac fuel metabolism.

Because PGC-1\(\alpha\) is a direct downstream target of COUP-TFII in adipocytes\(^{20}\), it is likely that the same regulatory mechanism also operates in the heart. Using chromatin immunoprecipitation (ChIP)-quantitative PCR (qPCR) assays, we found that COUP-TFII indeed binds to a previously identified intronic COUP-TFII-binding site in adipocytes (Fig. 2e)\(^{20}\). This result confirms that PGC-1\(\alpha\) is likely a direct target of COUP-TFII in the heart and that the mechanism of action by which COUP-TFII regulates PGC-1\(\alpha\) is conserved among various cell types. In addition to PGC-1\(\alpha\), COUP-TFII also directly binds to ERR\(\alpha\),
ERRγ and PPARα genes in ChIP assays (Fig. 2e). Collectively, our results indicate a direct regulatory role of COUP-TFII for these master metabolic regulators.

Development of mitochondrial dysfunction. We next examine whether mitochondria are dysfunctional in the OE hearts, based on downregulation of the expression of many genes that encode components in complexes I, II, III and IV of the mitochondrial ETC (Fig. 3a). Transcript analysis further confirmed suppression of ETC gene expression 9 days post induction of COUP-TFII expression when the OE hearts are at the early phase of heart failure (Supplementary Fig. 3a). The effect of altered ETC gene expression is manifested in the reduction of enzyme activities of complexes I, II, III and IV in isolated mitochondria of OE hearts (Fig. 3d). Control experiments carried out in a reduced condition on day 4 hearts. N = 3 for each genotypes. Diagram on the right depicts fuel utilization genes affected by COUP-TFII OE. Green marks downregulated genes and red labels upregulated genes in response to increased COUP-TFII expression. Expression of genes with the white background is not altered by COUP-TFII. *P<0.05; **P<0.01; ***P<0.001 (t-test) between CTRL and OE. Error bars denote the s.e.m.

Figure 2 | Impaired fuel utilization in COUP-TFII OE ventricles. (a) Relative mRNA levels by qRT-PCR in days 4, 9 and 16 ventricles. N=5 (CTRL) and 7 (OE). Colour-coded mRNA levels depict relative folds of OE over control hearts. (b–d) Measurement of oleate oxidation (b), glucose oxidation (c) and oxygen consumption (d) rates in isolated whole hearts at day 9 via Langendorff preparation. N = 5 (CTRL) and 7 (OE). (e) ChIP-qPCR analysis on day 4 hearts. N = 3 for each genotypes. Diagram on the right depicts fuel utilization genes affected by COUP-TFII OE. Green marks downregulated genes and red labels upregulated genes in response to increased COUP-TFII expression. Expression of genes with the white background is not altered by COUP-TFII. *P<0.05; **P<0.01; ***P<0.001 (t-test) between CTRL and OE. Error bars denote the s.e.m.

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To confirm that the observed alterations of the mitochondrial metabolism in the heart are caused by COUP-TFII overexpression and are not secondary to heart failure, we examine the expression of COUP-TFII target genes in earlier time point before onset of heart failure. Day 4 is likely the earliest time point for induction of COUP-TFII expression subsequent to 3-day treatment of tamoxifen before the exhibition of defects in the heart function. As anticipated, echocardiogram results indeed show no significant difference in ventricular chamber size, wall thickness and ejection fraction between CTRL and OE hearts (Supplementary Fig. 3e–g), indicating that day 4 OE hearts are structurally and functionally comparable to CTRLs. Results of qRT–PCR analyses on day 4 hearts reaffirm that suppression of gene expression by COUP-TFII precedes the development of...
Pink1 these genes. The reduced expression of indicating that COUP-TFII may directly control the expression of Mfn1 and Mfn2 were reduced in isolated cardiomyocytes of OE seen in the expression of fission genes Fis1 (Fig. 4b and Supplementary Fig. 4a), whereas no changes were for fusion and regeneration of healthy mitochondria Pink1 exhibit a roundish shape with reduced electron density and mitochondrial respiration rate is also lower in day 4 OE hearts than that of CTRLs (Supplementary Fig. 3g), corroborating the notion that COUP-TFII is likely to directly regulate the expression of these genes. Importantly, the mitochondrial respiration rate is also lower in day 4 OE hearts than that of CTRLs (Supplementary Fig. 3h), consistent with the COUP-TFII-dependent changes of gene expression. Taken together, our data demonstrate that regulation of mitochondrial gene expression and function by COUP-TFII occurs at the time when OE hearts are structurally and functionally similar to the CTRL hearts. These findings are indicative of the facts that dysregulation of mitochondrial function in the OE mice is a result of COUP-TFII signalling rather than a secondary consequence of heart failure.

