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Lack of MTTP activity in pluripotent stem cell-derived hepatocytes and cardiomyocytes abolishes apoB secretion and increases cell stress

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Article

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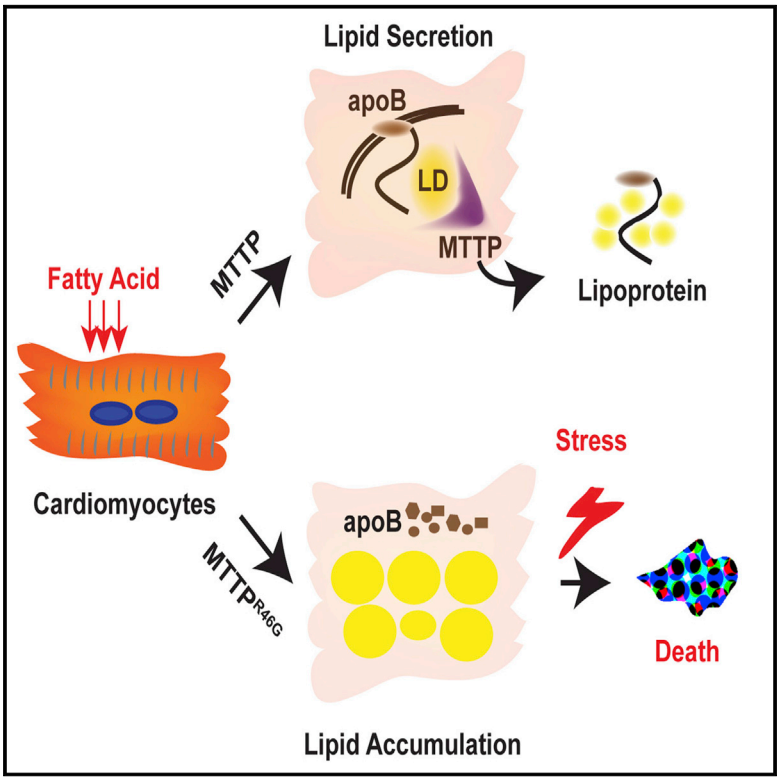
Graphical Abstract



Highlights

- Disease modeling of abetalipoproteinemia (ABL) using patient-specific iPSCs
- *MTTP* mutation correction by CRISPR/Cas9 rescues the ABL phenotype
- Cardiomyocytes carrying an MTTP mutation are hypersensitive to metabolic stress

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In Brief

Liu et al. use patient-specific iPSCs and CRISPR/Cas9 genome editing to uncover the functional consequences of *MTTP* mutations in human hepatocytes and cardiomyocytes. They find that *MTTP* is required for apoB secretion and that its absence results in increased cell stress in cardiomyocytes.

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Lack of MTTP Activity in Pluripotent Stem Cell-Derived Hepatocytes and Cardiomyocytes Abolishes apoB Secretion and Increases Cell Stress

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SUMMARY

Abetalipoproteinemia (ABL) is an inherited disorder of lipoprotein metabolism resulting from mutations in microsomal triglyceride transfer protein (MTTP). In addition to expression in the liver and intestine, MTTP is expressed in cardiomyocytes, and cardiomyopathy has been reported in several ABL cases. Using induced pluripotent stem cells (iPSCs) generated from an ABL patient homozygous for a missense mutation ($MTTP^{R46G}$), we show that human hepatocytes and cardiomyocytes exhibit defects associated with ABL disease, including loss of apolipoprotein B (apoB) secretion and intracellular accumulation of lipids. $MTTP^{R46G}$ iPSC-derived cardiomyocytes failed to secrete apoB, accumulated intracellular lipids, and displayed increased cell death, suggesting intrinsic defects in lipid metabolism due to loss of MTTP function. Importantly, these phenotypes were reversed after the correction of the $MTTP^{R46G}$ mutation by CRISPR/Cas9 gene editing. Together, these data reveal clear cellular defects in iPSC-derived hepatocytes and cardiomyocytes lacking MTTP activity, including a cardiomyocyte-specific regulated stress response to elevated lipids.

INTRODUCTION

Abetalipoproteinemia (ABL) is an autosomal-recessive disorder caused by mutations in the gene *MTTP*, which encodes the endoplasmic reticulum (ER)-resident lipid transfer protein microsomal triglyceride transfer protein (MTTP). MTTP functions to promote lipid transfer to apolipoprotein B (apoB) within the ER, facilitating its secretion by the enterocyte and hepatocyte (Wetterau et al., 1990, 1991). Genetic deficiency in MTTP results in undetectable apoB-containing lipoproteins in plasma due to the inability to secrete them. While hepatic steatosis is a known complication of ABL, primary hepatocytes from patients with ABL have never been characterized.

Both MTTP and apoB are expressed in human and mouse heart tissue, suggesting that the heart has the capacity to secrete lipoproteins (Borén et al., 1998; Nielsen et al., 1998, 1999). *Mttp* null mice have elevated triglyceride stores in the heart (Bjorkegren et al., 2001). However, it is not known whether cardiac lipid metabolism is affected in patients with ABL. Interestingly, some patients with ABL disease exhibit cardiac arrhythmias and heart failure (Dische and Porro, 1970; Gregg and Wetterau, 1994; Ledmyr et al., 2004; Sobrevilla et al., 1964; Zamel et al., 2008). These findings are consistent with the concept that MTTP-mediated secretion of apoB is a possible mechanism for protecting against cardiomyocyte lipid overload.

In order to define the cell-intrinsic roles for MTTP in hepatocytes and cardiomyocytes, induced pluripotent stem cells were generated from an ABL patient and control subjects and differentiated into hepatocytes and cardiomyocytes. As

expected, ABL induced pluripotent stem cell (iPSC) hepatocytes failed to secrete apoB and accumulated lipids. Interestingly, compared with control iPSC cardiomyocytes, iPSC cardiomyocytes from the ABL patient failed to secrete apoB, accumulated intracellular lipids, and responded poorly after stress induction through increased apoptosis. These data provide evidence that MTTP in human cardiomyocytes facilitates lipid export, leading to protection from cellular stress in the setting of lipid overload.

RESULTS

Generation of MTTP^{R46G}-Specific and Control iPSCs

We generated iPSCs from an ABL patient homozygous for a rare missense mutation in the *MTTP* gene (136C > G) and matched control subjects using standard procedures (Yang et al., 2012). This missense mutation occurs in the N-terminal region of MTTP, causing an amino acid switch from arginine to glycine at residue 46 (MTTP^{R46G}) (Figures S1A and A1B). Clinically, the patient with the MTTP^{R46G} mutation had undetectable plasma apoB, very low plasma cholesterol and triglyceride levels (Table S1), steatorrhea, microvesicular steatosis, spinocerebellar degeneration, and retinopathy, all consistent with classic ABL (Miller et al., 2014; Walsh et al., 2015; Zeissig et al., 2010). Three different iPSC lines were established from this patient and control subjects (Table S2). These iPSCs expressed high levels of pluripotency markers, including NANOG, OCT4, and SOX2 (Figure S1C). Quantitative flow cytometry demonstrated over 90% of the MTTP^{R46G} iPSCs express the cell-surface pluripotency markers SSEA-4 and TRA-1-60 (Figure S1D).

