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Perturbation of NCOA6 Leads to Dilated Cardiomyopathy

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SUMMARY

Dilated cardiomyopathy (DCM) is a progressive heart disease characterized by left ventricular dilation and contractile dysfunction. Although many candidate genes have been identified with mouse models, few of them have been shown to be associated with DCM in humans. Germline depletion of *Ncoa6*, a nuclear hormone receptor coactivator, leads to embryonic lethality and heart defects. However, it is unclear whether *Ncoa6* mutations cause heart diseases in adults. Here, we report that two independent mouse models of NCOA6 dysfunction develop severe DCM with impaired mitochondrial function and reduced activity of peroxisome proliferator-activated receptor δ (PPARδ), an NCOA6 target critical for normal heart function. Sequencing of NCOA6-coding regions revealed three independent nonsynonymous mutations present in 5 of 50 (10%) patients with idiopathic DCM (iDCM). These data suggest that malfunction of NCOA6 can cause DCM in humans.

INTRODUCTION

Dilated cardiomyopathy (DCM), characterized by cardiac enlargement and systolic dysfunction, is the most common form of cardiomyopathy, accounting for up to 30%–40% of all heart failure cases (Towbin and Bowles, 2002). Although DCM has diverse causes, approximately half of cases are idiopathic (iDCM). Clinical investigations have shown that approximately 40% of patients with iDCM exhibit the patterns of autosomal-dominant inheritance, implying that genes play an important role in DCM pathogenesis (Hershberger et al., 2010). Hence, the search for novel susceptibility loci is a major challenge in DCM research.

Nuclear receptor coactivator 6 (or NCOA6, also known as ASC-2, NRC, TRBP, PRIP, and RAP250) is ubiquitously expressed in diverse tissues including the heart and stimulates the transcriptional activity of various transcription factors and nuclear hormone receptors (NRs) by coordinating with cofactors (Mahajan and Samuels, 2008). NCOA6 possesses two LXXLL motifs required for interaction with NRs. The first LXXLL motif of NCOA6 can interact with almost all liganded NRs, whereas more restricted NRs can bind to the second LXXLL motif. In mice, germline mutation of *Ncoa6* induces growth retardation and developmental defects in the heart, liver, brain, and placenta, resulting in embryonic lethality between embryonic days 8.5 (E8.5) and 12.5 (Antonson et al., 2003; Kuang et al., 2002; Mahajan et al., 2004; Zhu et al., 2003). Overexpression of the dominant-negative version of the gene (*DN1*) incompletely suppresses endogenous NCOA6 activity, allowing mice to survive, but to develop several defects including cataract, atrial thrombosis, and hypertrophy at the later stage (Kim et al., 2002).

Peroxisome proliferator-activated receptors (PPARs) are the members of the NR superfamily that regulate fatty acid metabolism and mitochondrial function (Barger and Kelly, 2000; Wang et al., 2010). The transcriptional activity of *PPARs* is elevated or induced upon ligand binding and is regulated further by coactivators and corepressors (Guan et al., 2005; Viswakarma et al., 2010). PPARδ (also called PPARβ) is expressed at high levels in the heart and is essential for normal heart function (Cheng et al., 2004). In a mouse model, cardiomyocyte-specific

PPARδ knockout leads to DCM; however, whether an association between *PPARδ* polymorphisms and DCM exists in humans remains unknown.

RESULTS

Overexpression of *DN1* Causes DCM in Mice

Germline *Ncoa6* deficiency results in embryonic lethality with several developmental defects (Antonson et al., 2003; Kuang et al., 2002; Mahajan et al., 2004; Zhu et al., 2003). We produced transgenic (Tg) mouse lines overexpressing DN1 (849–929 residues containing an N-terminal LXXLL-1 motif of NCOA6, Figures S1A and S1B) that are able to reach adulthood, although these animals are prone to diverse defects (Kim et al., 2002). Among nine DN1-Tg lines, four founder mice (#71, #84, #87, and #104) expressed DN1 in their hearts (representative expression of DN1 is shown for founder #87 in Figure S1C). The progenies of founder #87 died prematurely at 20 weeks old (Figure S1D). Anatomical examination revealed cardiac enlargement and increased heart-to-body weight ratios with frequent pericardial effusion (Figures S1E–S1H). The progenies of founders #71, #84, and #104 exhibited similar phenotypes, which were absent in wild-type (WT) and mutant DN1-Tg mice in which the LXXLL-1 motif had been mutated to LXXAA (Kim et al., 2002). These phenotypes closely resembled those of DCM (Towbin and Bowles, 2002). Given that DN1 would be expected to compete with the binding of other coactivators for liganded NRs and multiorgan defects were found in DN1-Tg mice, it is unclear whether DN1 specifically blocks the activity of endogenous NCOA6 and causes DCM through cardiac cells only. For instance, we found multiple phenotypes such as fatty liver, thymic and spleen atrophy, lung hypoplasia, kidney hyperplasia, and brain defects in DN1-Tg mice (Kim et al., 2002).