Interestingly, we found that mitochondria of day 9 OE hearts exhibit a roundish shape with reduced electron density and increased distance between cristae (Fig. 4a), suggesting an accumulation of unhealthy mitochondria. Consistent with the morphological findings, we observed a reduction in the expression of key mitochondrial dynamics genes in OE hearts, including Pink1 for removal of damaged mitochondria and Opa1, Mfn1 and Mfn2 for fusion and regeneration of healthy mitochondria (Fig. 4b), whereas no changes were seen in Supplementary Fig. 4a), indicating that COUP-TFII may directly control the expression of these genes. The reduced expression of Pink1 and Mfn2, two of three major members of the mitochondrial quality control pathway, in the OE hearts may disrupt the mitochondrial dynamics and contribute to the accumulation of damaged mitochondria as seen in the electron micrograph (Fig. 4a). Collectively, these findings suggest that increased COUP-TFII perturbs mitochondrial homeostasis through suppressing the expression of mitochondrial dynamics genes.

Results from ChIP-qPCR assays further reveal in vivo binding of COUP-TFII on genomic loci of the mitochondrial dynamics genes Pink1, Opa1 and Mfn2 of adult hearts (Fig. 4c), suggesting that these genes are direct targets of COUP-TFII. Next, we used a conserved COUP-TFII-binding site of the Pink1 gene as an example to demonstrate direct regulation of Pink1 by COUP-TFII through these cis-acting elements. This COUP-TFII-binding site is located between 93 and 105 base pairs upstream of the transcription start site, within a stretch of highly conserved nucleotides between human and mouse. This site is surrounded by open chromatin as evident by the high DNaseI sensitivity (Fig. 4d). A stretch of conserved nucleotides from this region that contain the wild-type (Pink1_WT) or mutant (Pink1_mu) COUP-TFII-binding sites was cloned in front of a TATA-promoter-driven luciferase reporter for luciferase assay in C2C12 cells. The COUP-TFII-binding site shows reduced reporter activity compared with that of the mutant site (Fig. 4e), suggesting a repressive role in the regulation of Pink1 gene expression. Taken together, the results indicate that binding of COUP-TFII at genomic loci represses expression of mitochondrial dynamics genes.

The COUP-TFII regulatory network in human. The pivotal role of COUP-TFII in regulation of mitochondrial and metabolic
genes observed in mice, here, also occurs in humans, as supported by publicly available data from patient specimens (Fig. 5). COUP-TFII activity was scored and ranked in RNA profiles of 102 human ventricles (GSE5406) based on the mouse COUP-TFII gene signature (Fig. 5, top panel). A higher COUP-TFII signature indicates a stronger COUP-TFII activity in a sample. We first observed a positive and significant correlation between COUP-TFII levels and activities in these human samples (Fig. 5). This result supports the notion that the COUP-TFII regulatory network identified in mice also operates in the human heart. In addition, there is a positive correlation between COUP-TFII activity and levels of energy metabolic genes, including fatty acid catabolic genes FABP3, MLYCD, ACADVL, ACADM, ACADS and ACA2, glucose metabolic genes SLC2A4, PFKM, LDHB and PDK2, and major metabolic regulators ERRα, ERRγ and PPARα (Fig. 5). The compensatory effect of increasing expression of the glycolytic gene ENO1 is also observed in the human heart (Fig. 5), further supporting the presence of a conserved regulatory mechanism for cardiac metabolism. In summary, expression of energy metabolism genes is suppressed when COUP-TFII levels are elevated in the human hearts.