Recapitulation of the ABL Phenotype in Mutant MTTP^{R46G} Hepatocytes

To determine the effect of the MTTP^{R46G} mutation on apoB secretion in hepatocytes, we differentiated MTTP^{R46G} and control iPSCs into hepatocytes using a standard protocol (Si-Tayeb et al., 2010). After 20 days, the majority of the differentiated cells from control and MTTP^{R46G} iPSCs were positive for the hepatocyte markers HNF4α and ASGPR1 (Figure S2A). Expression of hepatic genes, including *ALB*, *AFP*, *HNF4*α, and *ASGPR1*, were at levels similar to those of the hepatic cell line Huh7 (Figure S2B). Additionally, functional synthesis and secretion of albumin were equivalent between control and MTTP^{R46G} hepatocytes (Figures S2C and S2D).

Hepatocytes derived from MTTP^{R46G} iPSCs expressed levels of the *MTTP* gene and MTTP protein comparable to those of differentiated hepatocytes from control iPSCs (Figures 1A and 1B). However, as shown in Figure 1C, lysate from control hepatocytes displayed triglyceride transfer in an MTTP activity assay, whereas lysate from the MTTP^{R46G} iPSC hepatocytes had no detectable MTTP activity (Figure 1C). Thus, the MTTP^{R46G} mutation abolished MTTP triglyceride transfer activity, consistent with the phenotypes of ABL in the patient. The apoB mRNA level was normal in MTTP^{R46G} hepatocytes, but there was an almost complete absence of intracellular and extracellular secreted apoB protein (Figures 1D–1F). Because poorly lipidated apoB is known to undergo proteasomal degradation (Fisher et al., 1997; Yeung et al., 1996), we inhibited this process with N-acetyl-leucyl-leucyl-norleucinal (ALLN) (Sakata and Dixon, 1999). After a 1-hr pre-treatment with ALLN followed by a 20-min pulse with ^{35}S methionine/cysteine, a significant amount of new apoB protein was synthesized in MTTP^{R46G} hepatocytes, whereas control cells exhibited only a moderate elevation in newly synthesized apoB, indicating substantial proteasomal degradation of apoB in the mutant MTTP^{R46G} hepatocytes (Figures 1G and 1H). When these cells were chased in label-free media, control cells exhibited a slower decrease in newly synthesized cellular apoB levels that reached 30% of initial levels after a 120-min chase, whereas MTTP^{R46G} hepatocytes had a dramatic reduction in apoB after just a 30-min chase, with only 9% of the initially labeled protein remaining after a 60-min chase (Figure 1I).

Hepatosteatosis is commonly observed in both ABL and mouse models representing loss of MTTP (Chang et al., 1999; Raabe et al., 1999). We examined whether iPSC-derived hepatocytes from the MTTP^{R46G} patient exhibited lipid accumulation as assessed by Oil Red O staining. A significant amount of large lipid droplets were observed in MTTP^{R46G} hepatocytes, whereas control cells were nearly free of large intracellular lipid droplets (Figure 2A). This is supported by a quantitative increase in triglycerides and cholesterol in MTTP^{R46G} iPSC-derived hepatocytes (Figures 2B and 2C). Oleic acid (OA) treatment, which stimulates neutral lipid synthesis and secretion in hepatocytes, also resulted in a significant increase in cellular triglyceride and cholesterol levels in MTTP^{R46G}-derived hepatocytes compared to control cells (Figures 2B and 2C). Labeling cells with [^{3}H]-OA showed that the increase in triglyceride (TG) from OA treatment was due to defective secretion from hepatocytes (Figures 2D and 2E). Taken together, MTTP^{R46G} iPSC-derived hepatocytes exhibit the hallmark features of ABL, including the absence of apoB secretion, excess intracellular lipid storage, reduced hepatic lipid secretion, and excess intracellular lipid storage, presumably due to loss of MTTP lipid transfer activity.

Correction of the C136G Mutation in *MTTP* by Gene Editing Rescues the ABL Phenotype in MTTP^{R46G} iPSC-Derived Hepatocytes

We corrected *MTTP* C136G mutation causing the R46G mutant using a CRISPR/Cas9 gene editing approach (Figure 3A) (Ran et al., 2013). We obtained two clones bearing the corrected allele as confirmed by DNA sequencing (Figure 3B). These corrected iPSC lines expressed similar levels of pluripotency markers as well as hepatocyte markers upon differentiation (Figures 3C and 3D). Newly synthesized apoB was examined in the corrected lines using [^{35}S] methionine/cysteine 2-hr labeling experiments. In contrast to the mutant MTTP^{R46G} line, levels of cellular and secreted apoB were mostly or partially normalized by the gene correction, indicating that the MTTP^{R46G} mutation caused the decrease in apoB stability in hepatocytes (Figures 3E–3G). Moreover, Oil Red O staining revealed a decrease in lipid droplet accumulation in the differentiated hepatocytes from the corrected line in comparison to the MTTP^{R46G} line (Figure 3H).

Cardiomyocyte-Intrinsic Defects due to Expression of the MTTP^{R46G} Protein

While liver and intestine are the major sites of *MTTP* expression, human and mouse heart also expresses MTTP proteins (Nielsen