Cardiomyocyte-Specific Ablation of NCOA6 Leads to DCM in Mice

To confirm the heart-specific function of NCOA6, we generated cardiomyocyte-specific *Ncoa6*-deficient (Δ/Δ) mice by employing floxed *Ncoa6* alleles (f/f) and a Cre recombinase transgene specific for differentiated cardiomyocytes under the control of the α-myosin heavy-chain promoter (α-*MHC-Cre*-Tg) (Agah et al., 1997; Zhu et al., 2003). Compared to control mice (f/f), NCOA6 mRNA and protein levels were significantly decreased in a heart-restrictive manner in Δ/Δ mice (Figures 1A and 1B). These mice demonstrated Mendelian inheritance and showed no significant cardiac developmental defects. Consistent with the phenotypes observed in DN1-Tg mice, Δ/Δ mice showed premature death, increased heart weight-to-body weight ratios, and cardiac dilatation (Figures 1C, 1D, and S2A–S2D). The increased heart weight was caused by atrial thrombi, whereas there was no alteration in the weight of the ventricle (Figure S2C). Histological examination of the hearts of Δ/Δ mice revealed common features of DCM, including enlarged ventricular cavities with thin walls and reactive myocardial fibrosis (Figures 1D, 1E, and S2D). Notably, *Ncoa6* heterozygous knockout mice (Δ/+) also exhibited the characteristics of DCM with late onset (Figures S2E–S2I), indicating that *Ncoa6* haploinsufficiency is sufficient to precipitate DCM in mice. Consistent with the previous reports by Agah et al. (1997) and Cheng et al. (2004), we could not find any significant differences of cardiac functions in α-*MHC-Cre*-Tg mice, compared to the f/f mice (data not shown). These results demonstrate that loss or insufficiency of NCOA6 function directly causes DCM.

Comparative transthoracic echocardiographic analysis revealed severe dilatation of the left ventricle (LV) and decreased contractility without a significant alteration in heart rate in Δ/Δ mice at 4 months of age (Figure 1F). Left ventricular end-systolic dimensions (LVESDs) and end-diastolic dimensions were enlarged 1.72- and 2.35-fold, respectively, compared to control mice (Figure 1G). Ejection fraction (EF) and fractional shortening, two direct measures of cardiac contractile function, were profoundly diminished compared to control mice (Figures 1H and S2J). Impaired cardiac function was also evident in Δ/+ and DN1-Tg mice (Figures S1I–S2L). Increased expression of cardiac hypertrophy markers, including atrial natriuretic peptide (*Anp*) and α- to β-*MHC* isoform switching (Reiser et al., 2001), were found in 12- and 20-week-old, but not 4-week-old, Δ/Δ mice (Figures S2M–S2O). Furthermore, the hearts of 12-week-old mice displayed hypertrophic phenotype (Figure S2P), suggesting that concentric hypertrophy transiently proceeds prior to the onset of DCM phenotype. Thus, *Ncoa6* defects progressively compromise overall cardiac function in mice.

Impaired Cardiac Ultrastructure and Mitochondrial Function in *NCOA6*-Deficient Mice

To examine potential mechanisms in the development of DCM, ultrastructural examination of cardiac tissues with transmission electron microscopy (TEM) was conducted and showed disarray of sarcomeres and mitochondria in the hearts of Δ/Δ mice (Figure 2A). The number of mitochondria was significantly decreased in the hearts of Δ/Δ mice (Figure 2B). In addition, reduced mitochondrial content was confirmed by measuring DNA, RNA, and respiratory chain protein levels, and impaired activity of mitochondrial complex II was evident in Δ/Δ and Δ/+ mice (Figures 2C–2F). These results suggest that NCOA6 is essential for the maintenance of normal mitochondrial function in cardiomyocytes.

NCOA6 Deficiency Impairs PPARδ Activity in Cardiomyocytes

Because PPARδ, one of the major targets of NCOA6, is crucial for mitochondrial biogenesis and cardiomyocyte-specific knockout of *Pparδ* causes spontaneous DCM in mice (Cheng et al., 2004; Wang et al., 2010), we hypothesized that depletion of NCOA6 alters PPARδ activity. To further explore the role of NCOA6 in PPARδ-mediated transactivation, we measured transcript levels of PPARδ targets in the mouse heart. Levels of key molecules involved in fatty acid oxidation (Cheng et al., 2004), such as *muscle-type carnitine palmitoyltransferase-1* (*Mcpt-1*), *Cd36*, *Fatp1*, *pyruvate dehydrogenase lipoamide kinase, isozyme 4* (*Pdk4*), and *uncoupling protein 3* (*Ucp3*), were considerably decreased even though no significant difference in *Pparδ* transcript levels was observed between Δ/Δ and f/f hearts (Figures 3A–3C). Similar to cardiomyocyte-specific knockout of *Pparδ* (Cheng et al., 2004), Δ/Δ mouse showed cardiac lipid accumulation (Figure S3A). Collectively, these data suggest