**COUP-TFII reduction attenuates disease progression.** The calcineurin transgenic mice develop DCM and expression of COUP-TFII is elevated in these mice (Supplementary Fig. 1b,c). We therefore utilized this model to test whether reducing COUP-TFII dosage ameliorates disease progression. The COUP-TFII floxed and Myh6-MerCreMer alleles were bred into the CnTg background to allow genetic removal of one copy of the COUP-TFII gene in cardiomyocytes in 7-week-old animals through tamoxifen-induced, cre recombinase-mediated gene excision (Fig. 6a). The majority of mice carrying the calcineurin transgene in a COUP-TFII*lox/+* background (CnTg; COUP-TFII*lox/+*, CnTg/F+) exhibit an accelerated rate of sudden death after 12 weeks of age, when compared with mice of CTRL groups (CnTg/F+) and Myh6-MerCreMer; COUP-TFII*lox/+* (Cre/F+) shown in Fig. 6b. Removing one copy of the COUP-TFII gene in cardiomyocytes in the calcineurin transgenic background (CnTg; Myh6-MerCreMer; COUP-TFII*lox/+*, CnTg/CyF+) substantially reduces COUP-TFII protein levels (Supplementary Fig. 5a) and leads to an increase in overall survival rate when compared with the CnTg/F+ group over a 20-week period.
negative correlation. Before the treatment (Supplementary Fig. 5b,c), consistent with the transgene already developed eccentric hypertrophy at 6 weeks, Red denotes positive correlation (Supplementary panel). Direction and statistical significance of correlation between genes of interest in individual samples corresponding to the order in the top manifestation of the signature. (Bottom panel) Heat map shows levels of transcriptional signature of COUP-TFII, with samples ordered based on samples.

Figure 5 | A COUP-TFII-dependent regulatory network in human heart samples. (Top panel) Expression array data of 86 failing and 16 non-failing human left ventricular myocardium from GSE5406, for genes in a transcriptional signature of COUP-TFII, with samples ordered based on manifestation of the signature. (Bottom panel) Heat map shows levels of genes of interest in individual samples corresponding to the order in the top panel. Direction and statistical significance of correlation between expression of genes of interest and COUP-TFII signature are noted at side. Red denotes positive correlation (P<0.05, Pearson’s) and blue denotes negative correlation.

This demonstrates that lowering COUP-TFII dosage prolongs survival under intense cardiac stress.

Echocardiography imaging before the removal of one copy of COUP-TFII (6 weeks old) and post-removal (12 weeks old) was performed to examine structural and functional alterations of the heart. The choice of the 12-week time point is based on previous observations that CnTg/F+ mice develop DCM, exhibiting a greatly increased inner chamber size (Fig. 6c) and reduced RWT (Fig. 6d), compared with CTRL groups of the same age or within the group at age of 6 weeks (Fig. 6c,d and Supplementary Fig. 5c). In contrast, removal of an allele of COUP-TFII in CnTg/Cre/F+ mice partially suppresses the dilation (Fig. 6c), and they maintain RWT levels similar to that of 6-week-old mice (Fig. 6d). This result suggests that hearts of calcineurin transgenic mice in which one copy of COUP-TFII is removed are still able to compensate for stress via a hypertrophic response rather than progressing towards DCM. However, removal of just one copy of COUP-TFII failed to rescue the declining contractility (Supplementary Fig. 5d). Taken together, our results indicate that reducing COUP-TFII dosage is able to improve survival rates in stressed hearts by partially attenuating the disease progression towards DCM.