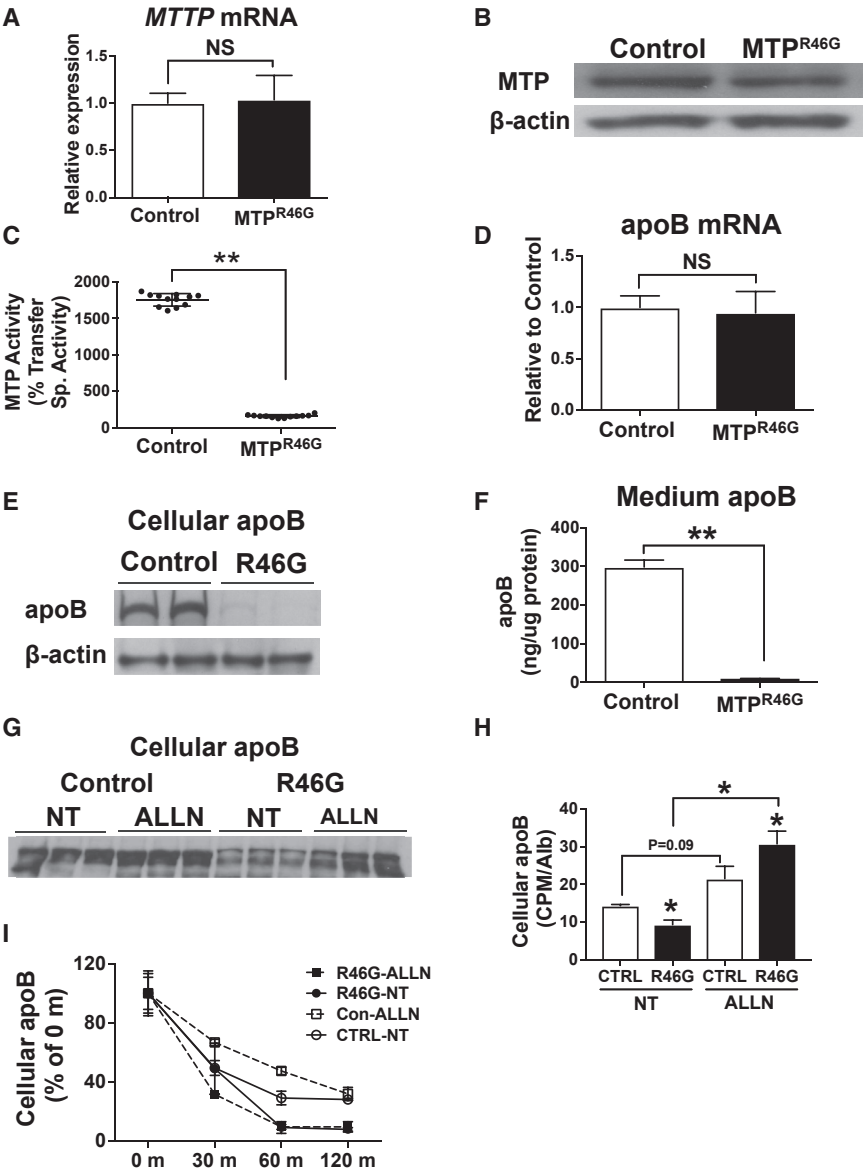

Figure 1. Abolished apoB Secretion in Mutant MTTP^{R46G} Differentiated Hepatocytes

(A and B) Quantification of messenger RNA of *MTTP* (A) and MTTP protein (B) by real-time PCR and western blot, respectively.

(C) Microsomal TG transfer activity was measured.

(D and E) Quantification of messenger RNA (D) and protein (E) of apoB 100 by real-time PCR and western blot, respectively.

(F) ApoB protein secreted into media after a 16-hr incubation was measured by ELISA.

(G) Newly synthesized cellular apoB was analyzed by immunoprecipitation and SDS-PAGE after a 20-min label with [^{35}S]methionine/cysteine in the presence or absence of ALLN.

(H) Quantification of newly synthesized apoB protein with normalization to albumin after a 20-min pulse with [^{35}S]methionine/cysteine.

(I) Pulse-chase of newly synthesized apoB for 30, 60, and 120 min. Graph shows the percentage relative to initial amount of apoB at the end of the label.

*$p < 0.05$ **$p < 0.01$. Values are means of three independent experiments.

et al., 1998). However, the role MTTP plays in cardiac lipid metabolism remains unclear. To examine the role of MTTP in human cardiomyocytes, we differentiated both control and MTTP^{R46G} iPSCs into cardiomyocytes using a previously published protocol (Laflamme et al., 2007; Shiba et al., 2012). Synchronic beating from induced cardiomyocytes was observed in control, MTTP^{R46G}, and corrected lines by day 14 of differentiation. All cells expressed equivalent levels of cardiomyocyte markers, including *MYH6*, *MYH7*, *MLC2a*, and *MLC2v* (Figure S3).

Similar levels of MTTP mRNA and MTTP protein were observed in control, MTTP^{R46G}, and correction cardiomyocytes (Figures S4A and S4B). We next examined the effect of lipid loading on all cell lines. Interestingly, both OA and palmitic acid (PA) induced apoB transcription in control, MTTP^{R46G}, and corrected cells (Figure 4A). However, apoB secretion was only detectable in control cardiomyocytes and the gene-corrected line, whereas MTTP^{R46G} cells had little to no detectable apoB secretion after OA or PA treatment (Figure 4B). OA and PA treatment also induced increased neutral lipid accumulation in MTTP^{R46G} compared to control cardiomyocytes (Figures 4C, 4D, and S4C), which is further supported by a significant increase in TG synthesis in MTTP^{R46G}-derived cardiomyocytes (Figure 4E). This also led to a decrease in secreted TGs in the MTTP^{R46G}-derived cardiomyocytes (Figure 4F). While the amounts of apoB and TG secreted are dramatically lower in cardiomyocytes than in hepatocytes, there was still a significant decrease observed in MTTP^{R46G} relative to control cardiomyocytes. In addition, the TG:CE (cholesteryl ester) ratio of lipids secreted from cardiomyocytes and hepatocytes differs, suggesting less TG-rich particles produced by cardiomyocytes (Figure S4D). This is consistent with the previous findings that the apoB-containing lipoproteins secreted from mouse and human hearts are not TG rich but rather have a density consistent with a more cholesterol-rich low-density lipoprotein (LDL) particle (Borén et al., 1998). Correction of the R46G mutation restored apoB and TG secretion and lipid accumulation to those of control cardiomyocyte levels (Figures 4B–4F). These results demonstrate that human cardiomyocytes require MTTP for secretion of apoB and lipid and in the setting of genetic MTTP deficiency are vulnerable to lipid accumulation.

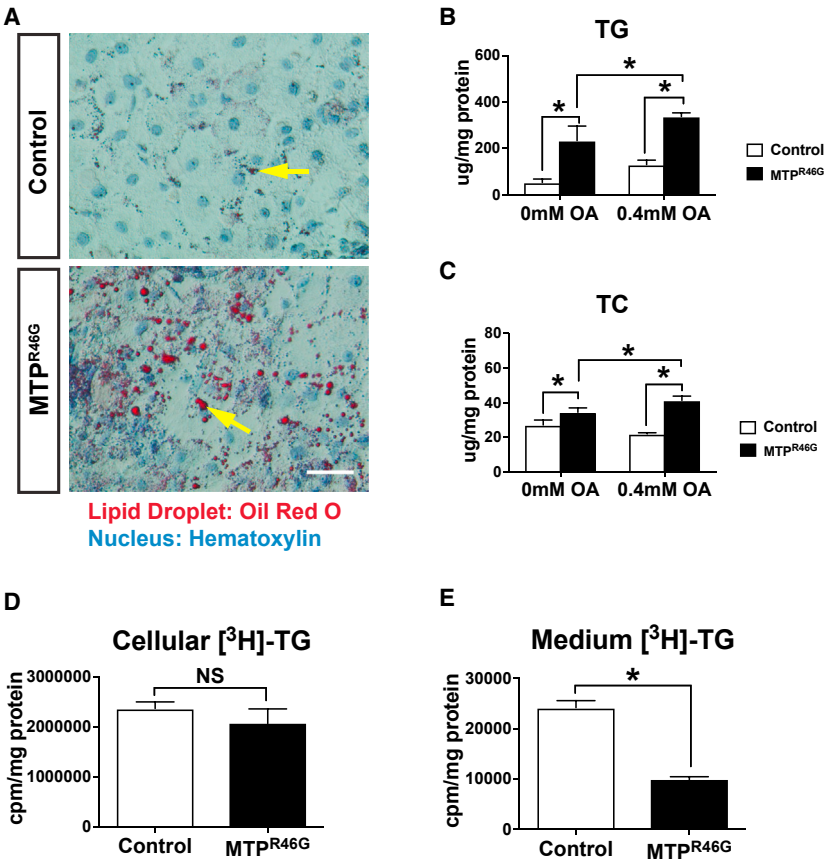

Figure 2. Accumulated Lipid Droplets in Mutant MTTP^{R46G} Differentiated Hepatocytes

(A) Representative images of lipid droplets stained with oil red O. Yellow arrowheads indicate red positive lipid stains. Hematoxylin stains the nuclei blue.