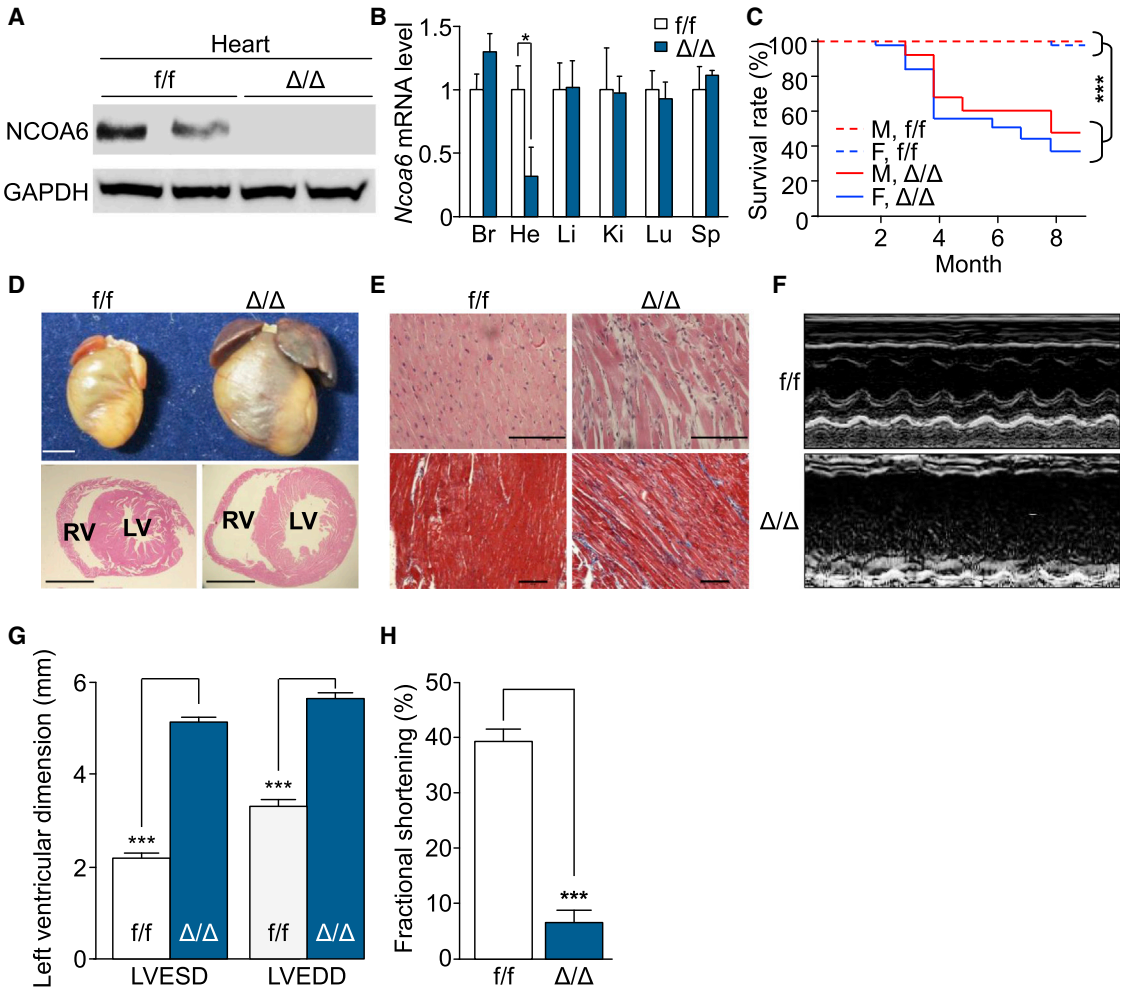

Figure 1. Premature Death and Impaired Cardiac Function in Cardiomyocyte-Specific *Ncoa6* Knockout Mice

(A) Western blot analysis of NCOA6 in the hearts of 1-month-old f/f and Δ/Δ male mice. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

(B) Real-time quantitative PCR analysis of *Ncoa6* mRNA in various organs of 1-month-old f/f and Δ/Δ male mice. Br, brain; He, heart; Li, liver; Ki, kidney; Lu, lung; Sp, spleen. *Ncoa6* mRNA levels were normalized to those of *Gapdh*. Graphs show mean ± SD. *$p < 0.05$.

(C) Kaplan-Meier survival curves of f/f (male, n = 34; female, n = 34) and Δ/Δ (male, n = 21; female, n = 39) mice. Genders and genotypes are labeled inside the plot. M, male; F, female. ***$p < 0.001$.