Discussion

Hearts of DCM exhibit mitochondrial dysfunction4,27, which are proposed to increase oxidative stress, compromise high-energy phosphate production, and eventually lead to contractile dysfunction1. Normally, oxidative phosphorylation produces ROS as a by-product, which are removed by endogenous antioxidants in healthy mitochondria. However, dysfunctional mitochondria may generate excessive ROS to trigger and augment cellular oxidative stress, and lead to pathogenesis of heart failure6,28,29. In the present study, we found that adult hearts with elevated COUP-TFII levels produce excessive ROS in the mitochondria. The excessive ROS inflict damages on mitochondria, as evidenced by structural abnormality, decreased activities of oxidation-sensitive aconitase and reduced OCRs, eventually leading to contractility reduction and chamber dilation. Our findings are in line with previous studies that demonstrate the key role of excessive mitochondrial ROS production in the development of cardiac dysfunction. For example, increasing ROS levels by knocking out Sod2, a principal ROS scavenger located in mitochondrial matrix, leads to heart failure30. Conversely, normalizing mitochondrial ROS levels by administering synthetic Szeto-Schiller peptide SS-31 or mitochondrial targeted catalase in hearts ameliorates stress-induced cardiac dysfunction31,32. Thus, our study links increased COUP-TFII expression, as observed in a subset of DCM patients, to excessive ROS production in adult hearts.

Functioning as a transcription factor, COUP-TFII regulates multiple genes that control mitochondrial ROS levels. COUP-TFII represses expression of many ETC genes in both direct and indirect manners, resulting in decreased activities of ETC enzyme complexes. Modifications of ETC complexes have been shown to disturb electron transportation along the ETC and divert electrons to oxygen for excess ROS production6,33,34. Therefore, our findings suggest that COUP-TFII-mediated defects promote ROS production. At the same time, COUP-TFII overexpression also compromises the endogenous antioxidant capacity by directly repressing the Sod2 gene. Collectively, the increased oxidative stress observed in COUP-TFII overexpression hearts is likely the result of the combined effect of increased ROS production and decreased ROS detoxification.

Mitochondrial dynamics emerge as an important mechanism in maintaining normal cardiac function by revitalizing mitochondrial targeted catalase in hearts ameliorates stress-induced cardiac dysfunction31,32. Thus, our study links increased COUP-TFII expression, as observed in a subset of DCM patients, to excessive ROS production in adult hearts.

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Normal human specimens, in which COUP-TFII activities inversely regulatory mechanisms find further support in the analyses of for oxidative phosphorylation. These COUP-TFII-dependent of regulating mechanisms to control the essential machinery ETC genes, indicating that COUP-TFII utilizes two layers show that COUP-TFII can directly modulate expression of key place COUP-TFII upstream of this network. Importantly, we now

ROS production and cardiomyopathy24,38,39. Thus, the reduced or indicated time points. (a) Kaplan–Meier curve of mice with denoted genotypes over a 20-week observation period. (c) Left ventricular interior dimension at diastole at indicated time points. (d) Left ventricle relative wall thickness. Genotypes are denoted at bottom. N = 10 (F⁺), 10 (Cre/F⁺), 5 (CnTg/F⁺) and 11 (CnTg/Cr/Cr/F⁺). *P < 0.05; NS, P > 0.05 (t-test) between denoted groups. Error bars denote the s.e.m.

Figure 6 | COUP-TFII haploinsufficiency partially rescues calcineurin transgenic mice. (a) Experimental design. Echo, echocardiography.