(B and C) Cellular TG (B) and total cholesterol (TC) (C) content was determined by enzymatic assays and normalized to total cellular protein as measured by BCA assay.

(D and E) Cells were incubated with [^{3}H]-OA(5 μCi/mL) + 0.8 mM OA for 4 hr. Cellular (D) and medium (E) TG were extracted, separated by TLC, and quantified by liquid scintillation counting. TG counts are normalized to protein contents determined by Lowry protein assay (n = 4).

*$p < 0.05$ Values are means for three independent experiments. Scale bar, 400 μm.

used as an in vitro model of ischemia reperfusion (IR) injury. Cells were subjected to PA, hypoxia and reoxygenation (H/R), or H/R+PA treatment. While H/R+PA triggered an apoptotic response in all cell lines, a more pronounced response was noted in MTTP^{R46G} cells relative to controls (Figures 5E and 5F). This differential sensitivity in MTTP^{R46G} cardiomyocytes to H/R+PA was normalized in the gene-corrected line (Figures 5E and 5F). This suggests PA-induced overload of intracellular lipid in cardiomyocytes sensitizes stress responses to multiple metabolic stresses. Expression of genes associated with cardiac dysfunction and failure was significantly higher in MTTP^{R46G} cardiomyocytes than in control cardiomyocytes after treatment of sunitinib together with PA (Figure 6A). However, correction of the *MTTP* C136G mutation normalized the expression of genes after PA treatment, suggesting a protective role of MTTP during lipid-induced stresses in cardiomyocytes. ANP and BNP expression was also significantly upregulated in MTTP^{R46G} cells after hypoxic treatment, suggesting a protective role of MTTP in cardiomyocytes. Importantly, hypoxic stress was normalized by correction of the *MTTP* C136G mutation (Figure 6B).

Hypersensitivity of MTTP^{R46G} Cardiomyocytes to Metabolic Stresses

Altered metabolism of free fatty acids and lipid accumulation in the myocardium can cause myocardial dysfunction and cardiomyocyte apoptosis (Chiu et al., 2001; Christoffersen et al., 2003). Increased cardiac apoB secretion has been shown to ameliorate cardiac dysfunction in dietary and genetic mouse models of lipid overload (Bartels et al., 2009; Yokoyama et al., 2004). Therefore, we examined whether loss of MTTP function altered the response of mutant MTTP^{R46G} cardiomyocytes to multiple metabolic stresses. MTTP^{R46G} and control cells treated with sunitinib, a receptor tyrosine kinase inhibitor that has cardiac cytotoxicity (Force and Kolaja, 2011; Orphanos et al., 2009), exhibited a similar dose-dependent increase in apoptosis (Figures S5A and S5B). However, when sunitinib was added in the presence of PA to induce lipid synthesis, a significant increase in apoptosis, evaluated by TUNEL and cleaved caspase-3 staining, was noted in MTTP^{R46G} cardiomyocytes relative to control cells (Figures 5A–5D). The increased apoptosis in MTTP^{R46G} cardiomyocytes was normalized to control levels with correction of the MTTP^{R46G} mutation (Figures 5A–5D). These results suggest an overload of intracellular lipid content reduces the tolerance toward the combined effects of multiple pharmacological stressors in MTTP^{R46G} cells.

We next tested cardiomyocytes stress tolerance with hypoxia followed by reoxygenation, another metabolic stressor that is

DISCUSSION

The present study uses patient-specific iPSCs generated from a patient with ABL with a missense mutation (MTTP^{R46G}) in both alleles of the *MTTP* gene. Differentiation of the iPSCs to hepatocytes produced the expected phenotype of abolished apoB production and accumulation of intracellular lipid, phenotypes that were corrected by genome editing of the causal MTTP mutation. Moreover, cardiomyocytes derived from the ABL iPSCs displayed impaired apoB secretion, lipid accumulation, and increased sensitivity to cellular stress. These results are consistent with a role for MTTP in promoting secretion of apoB in hepatocytes and cardiomyocytes and protecting cardiomyocytes from cellular stress.