(D) Gross morphology (top) and histological examinations (bottom; hematoxylin and eosin staining [H&E]) of hearts from 4-month-old female f/f and Δ/Δ mice. RV, right ventricle. Scale bars, 2.5 mm.

(E) H&E (top) and Masson's trichrome staining (bottom) of LVs from 4-month-old female mice. Scale bars, 100 μm.

(F–H) Representative profiles of M-mode echocardiographic analyses (F) and quantitative representations of LVESD and left ventricular end-diastolic dimension (LVEDD) (G) and percent fractional shortening (H) of f/f (male, n = 3; female, n = 4) and Δ/Δ (male, n = 3; female, n = 3) mice. Graphs show mean ± SD. ***$p < 0.001$. See also Figures S1 and S2.

that *NCOA6* deficiency results in dysfunctional activity of PPARδ and fatty acid metabolism. Interestingly, increased expressions of estrogen receptor alpha (ERα) and its target genes were found in the Δ/Δ mouse hearts (Figures S3B–S3D).

We also found that Cre-mediated *Ncoa6* deficiency attenuated the induction of PPARδ targets by the selective PPARδ agonist GW501516 (GW) in neonatal primary cardiomyocytes (Figures 3D–3G). In addition, in the AC16 human cardiomyocyte cell line, small hairpin RNA (shRNA)-mediated NCOA6 knockdown (Figures 3H and 3I) reduced GW-induced activation of PPARδ and its target genes (Figures 3J–3M), indicating that PPARδ requires NCOA6 for optimal transactivation of its target genes.

Nonsynonymous Mutations of *NCOA6* in Patients with iDCM

To find the relevance of NCOA6-mediated DCM pathogenesis to human iDCM, we screened the entire *NCOA6*-coding sequence in patients with iDCM and identified four nonsynonymous substitutions in 8 out of 50 patients with iDCM (c.1038C > G, P239R;

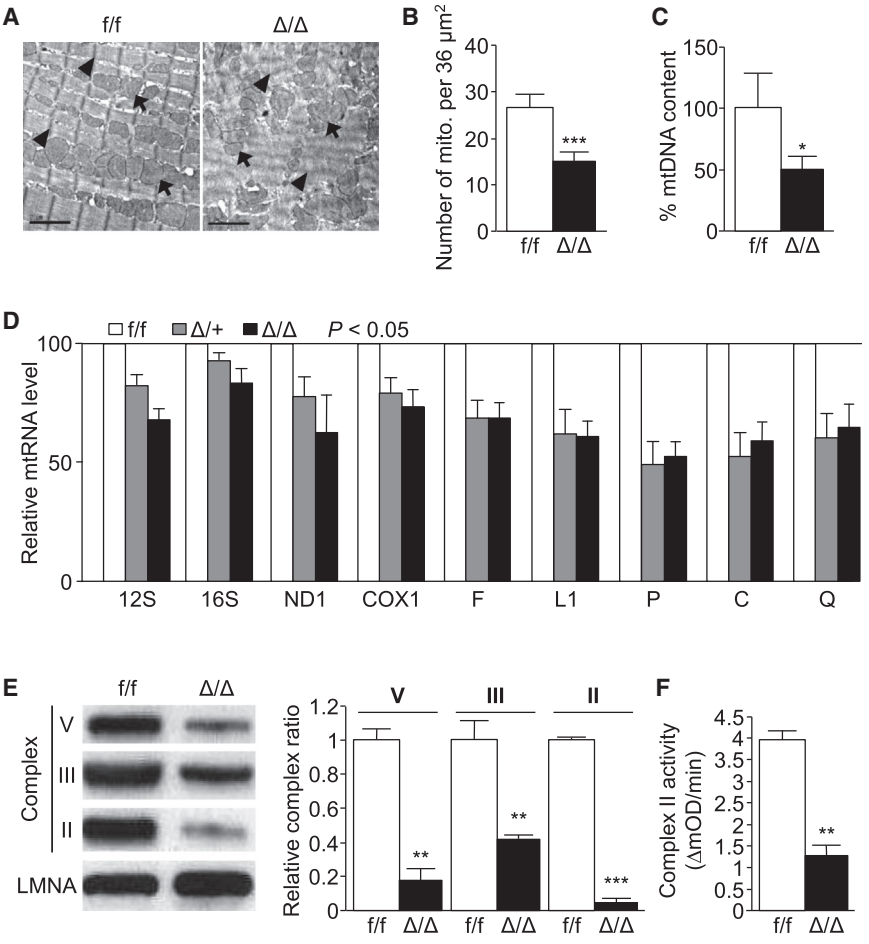

Figure 2. Cardiomyocyte-Specific *Ncoa6* Deficiency Impairs Cardiac Ultrastructures and Mitochondrial Function in Mice

(A) TEM analysis of LVs from 3-month-old f/f and Δ/Δ female mice. Scale bars, 2 μm. Arrowheads point to Z discs; arrows point to mitochondria.