(b) Kaplan–Meier curve of mice with denoted genotypes over a 20-week observation period. (c) Left ventricular interior dimension at diastole at indicated time points. (d) Left ventricle relative wall thickness. Genotypes are denoted at bottom. N = 10 (F⁺), 10 (Cre/F⁺), 5 (CnTg/F⁺) and 11 (CnTg/Cr/Cr/F⁺). *P < 0.05; NS, P > 0.05 (t-test) between denoted groups. Error bars denote the s.e.m.

phenotype. The reduced expression of mitochondrial inner membrane fusion gene Opal in the OE hearts could be part of the explanation, as Opal deficiency confers a mitochondrial dysfunction phenotype as strong as the combined deficiency of Mfn1 and Mfn2 in MEF cells36. In addition, increased COUP-TFII may impair mitochondrial quality control that depends on the Pink1-Mfn2 pathway24,37. Previous studies show that loss of Pink1 or Mfn2 results in mitochondrial dysfunction, increased ROS production and cardiomyopathy24,38,39. Thus, the reduced levels of Pink1 and Mfn2 in the OE hearts might lead to pathological accumulation of damaged mitochondria that further contribute to the increase of ROS production.

Transcriptional regulation of mitochondrial ETC genes by the PGC-1 network has been previously reported10–13 and our results place COUP-TFII upstream of this network. Importantly, we now show that COUP-TFII can directly modulate expression of key ETC genes, indicating that COUP-TFII utilizes two layers of regulating mechanisms to control the essential machinery for oxidative phosphorylation. These COUP-TFII-dependent regulatory mechanisms find further support in the analyses of human specimens, in which COUP-TFII activities inversely correlate with mRNA levels of multiple ETC genes as well as ERRα, ERRγ and PPARα, whereas failing hearts exhibit higher COUP-TFII and lower PGC-1α/ERR activities40. Notably, PGC-1α has been shown to serve as an upstream regulator in promoting Sod2 expression41, and is negatively regulated by COUP-TFII in the hearts. These findings suggest that COUP-TFII may also compromise the ROS detoxifying system through suppressing the PGC-1α-Sod2 pathway.

Impaired cardiac energy homeostasis is a salient feature of heart failure42. In response to a variety of stresses, hearts switch fuel substrate preference from fatty acids to carbohydrates and its proposed mechanism is alteration of expression of cardiac metabolic genes43,44. Emerging evidence reveals that failing hearts suffer impairment in this substrate selection flexibility14,45–47. In our mouse model, increased COUP-TFII reduced fatty acid oxidation and reduced glucose usage. This phenotype may be attributed to repressed expression of the PGC-1 network genes and key glucose utilization genes Glut4, HK2 and Pfkmby COUP-TFII. Collectively, our COUP-TFII OE mice exhibit a cardiac metabolic phenotype that resembles loss of flexibility of substrate selection in advanced DCM. We propose that alteration of expression of key genes for both fatty acid and glucose metabolism by COUP-TFII underlies the development of the metabolic inflexibility.

In summary, our data indicate that overexpression of COUP-TFII in hearts results in defects in ETC, accumulation of damaged mitochondria and reduced mitochondrial ROS scavenging capacity. All these defects result in the accumulation of oxidative stress and eventually lead to heart failure. Importantly, alterations of gene expression are already observed at day 4 when OE and CTRL hearts are structurally and functionally similar to each other. Therefore, the observable phenotypes are due to COUP-TFII, not secondary to heart failure.

Methods

Animals. Mice carried CAG-S-COUP-TFII allele and COUP-TFII flox allele were described previously14,48. The cardiomyocyte-specific, tamoxifen inducible cre driver Myh6-MerCreMer (Myh6-MCM)39 and calcineurin transgenic mice Tg(Myh6-Ppp3Ca)25 were acquired from the Jackson Laboratory (Stock number 009075). All animals used here are male and are between 2 and 4 months old unless otherwise indicated. All animal experiments adhered to the Guidelines of the Institutional Animal Care and Use Committee of the Baylor College of Medicine and were conducted within the scope of approved animal protocols.

qRT-PCR. Reverse transcription reactions were carried out using the Transcriptor First Strand cDNA Synthesis kit (Roche, 04379012001) to make cDNA according
to the manufacturer's guide. Quantitative PCR (qPCR) analysis was performed on Applied Biosystems StepOnePlus using the FastStart Universal SYBR Green Master Mix (Roche, 4913859001) or the Taqman Universal Master Mix II (Invitrogen, 4440040). Each cycle of the qPCR consists of 95 °C, 15 s, for denaturing and 60 °C, 60 s, for annealing and extension with total 40 cycles performed. Primer sequences are included in Supplementary Table 1.