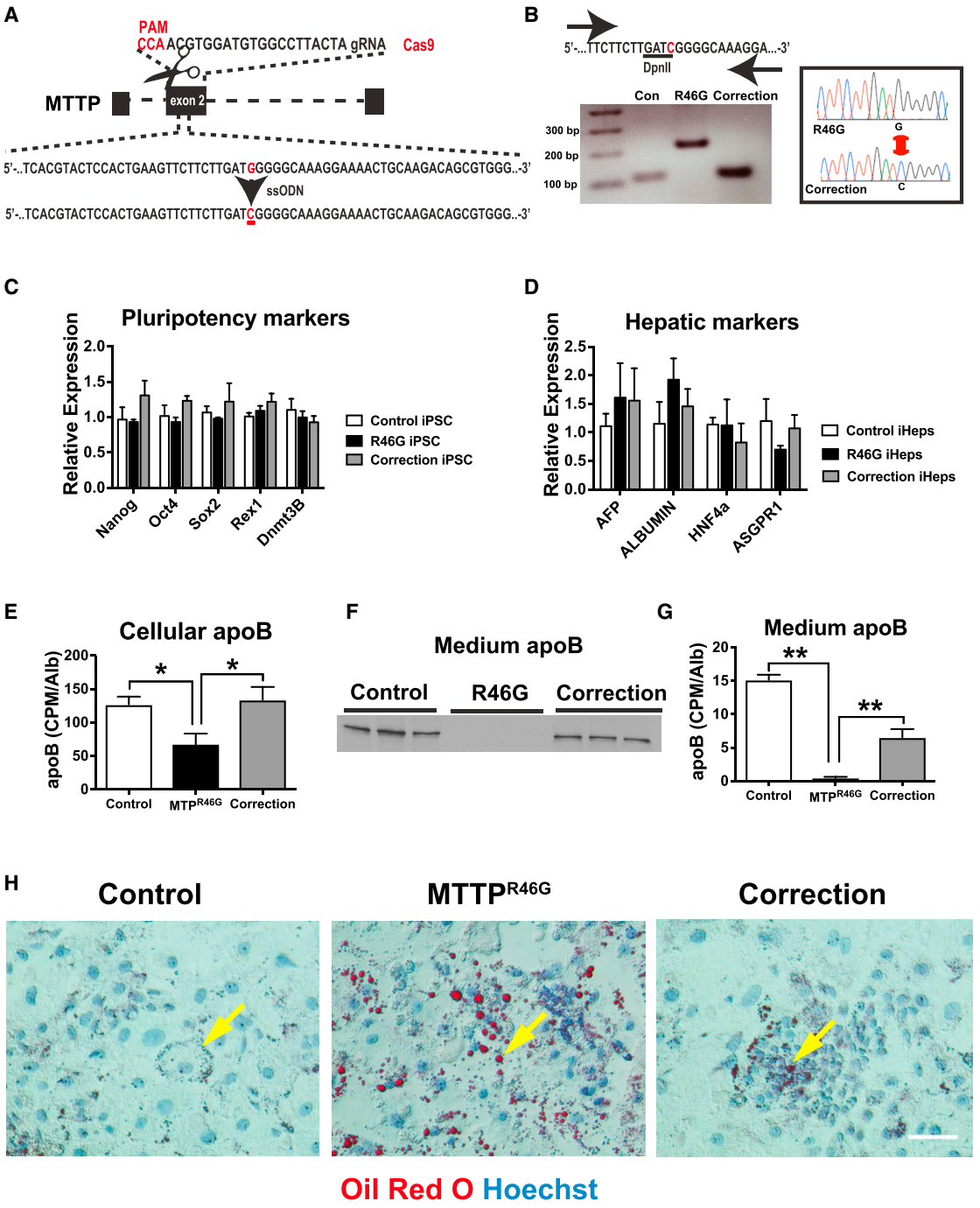

Figure 3. Correction of C136G in *MTTP* Rescues the ABL Phenotype

(A) Schematic strategy for correction of C136G in *MTTP* by CRISPR/Cas9.

(B) An iPSC from an ABL patient was transfected with plasmids containing guide RNA and Cas9. Genomic DNA was extracted from GFP+ colonies and subjected to PCR amplification. Subsequent DpnII digestion was applied to identify the positively targeted clones.

(C) The corrected iPSC lines were tested for expression of pluripotency markers by real-time PCR.

(D) Expression of hepatic genes was analyzed by real-time PCR in hepatocytes derived from the corrected iPSC lines.

(E–G) Amount of newly synthesized apoB in the cell or secreted in the medium was measured at the end of a 2-hr label with [^{35}S]methionine/cysteine.

(F) Autoradiography for apoB in the media.

(H) Cellular lipid accumulation by oil red O staining following rescue of the C136G *MTTP* mutation by CRISPR/Cas9. Scale bar, 400 μm.

*$p < 0.05$. **$p < 0.01$. Values are means ± SD from three independent experiments.

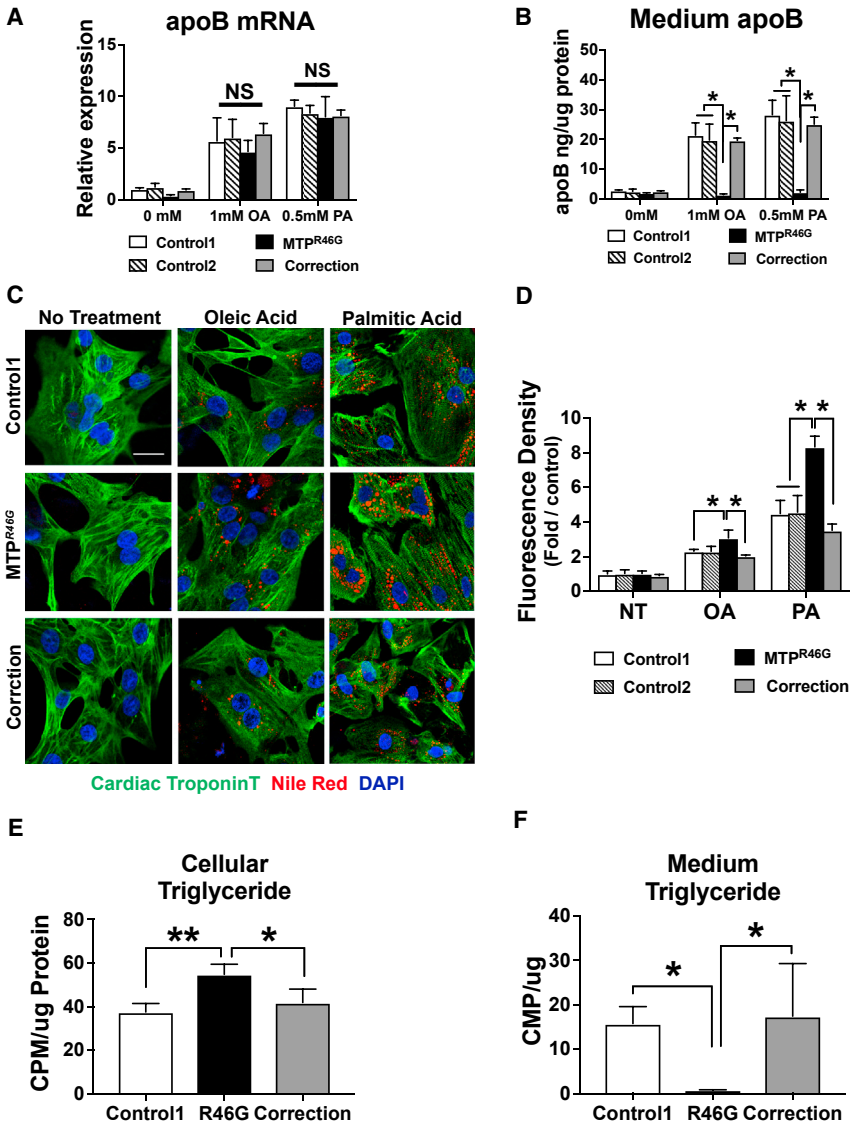

Figure 4. Abolished apoB and Elevated Lipid Storage upon Fatty Acid Treatment in Cardiomyocytes Was Rescued by Correction of MTTP^{R46G}

(A and B) ApoB transcript (A) and medium (B) apoB protein upon fatty acids treatment were quantified by real-time PCR and ELISA, respectively. Cells were incubated with oleic acid (OA; 1 mM) or palmitic acid (PA; 0.5 mM) for 48 hr.

(C) Representative images of neutral lipid staining in cardiomyocytes. Cardiac troponin T stains cardiac muscle fiber proteins green. Nile red stains lipid droplet red. DAPI stains nuclei blue.

(D) Mean fluorescence intensity of Nile red was analyzed by ImageJ. Fold relative to control is shown.

(E and F) Newly synthesized cellular TG content is quantified in cardiomyocytes (E) and in the culture media (F) following a 24-hr label with [^{14}C] oleic acid and lipid extraction. TG counts are normalized to protein contents determined by Lowry protein assay.

*$p < 0.05$. Values are means for three independent experiments. Scale bar, 25 μm.