(B and C) Quantification of the number of mitochondria (mito.) per 36 μm^2 calculated from three independent TEM images (B) and mtDNA contents (C) by Southern blot analyses in the hearts of 3-month-old male and female mouse ventricles (n = 3 for each genotype). Graphs show mean ± SD. *p < 0.05; ***p < 0.001.

(D) Analyses of mitochondrial rRNAs (12S and 16S), mRNAs (*ND1* and *COXI*), and tRNAs (F, L1, P, C, and Q) by northern blot. p < 0.05 for all mitochondrial RNAs (mtRNAs). LVs of 3- to 5-month-old male and female mice (n = 3 for each genotype) were used.

(E) Analysis of mitochondrial respiratory chain complexes II, III, and V by western blot (left) and its quantitative ratios (right) of each complex to lamin A (LMNA). LVs of 3- to 5-month-old male and female mice (n = 3 for each genotype) were used. LMNA was used as a loading control. Graphs show mean ± SD. **p < 0.01; ***p < 0.001.

(F) Analysis of mitochondrial complex II activity in 2- to 3-month-old mice (male, n = 3; female, n = 4 for each genotype). mOD, mitochondrial optical density. Graphs show mean ± SD. **p < 0.01.

c.2430G > A, G703E; c.2618A > T, M766L; and c.3848A > G, T1176A). P239R was found in both patients without DCM and patients with iDCM, indicating that this is not a DCM-causing mutation. Rather, it appears to be a SNP that is specific to the Korean population. This SNP is not found among American populations (Table S1) (Fu et al., 2013). G703E and M766L mutations were found in one patient each, and three patients were harboring the T1176A mutation (total 10%; Table S1). These 3 mutations were absent in 403 Korean healthy subjects, and only 1 subject showed T1176A mutation among the 6,502 American populations (Figures 4A and 4B; Table S1) (Fu et al., 2013), suggesting that 3 variants are potent DCM-related mutations in humans. G703E and T1176A were located in regions of the *NCOA6* gene that are highly conserved across species (Figure 4C), whereas *Macaca mulatta* encodes leucine rather than methionine at human M766 position, suggesting that M766L substitution might not be harmful. All five patients possessed one WT allele.

The effects of each *NCOA6* mutation on the protein conformation were estimated with the protein structure-prediction software (Buchan et al., 2010; Cheng et al., 2005). The G703E substitution appeared to induce remarkable structural deformations by transforming coiled-coil and β sheet structures into an α helix (Figure S4A). Furthermore, in silico evaluation with PolyPhen-2 and SIFT software showed a potentially deleterious effect of G703E on NCOA6 function: PolyPhen score of 0.999; SIFT score of 0.006 (Adzhubei et al., 2010; Ng and Henikoff, 2003). Although M766L and T1176A substitutions were predicted to induce no structural change and be benign (PolyPhen-2 score of 0 in both, and SIFT score of 0.039 and 0.289 in M766L and T1176A, respectively), we could not exclude the possibility that M766L and T1176A substitutions are also deleterious for NCOA6 functions. T1176A transcript level was similar to those of the *NCOA6* WT and its other mutants, but its protein level was significantly low (Figures S4B and S4C), though its mechanism is inconclusive.

Because depletion of *Ncoa6* leads to decreased transcriptional activity of PPARδ in mice, we examined whether NCOA6 variants alter the activity PPARδ. In fact, overexpression of G703E and T1176A significantly suppressed the activation of a PPARδ and its targets including *CD36* and *FATP1* in the AC16 parental and *NCOA6* knockdown cells upon the treatment with GW (Figures 4D–4F and S4D). However, no remarkable difference was observed between WT and M766L mutant NCOA6, implying that M766L may not be a pathogenic mutation for DCM, though further investigation is required to clarify its physiological effect. Altogether, these data suggest that two substitutions (G703E and T1176A, if M766L is not) perturb NCOA6-PPAR signaling via distinct mechanisms: G703E causes structural and functional defects, whereas T1176A reduces the expression of PPARδ targets possibly by destabilizing NCOA6 proteins.