**Antibodies.** Anti-COUP-TFII antibodies were purchased from Perseus Proteomics (H7147, 1:2,000 dilution) or from Cell Signaling (#4634, 1:1,000 dilution). The Pimp1 kinase is from Abcam (ab23707, 1:1,000 dilution). The Mfn1 antibody is from from Abcam (ab56889, 1:500 dilution). The Hsp60 antibody is from BD Transduction (#611563, 1:5,000 dilution).

**Cell line.** C2C12 (ATCC CRL-1772) are acquired from American Type Culture Collection.

**Expression array analysis.** Whole ventricles were collected from 2-month-old animals comprising three Myh6-MCM and three Myh6-MCM; CAG-S-COUP-TFII mice. RNA extraction was performed using Trizol and Qiagen RNeasy according to the manufacturer's guides. Affymetrix mouse genome 430 2.0 chips were used in this study. The BCM Genomic and RNA Profiling Core Lab performed the probe generation and subsequent microarray hybridization on Affymetrix mouse genome 430 2.0 chips. Acquired raw data were first processed by the GeneSpring GX software with summarization of probe intensities, Quantile normalization and median of all samples for baseline transformation. The resulting entity list was filtered on expression (50.0–35272.484) in raw data followed by statistical analysis using one-way analysis of variance with corrected P value cutoff at 0.05, asymptotic for P value computation and Benjamini–Hochberg for multiple testing correction. Calculated against the CTRL atria group, entities from the mutant atria and CTRL ventricles having absolute fold change greater than 1.2 were considered differentially expressed. The entity lists were subsequently annotated using Affymetrix annotation and analysed by Ingenuity Pathways Analysis (Ingenuity Systems). The NCBI accession number for the expression array study reported in this paper is GSE63759.

The publicly available human array data sets GSE1869 and GSE4506 were scored for manifestation of the mouse model-derived COUP-TFII gene signature, using published methods. A gene signature score (also known as a '1-score') was defined for each external profile as the two-sided t-statistic comparing, within the profile, the average of the COUP-TFII-induced genes with the average of the COUP-TFII-repressed genes. In this way, the t-score contrasted the patterns of the 'COUP-TFII-induced' genes against those of the 'COUP-TFII-repressed' genes, could be used to derive a single value denoting coordinate expression of the two gene sets. Where multiple probes in GSE4506 referred to the same gene, the probes with the same name were taken to represent the gene genes were then centred to standard deviations from the mean across the sample profiles. Expression patterns were visualized as heat maps using Java TreeView. We analysed data from non-ischaemic failing hearts primarily because of the scope of the present study. For GSE1869, we used data from 6 'nonfailing' and 13 'Nonischemic, Pre-LVAD Patient group' hearts for analysis. We exclude the 'Nonischemic, No-LVAD Patient group' because the expression profile of this group exhibits similar pattern with the nonfailing group. For GSE4506, we used data from 16 'human nonfailing LV myocardium' samples and 86 'human failing LV myocardium, Idiopathic' specimens for analysis.