MTTP plays a critical role in intracellular assembly of apoB-containing TG-rich lipoproteins in both liver and intestine (Young, 1990). The *MTTP* gene is expressed not only in the intestine and liver but also in the heart (Berriot-Varoqueaux et al., 2000; Hussain et al., 2012). Expression and secretion of apoB lipoproteins has been demonstrated from human heart biopsy specimens (Borén et al., 1998; Nielsen et al., 1998, 1999). However, the physiological role of this cardiac expression and secretion of lipoproteins remains to be determined. A role for MTTP in promoting secretion of apoB in the human heart has been previously proposed to protect the heart against toxic load of lipid accumulation (Bartels et al., 2009). Heart-specific *MTTP* knockout mice had elevated cardiac TG levels (Bjorkegren et al., 2001). Although cardiac expression of *MTTP* is low (Aminoff et al., 2010), its expression increases in the ischemic myocardium. Interestingly, reduced myocardial expression of *MTTP* in hypoxic hearts is associated with increased myocardial lipid (Ledmyr et al., 2004; Nielsen et al., 2002). A genetic variant associated with reduced *MTTP* expression is associated with increased cardiac disease (Ledmyr et al., 2004). In addition, cardiomyopathy, arrhythmias, cardiomegaly, and cardiac failure have been described in ABL patients, though the patient in our study did not present with obvious cardiomyopathy (Dische and Porro, 1970; Sobrevilla et al., 1964; Zamel et al., 2008). While the etiology of myopathy is unclear in these cases, it could be related to muscle weakness caused by vitamin E deficiency resulting from lack of absorption in intestine. Our studies reveal a cardiomyocyte-intrinsic phenotype due to loss of MTTP activity, which

A number of mutations in MTTP have been identified that result in the ABL phenotype (Miller et al., 2014; Shoulders et al., 1993; Walsh et al., 2015). Many of these mutations lead to premature stop codons or defective splicing and result in reduced or absent expression of a full-length MTTP protein (Pons et al., 2011). Other missense mutations are not predicted to affect the TG transfer function of MTTP and may act through different mechanisms to cause ABL phenotypes (Al-Shali et al., 2003; Ohashi et al., 2000; Wang and Hegele, 2000). While there are several mouse models to study the function of MTTP, none of these examine the effect of human missense mutations (Bartels et al., 2009; Chang et al., 1999; Liang et al., 2014; Raabe et al., 1999; Tietge et al., 1999). Our studies clearly establish that the missense mutation MTTP^{R46G} abolishes MTTP activity and recapitulates the ABL phenotype, making the patient-specific iPSCs in this report a human model for studying the cellular effects of MTTP deficiency.

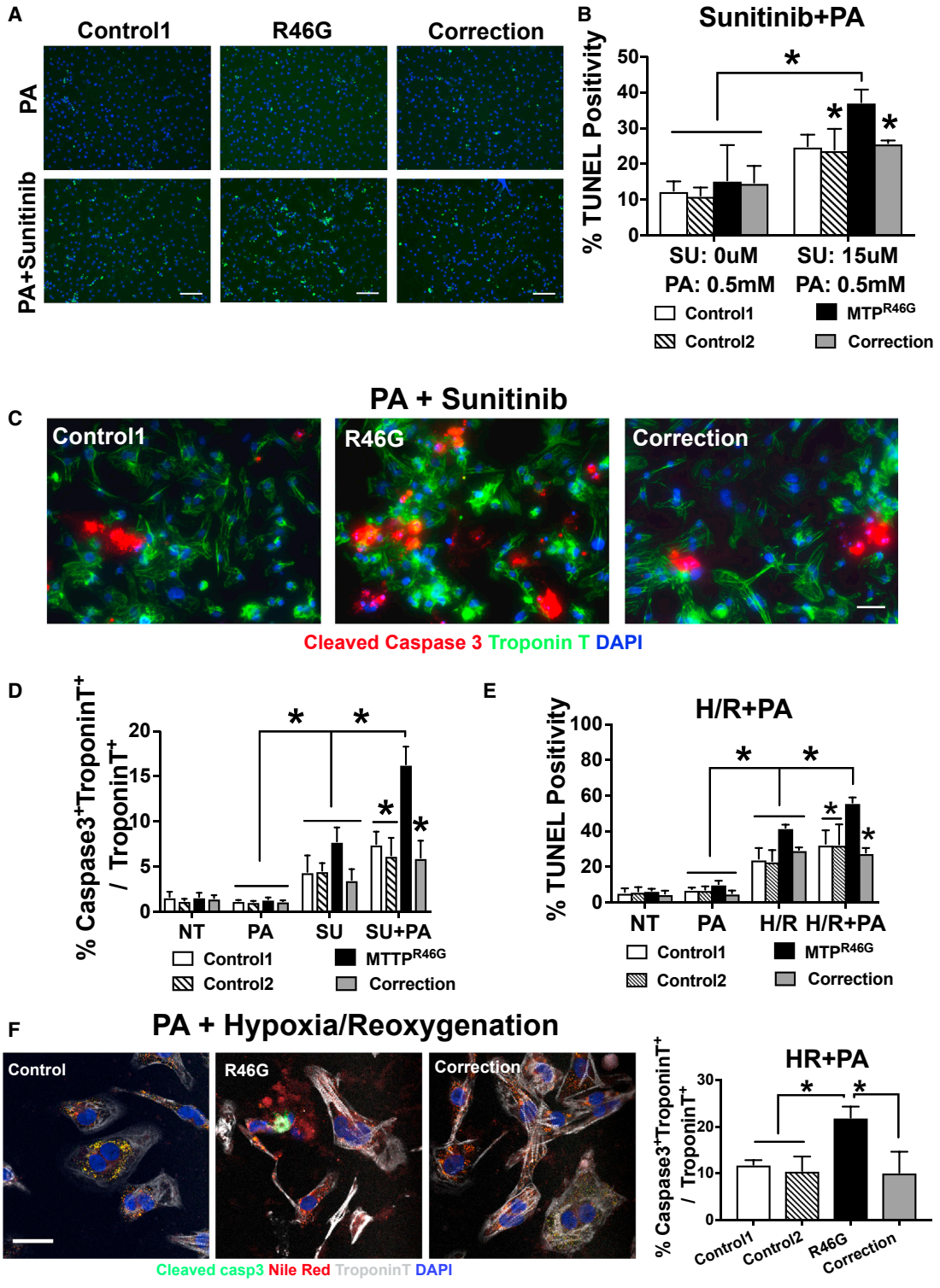

Figure 5. MTTP^{R46G} Cardiomyocytes Are Hypersensitive to Metabolic Stresses

(A) Representative images showing sunitinib (15 μM) and PA (0.5mM) induced apoptosis as visualized by TUNEL-positive cells (green). DAPI stains nuclei blue.

(B) Percentage of sunitinib-induced TUNEL positivity was quantified by cell counting using ImageJ (>1,000 nuclei were counted per genotype).

(C) Representative images showing expression of cleaved caspase-3 in sunitinib- and PA-treated cardiomyocytes. Troponin T, green; cleaved caspase-3, red; and DAPI, blue.

(D) Mean fluorescence density for cleaved caspase-3 was quantified using ImageJ. Fold change relative to control is shown.