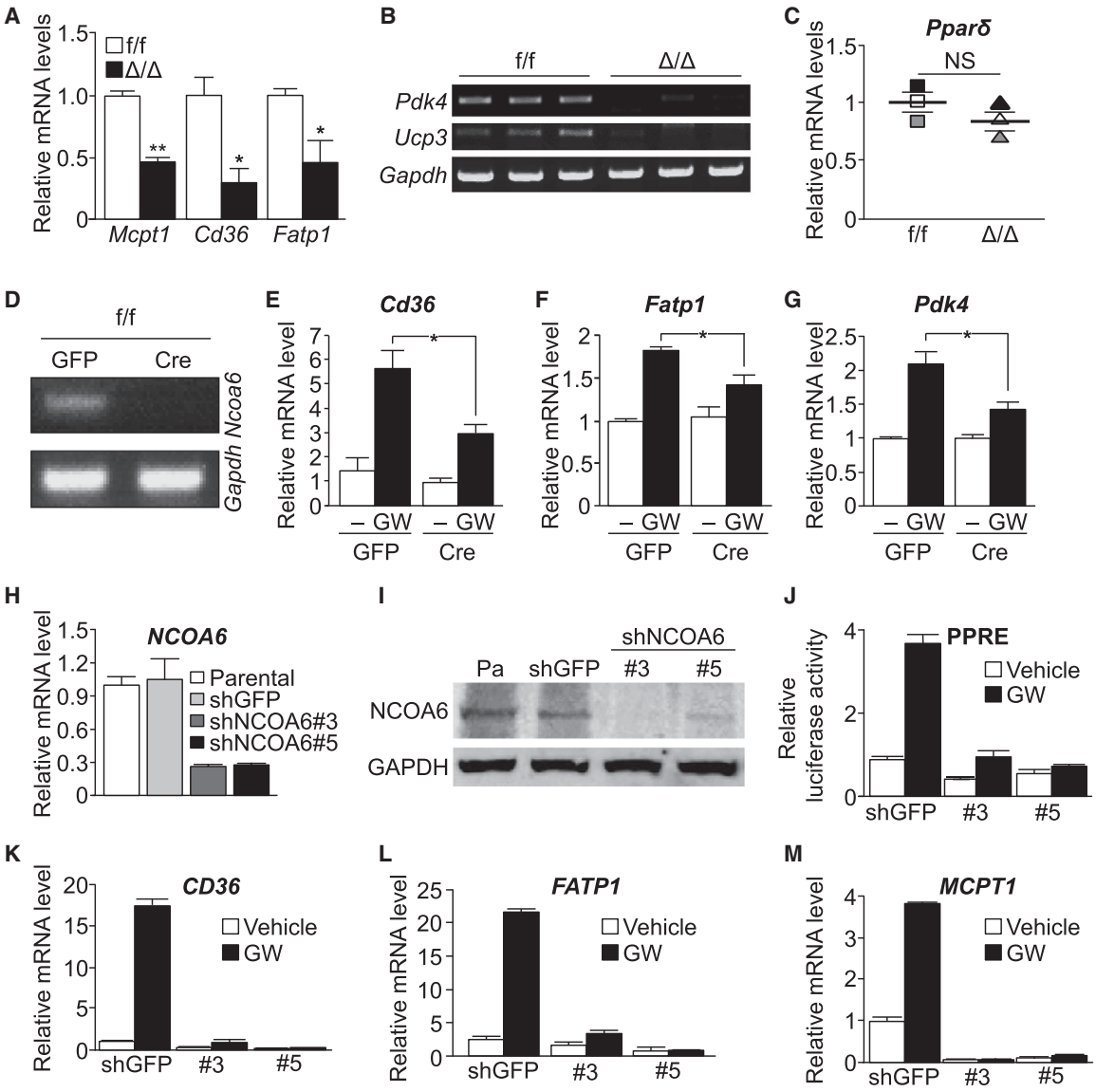

Figure 3. NCOA6 Is Required for Normal Transcriptional Activity of PPARδ

(A and B) Transcript levels of PPARδ targets in the LVs of f/f (white; male, n = 1; female, n = 2) and Δ/Δ (black; male, n = 1; female, n = 2) mice. Graphs show mean ± SD. *p < 0.05; **p < 0.01.

(C) mRNA levels of *PPARδ* in the hearts of f/f and Δ/Δ mice (n = 3 per each genotype). *Gapdh* was used as a loading control. NS, not statistically significant.

(D) Semiquantitative PCR analyses of *Ncoa6* in primary cardiomyocytes, transduced with lentivirus encoding GFP or Cre constructs, isolated from the f/f mouse heart.

(E–G) Transcript levels of *Cd36* (E), *Fatp1* (F), and *Pdk4* (G) in f/f primary cardiomyocytes containing GFP or Cre constructs. Treatment with GW lasted 48 hr. Graphs show mean ± SD. *p < 0.05.

(H–M) Transcript and protein levels of NCOA6 were examined by real-time quantitative PCR (H) and western blot (I). Parental (Pa), no transfection; shGFP, shRNA against *GFP*; shNCOA6 #3 and #5, independent shRNA constructs against *NCOA6*. (J) Relative PPAR response element (PPRE)-luciferase activity. Treatment with GW lasted 24 hr. (K–M) Transcript levels of *CD36* (K), *FATP1* (L), and *MCPT-1* (M) were measured by real-time quantitative PCR analyses after treatment with GW for 48 hr. Graphs show mean ± SD.

See also Figure S3.