**Mitochondrial respiration assay.** XF24 extracellular flux analyzer from Seahorse Biosciences was used to measure the rates of oxygen consumption. Mitochondria were isolated from appropriate tissue by homogenization in mitochondrial isolation buffer using a Dounce homogenizer ( Kontes) filtered through cheesecloth, and centrifuged at 1,500 g, then 9,000 g. XF24 cartridge was equilibrated with the calibration solution overnight at 37 °C. XF assay buffer (70 mM sucrose, 220 mM mannitol, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA, 0.2% BSA, 10 mM pyruvate and 5 mM malate) was prepared and pH adjusted to 7.2 on the day of the experiment. XF assay buffer was used to prepare mitochondrial assay reagents, 2.5 mM ADP, 2 μM oligomycin, 4 μM FCCP, 4 μM antimycin (final concentration). All the reagents were loaded in the ports as suggested by Seahorse Biosciences. OCRs were measured for 4 min with 30 s of mixing and expressed as pmol min⁻¹.

**ETC activity assay.** For enzymatic assays of respiratory chain complexes I–IV, sufficient amount of tissue or cells were collected. Potassium phosphate buffer (25 mM, pH 7.5) was added to a final volume of 300 μl and the samples were sonicated (5 x pulse at 60% power) using a Microson XL2000 Ultrasonic Cell Disruptor (Misonix). For experiments on isolated mitochondria, the mitochondria were also disrupted by sonication as described above. The spectrophotometric kinetic assays were performed at 30 °C in a volume of 175 μl using a monochromator microplate reader (Tecan M200). Complex I activity (NADH:ubiquinone oxidoreductase) was determined by measuring oxidation of NADH at 340 nm (using ferricyanide as the electron acceptor) in a reaction mixture of 25 mM potassium phosphate (pH 7.5), 0.2 mM NADH and 1.7 mM pyridine nucleotide (cytochrome c oxidase reductase, 10 μM cytochrome c at 550 nm in a reaction mixture of 25 mM potassium phosphate (pH 7.5), 20 mM succinate, 0.5 mM 2,6-dichlorophenol-indophenol, 10 μM rotenone, 2 μg ml⁻¹ antimycin A and 2 μM potassium cyanide. Complex IV activity (cytochrome c oxidase) was determined by measuring the reduction of cytochrome c at 550 nm in a reaction mixture of 25 mM potassium phosphate (pH 7.5), 20 μM reduced decylubiquinone, 15 μM cytochrome c, 10 μM rotenone and 2 mM potassium cyanide. Complex IV activity (cytochrome c oxidase) was determined by measuring the oxidation of cytochrome c at 550 nm in a reaction mixture of 10 mM potassium phosphate (pH 7.5) and 0.1 mM reduced cytochrome c. Citrate synthase activity was determined by measuring the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) at 412 nm, which is coupled to the reduction of acetyl-CoA by citrate synthase, in the presence of oxaloacetate. The reaction mixture consists of 10 mM potassium phosphate (pH 7.5), 100 μM 5,5'-dithiobis(2-nitrobenzoic acid), 50 μM acetyl-CoA and 250 μM oxaloacetate. All activities were calculated as nmol min⁻¹ per mg protein, and expressed as a percentage of control activity. Experiments were performed on six independent samples for each genotype.

**Aconitase assay.** The activity of mitochondrial aconitase was measured on the basis of conversion of citrate into α-ketoglutarate coupled with NADP reduction of the total protein. Activity was measured in the native state and after ‘reactivation’ by incubating mitochondria in ferrorosum ammonium sulfate for 5 min before performing the assay.

**Echocardiogram.** For transthoracic echocardiography, mice were anesthetized with 2% isoflurane in 95% O2. Body temperature was maintained on a heated platform, and electrocardiogram and temperature were continuously monitored. Cardiac function was assessed using a VisualSonics VeVo 770 Imaging System (VisualSonics) equipped with high-frequency 30 MHz probe.