(legend continued on next page)

may be critical in the setting of lipid overload. In particular, upon fatty acid treatment, apoB can be clearly detected in control cardiomyocytes and the conditioned media, whereas ABL cardiomyocytes had absent secreted apoB, lipid accumulated in the cell, and increased cellular TG levels. This leads to increased sensitivity to cytotoxic stresses, ultimately resulting in increased apoptosis in lipid-overloaded cardiomyocytes as well as increased sensitivity to hypoxia and rexoygenation. Together, our data suggest that low-level secretion of lipidated apoB by cardiomyocytes serves a different physiological function—not bulk secretion of lipids but rather secretion of particular lipids. Furthermore, this pathway in cardiomyocytes may not be physiologically relevant, except in times of stress and/or lipid overload. Importantly, the phenotypes we observed in both $MTTP^{R46G}$ hepatocytes and cardiomyocytes were rescued by CRISPR/Cas9-mediated gene correction. This strongly supports the contention that these phenotypes are due to the mutation in *MTTP*. Our studies provide new evidence to support a physiological role for MTTP expression in human cardiomyocytes to export apoB and excess lipid and protect the heart from stress.

Our studies reveal that coupling rigorous iPSC differentiation protocols for multiple cell lineages as well as gene correction allows for the discovery and analysis of new phenotypes caused by rare genetic mutations. Although our studies are limited to cells from one ABL patient, this human disease iPSC/CRISPR/Cas9 model allows for further resolution of the complex disease phenotype in ABL patients and favors for the development of therapies directed toward this rare genetic disorder as well as potentially other causes of cellular lipotoxicity.

EXPERIMENTAL PROCEDURES

Human Subjects

All human studies were approved by the University of Pennsylvania Human Subjects Research Institutional Review Board. The individuals were specifically recruited for this study and gave their informed written consent. The study was conducted at the Perelman School of Medicine at the University of Pennsylvania. Peripheral blood samples obtained from the subjects were used for general lipid measurements as well as generation of iPSC lines. See Table S1 for gender, age, and race of individuals used in this study.

Generation of Subject-Specific iPSCs and Differentiation into Hepatocytes and Cardiomyocytes

Subject-specific peripheral blood mononuclear cell (PBMC)-derived iPSCs were generated using Sendai viral vectors by the iPSC Core Facility at University of Pennsylvania as previously described (Yang et al., 2012, 2015). These cell lines have been deposited at WiCell Research Institute (http://www.wicell.org/home/stem-cell-lines/catalog-of-stem-cell-lines/collections/nhlbi-next-gen-rader.cmsx). The hepatocytes and cardiomyocytes were generated from iPSCs using standard protocols described previously (Cai et al., 2008; Laflamme et al., 2007; Mallanna and Duncan, 2013; Shiba et al., 2012).

ApoB Labeling

20 days after initiation of differentiation, newly synthesized apoB in iPSC-derived hepatocytes was labeled and traced using methods described previously (Yamaguchi et al., 2006). In brief, immunoprecipitation of proteins from each sample was carried out using antibodies against apoB (Calbiochem) or albumin (Sigma). Cell lysates or conditioned medium was mixed with NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris [pH 7.4], 0.5% Triton X-100, and 0.1% SDS) and an excess amount of various antisera. The mixture was incubated at 4°C for 1 hr. Protein A-agarose was added to the reaction solution, and the incubation was continued for additional 16 hr. The beads were washed with NET buffer, and proteins were released with sample buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, and 10% β-mercaptoethanol) by boiling for 5 min. Samples were resolved by gel electrophoresis followed by autoradiography. The total amount of apoB100 in the cell or media was normalized to the total amount of albumin protein in each sample.

ApoB Pulse-Chase Assay

Newly synthesized apoB was traced using the method described previously (Yamaguchi et al., 2006). Briefly, iPSC-derived hepatocytes were preincubated in serum-free DMEM without methionine/cysteine containing 1.5% BSA with or without 40 μg/mL ALLN (Calpain Inhibitor 1, Sigma) for 1 hr and then labeled with DMEM without methionine/cysteine containing 1.5% BSA with or without ALLN and 200 μCi/ml [^{35}S] methionine/cysteine for 20 min. After being washed, cells were incubated in serum-free DMEM plus 1.5% BSA containing 10 mM methionine and 3 mM cysteine for 10 or 120 min. The medium was collected, and cells were lysed at 10, 40, 70, or 130 min.

In other experiments, hepatocytes were preincubated for 1 hr and labeled with 150 μCi/mL [^{35}S]methionine/cysteine for 2 hr, after which media was collected and cells were lysed. The lysis buffer contained 62.5 mM sucrose, 0.5% sodium deoxycholate, 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 50 μg/mL leupeptin, 50 μg/mL pepstatin A, and 30 μL/mL protease inhibitor mixture (1 mM benzamidine, 5 mM EDTA, 100 U/mL aprotinin, and 10 mM HEPES [pH 8.0]). Conditioned medium was mixed with protease inhibitor mixture and 0.86 mM freshly made phenylmethylsulfonyl fluoride. Cell lysates and conditioned medium were used for immunoprecipitations.

TG and Total Cholesterol Measurement

Total TG and cholesterol contents were measured by enzymatic assays and normalized to protein as measured by bicinchoninic acid assay (BCA) (Bi et al., 2014). Briefly, iPSC-derived hepatocytes and cardiomyocytes were pretreated with or without OA or PA for 24 hr. Cells were washed with PBS, and pellets were obtained from centrifugation. Cell membrane was removed by sonication in RIPA buffer. Total TG and cholesterol contents were determined by enzymatic assays and normalized to protein as measured by BCA assay (Kohan et al., 2012). To measure newly synthesized TGs, cells were preincubated with [^{3}H]-OA (5 μCi/mL) in the presence of OA (0.8 mM) for 4 hr. To measure radiolabeled TG, lipids were extracted from medium and cells, fractionated by thin-layer chromatography (TLC), and quantified by scintillation spectroscopy. TG counts in cell and medium were normalized to total cellular protein (Chung et al., 2010)

MTTP Lipid Transfer Activity Assay

MTTP lipid transfer activity in iPSC-derived hepatocytes was determined using a method described previously (Athar et al., 2004).

Genome Editing by CRISPR/Cas9

Precise gene correction was performed by CRISPR/Cas9 following a published protocol (Ran et al., 2013). Briefly, single guide RNAs (sgRNAs) were designed using the MIT CRISPR Design Tool and cloned into the plasmid PX458 (Addgene plasmid ID: 48138). ssODNs were suspended in sterile H_2O and transfected into iPSCs using 4D-Nucleofector (Lonza) together with PX458-sgRNA. The nucleofected iPSCs were plated with mTeSR1 supplemented with 2 μM Thiazovivin (Sigma). After 24 hr, GFP+ cells were sorted

(E) Quantification of TUNEL positivity in response to hypoxia and reoxygenation and PA.
(F) Representative images showing expression of cleaved caspase-3 in hypoxia and reoxygenation- and PA-treated cardiomyocytes. Troponin T, white; cleaved caspase-3, green; Nile red, red; and DAPI, blue. Graph to the right shows mean fluorescence density for cleaved caspase-3 (quantified using ImageJ). Fold change relative to control is shown.
*$p < 0.05$. Values are means ± SD from three independent experiments. Scale bar represents 150 μm (A) and 40 μm (C).