DISCUSSION

Ncoa6 deficiency reduces the number of mitochondria in cardiac tissue. Cardiac abnormality of mitochondrial function is closely associated with DCM pathogenesis (Marin-Garcia et al., 1995). In addition, impaired expression of *Ncoa6* has been found in muscle fibers with a dysfunctional electron transport system (Herbst et al., 2013). Thus, *Ncoa6* mutations appear to induce a loss of or suppress biogenesis of mitochondria in the heart. NCOA6 is a critical component of the steady-state

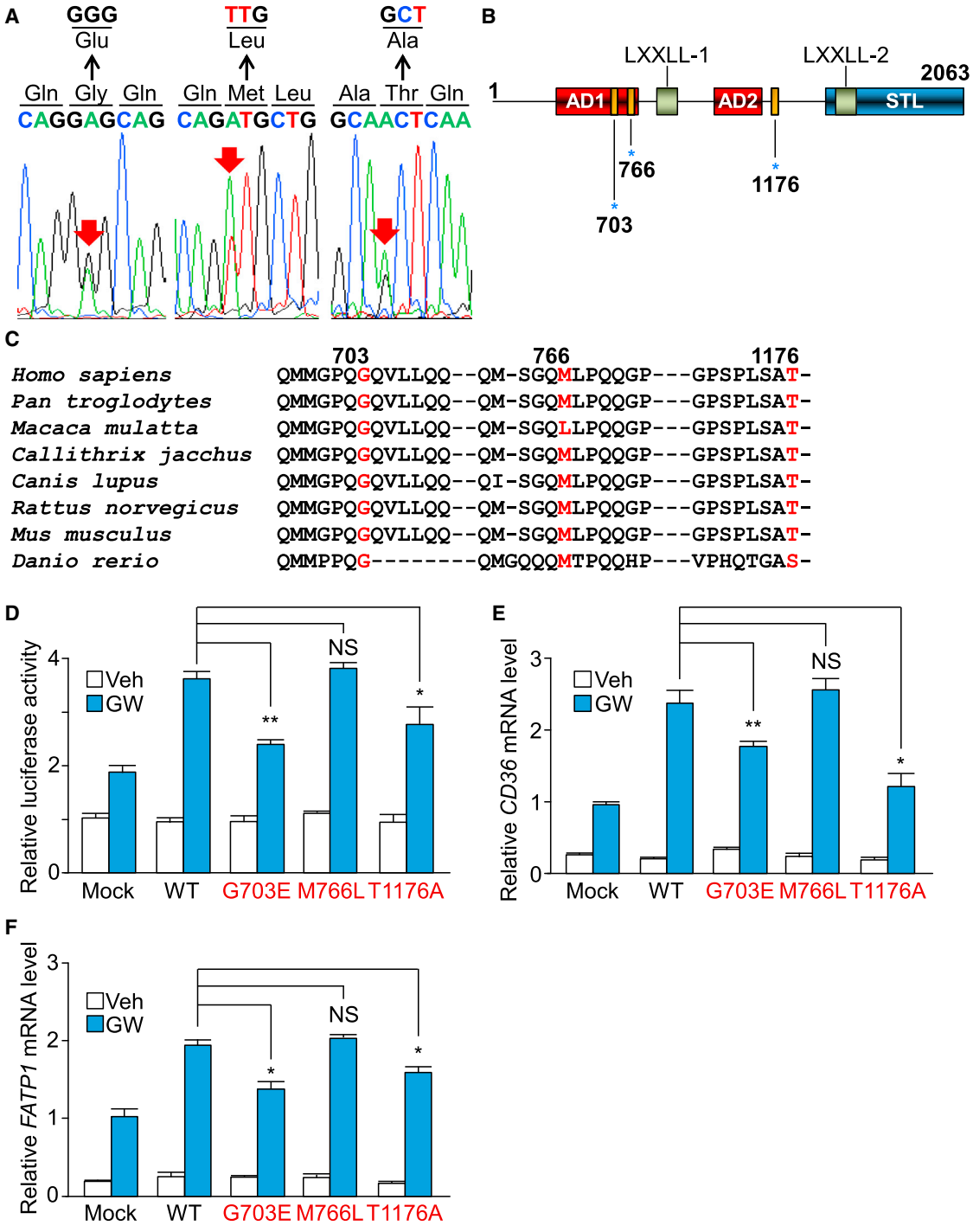

Figure 4. Nonsynonymous Mutations of *NCOA6* in Human Patients with iDCM Result in Impaired PPARδ Activity

(A) DNA sequences of *NCOA6* exons that were obtained from patients with iDCM. Red arrows indicate mutation sites. Glu, glutamate; Leu, leucine; Ala, alanine; Gln, glutamine; Gly, glycine; Met, methionine; Thr, threonine.

(B) Schematic representation of the human NCOA6 protein. AD, activation domain; STL, serine, threonine, and leucine-rich region. Blue asterisks indicate mutation sites.

(C) NCOA6 amino acid sequence alignment across eight species. Numerals indicate the positions of the amino acid mutations (red) in patients.