**Transaortic constriction.** The TAC surgery was performed as described on adult CD1 mice (strain code: 022, Charles River) of 8–10 weeks of age and between 20 and 25 g of weight. Mice were anesthetized with isoflurane (2–3%, inhalation) in an induction chamber and then intubated with a 20-G intravenous catheter and ventilated with a mouse ventilator (Minivent, Harvard Apparatus, Inc.). Anaesthesia was maintained with inhaled isoflurane (1–2%). A longitudinal 5-mm incision of the skin was made with scissors at midline of sternum. The chest cavity was opened by a small incision at the level of the second intercostal space 2–3 mm from the left sternal border. While opening the chest wall, the chest retractor was gently inserted to spread the wound 4–5 mm in width. The transverse portion of the aorta was bluntly dissected with curved forceps. Then, 6-0 silk was brought underneath the transverse aorta between the left common carotid artery and the brachiocephalic trunk. One 26-G needle was placed directly above and parallel to the aorta. The loop was then tied around the aorta and needle, and secured with a second knot. The needle was immediately removed to create a lumen with a fixed stenotic diameter. The chest cavity was closed by 0-0 silk suture. Sham-operated mice underwent similar surgical procedures, including isolation of the aorta, looping of aorta, but without tying of the suture. The pressure load caused by TAC was verified by the pressure gradient across the aortic constriction measured by echocardiography. Only mice with a pressure gradient > 15 mmHg were analysed for cardiac hypertrophy, echocardiography and gene analysis.

**Ex vivo cardiac metabolic analyses.** Mice were anaesthetized with chloral hydrate (400 mg kg⁻¹) and anaesthetized with heparin (50UI, i.v.). Hearts were rapidly excised, arrested in ice-cold Krebs–Henseleit buffer, and mounted on a Langendorff apparatus. Hearts were then perfused retrogradely at an afterload of 80 cm H2O with non-recirculating Krebs–Henseleit buffer containing glucose (5 mmol l⁻¹), sodium oleate (0.4 mmol l⁻¹) bound to bovine serum albumin (10g l⁻¹), EMD Millipore), NaH14CO3 (0.2 μCi ml⁻¹, solubilized in Krebs buffer (0.1 μCi ml⁻¹) and insulin (40 μU ml⁻¹; Eli Lilly and Company). The Krebs–Henseleit buffer was equilibrated with 95% O2–5% CO2 and maintained at a constant temperature of 37 °C. After an initial 30 min stabilization, rates of oleate and glucose oxidation were determined by quantitative collection of [14C]H2O and [14C]CO2, from the coronary effluent as previously reported. Myocardial oxygen consumption (MVO2; μmol min⁻¹) was calculated by measuring the arterio-venous O2 content difference with an YSI 5300A biological oxygen monitor.

**ChIP assay.** ChIP analysis of fresh cardiac tissues was performed according to a protocol published in the Nature Protocols. Chromatin DNA complexes were collected from mouse ventricles. The mouse monoclonal anti-FLAG antibody M2 (Sigma F1804) was used for the ChIP assay. DNA released from the precipitate was amplified using qPCR assay according to the identification of the ChIP-enriched DNA. qPCR analysis was performed on Applied Biosystems StepOnePlus using the FastStart Universal SYBR Green Master Mix (Roche, 4913852001). Each cycle of
the qPCR consists of 95 °C, 15 s for denaturing and 60 °C, 60 s, for annealing and extension performed with total 40 cycles. Primer sequences are included in Supplementary Table 1.

Oxyblot assay. The Oxyblot assay was performed on freshly isolated mitochondria from ventricles by using the Oxyblot protein oxidation kit from Millipore (S7150) according to the manufacturer’s guide.

Measurement of ATP content. ATP contents of isolated mitochondria were assessed by the ATPFluor kit from Perkin Elmer (6016943) according to the manufacturer’s instruction, and normalized with protein contents.

Statistical tests. Two-tailed t-test is used for statistical analyses unless otherwise indicated.

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Author contributions
S.P.W., M.J.T. and S.Y.T. designed the experiments. S.P.W. and C.Y.K. performed the experiments and data analysis. S.P.W., M.J.T. and S.Y.T. wrote the paper. C.J.C. performed the bioinformatics analysis. All other authors carried out or supervised various aspects of experimental data collection.

Additional information
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