(D–F) Relative luciferase activity of PPRE (D) and gene expression levels of *CD36* (E) and *FATP1* (F) in WT and mutant *NCOA6*-transfected AC16 cells with or without treatment with GW for 48 hr. Graphs show the mean of triplicate experiments ± SD. NS, not statistically significant; Veh, vehicle. *p < 0.05; **p < 0.01. See also Figure S4 and Table S1.

ASC-2 complex (ASCOM), which is essential for proper NR transactivation (Mahajan and Samuels, 2008). Because NCOA6 stimulates NR transactivation activity, it is reasonable to expect altered downstream signaling of NRs to play a role in the mechanism for DCM. Here, we provide the potent evidence linking abnormal NR signaling to human DCM pathogenesis. Consistent with previous studies reviewed by Mahajan and Samuels (2008), our results indicate that depletion of *NCOA6* expression results in remarkably reduced PPARδ activity both in vitro and in vivo.

Germline loss of *Ncoa6* leads to embryonic lethality with multiple organ dysfunction including the placenta (Antonson et al., 2003; Kuang et al., 2002; Zhu et al., 2003). The homozygous null mutation of *Ncoa6* precipitates cardiac developmental abnormalities at E10.5, suggesting that NCOA6 might still be essential for early cardiac development before this stage (Kuang et al., 2002). Because α-MHC-Cre starts to function at E14.5, α-MHC-Cre-mediated depletion of *Ncoa6* might not be sufficient to cause early cardiac developmental defects.

It appears that *NCOA6* mutations may disrupt diverse NR transactivation via an effect on ASCOM. In fact, another observation suggests that NR pathways other than PPARδ may be affected by *NCOA6* mutations (Mahajan and Samuels, 2008). Our findings imply that additional NRs might be influenced by NCOA6 impairment. In support of these findings, it has been shown that mutations in mixed-lineage leukemia (MLL) 2/4 and tryptophan (W)-aspartic acid (D) repeat domain (WDR) 5, which are also components of ASCOM, are likely to be involved in congenital heart disease (Zaidi et al., 2013). This evidence indicates that *Ncoa6* deficiency significantly alters the activity of the NRs, resulting in DCM.

A recent study in mice revealed that NCOA6 promotes ubiquitination-mediated degradation of ERα, whereas *Ncoa6* deficiency causes ERα accumulation in uterine stromal cells during the preimplantation period (Kawagoe et al., 2012). Consistent with these findings, we observed that the expression of ERα and its target genes was significantly increased in *Ncoa6* Δ/Δ mice prior to DCM pathogenesis. Interestingly, it is common for ERα expression to increase in patients with end-stage DCM (Mahmoodzadeh et al., 2006). Based on these lines of evidence, we hypothesized that the expression and/or function of *NCOA6* may be suppressed prior to the onset of clinical DCM manifestations, possibly accelerating the progression of the disease. Taken together, our findings will manifest stepping forward to a more accurate diagnosis of human DCM.

EXPERIMENTAL PROCEDURES

Animals

All animal experiments were performed in accordance with Korean Food and Drug Administration guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Yonsei University (YLARC 2008-0014). Mice that had been backcrossed to the FVB/NTac strain for at least nine generations were used and maintained in the specific pathogen-free facility of the Yonsei Laboratory Animal Research Center.

Primary Cardiomyocyte Culture

Primary cardiomyocytes were isolated as previously described by Crone et al. (2002).

Human Genomic DNA Mutation Analysis

Each subject was provided with written informed consent to participate in the study according to the guidelines of the institutional review board (4-2007-0234). Exon scanning of all *NCOA6* (NM_014071)-coding regions and splice junction sites was performed by direct sequencing.

Echocardiogram

Echocardiography was performed with the echocardiographic system (Vivid 7; GE Medical Systems) equipped with a 12 MHz transducer. Mice with shaved chests were anesthetized with isoflurane (Hana Pharmaceutical) and placed on a platform. End-systolic and end-diastolic dimensions were measured using M-mode echocardiogram imaging that was obtained at the papillary muscle level for the measurement of LV wall thickness and LV dimensions. We captured these images over more than ten cardiac cycles, and the data shown are averages from at least three cardiac cycles per image acquisition. These procedures were performed at least three times, and average values were determined for each mouse.

Statistical Analyses

Statistical significance was determined using the two-tailed Student's t test. Data were analyzed with GraphPad Prism (GraphPad Software). p values less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.07.027.

AUTHOR CONTRIBUTIONS

J.-i.R., C.C., and J.L. performed in vitro and in vivo experiments; J.O., D.-K.K., B.S.L., and S.-M.K. performed cardiac phenotyping; C.B.P. performed mitochondria-related experiments; J.-E.L. and J.H.L. analyzed human mutations; Y.S.G. performed TEM analysis; J.-i.R., C.C., Y.H.S., J.W.L., and H.-W.L. wrote the manuscript. H.-W.L. designed and supervised the project.

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