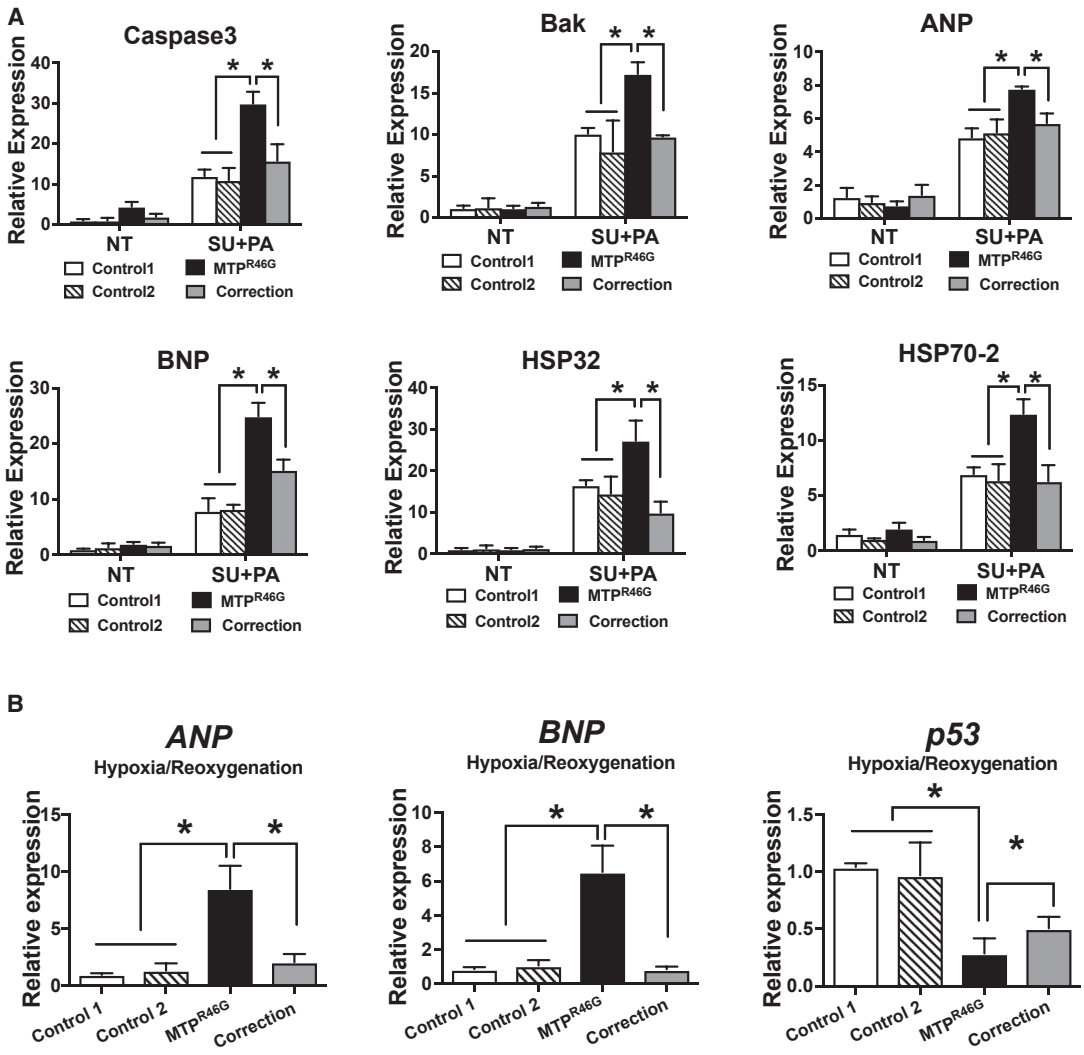

Figure 6. Increased Expression of Stress Response Genes in MTTP^{R46G} Cardiomyocytes

(A) Expression of stress associated genes, such as *Caspase3, Caspase9*, *Bak*, *ANP*, *BNP*, *Hsp32*, *and HSP70-2* in response to sunitinib (SU) and PA by real-time PCR.

(B) Expression of stress genes, including *Caspase3*, *p53*, *ANP*, *and BNP*, in response to H/R and PA.

*$p < 0.05$. Values are means ± SD from three independent experiments.

by FACSJazz (BD). Single GFP+ iPSCs were maintained in mTeSR1 and allowed to grow into colonies until manually picking for DNA extraction with Quick Extract DNA Extraction Solution (Epicentre). Then, DNA was subjected to PCR amplification around the cutting site and subsequent DpnII (New England Biolabs) digest to analyze successfully edited clones.

Lipid Droplet Staining

Oil red O (Sigma, O0625) and Nile red (Thermo Fisher Scientific) were used to label lipid droplets in iPSC-derived hepatocytes and cardiomyocytes according to the manufacturer's instructions. iPSC-derived hepatocytes were fixed with 4% paraformaldehyde for 15 min, followed by incubation with distilled water and subsequently with 60% isopropanol for 2 min and stained with a filtered 0.35% Oil Red O (Sigma) solution in 60% isopropanol for 10 min at room temperature. Then, cells were washed with sterile water and stained with hematoxylin solution for 1 min at room temperature. Images were analyzed under a light microscope. Lipid droplets appear red and nuclei appear blue.

To stain neutral lipids in iPSC-derived cardiomyocytes, cells at 20 days were treated with OA or PA separately for 24 hr. Fixation and permeabilization were performed using the same method as described above. Cardiomyocytes were co-stained with cardiac troponin T (Thermo Fisher Scientific) and Nile Red (Thermo Fisher Scientific), followed with Alexa 488 (Invitrogen). Images were acquired by confocal microscopy (Leica TCS SP8). Cardiac troponin T protein stains green, lipid droplets appear red, and nuclei stains blue.

TUNEL Assay

Cardiomyocytes derived from iPSCs were treated with sunitinib (0, 1.5 μM, and 15 μM) with or without palmitate (0.5 mM) for 18 hr. Cells were then fixed to determine apoptosis using the In Situ Cell Death Detection Kit (Roche, 11684795910) according to the manufacturer's protocol. Briefly, cells were fixed and permeabilized using the method described above. Cells were labeled with TUNEL enzyme mixture in a humidified environment at 37°C for 1 hr. DAPI solution was applied to stain total nuclei. Fluorescence microscopy was used to acquire images. Apoptotic cell nuclei stain green. Total nuclei

appear blue. Both nuclei were counted and calculated as the percentage of apoptotic index. Over 500 cells were counted in test samples.

Cleaved Caspase-3 Assay

Cardiomyocytes derived from iPSCs were treated with sunitinib (15 μM) with or without palmitate (0.5 mM) for 18 hr. Cells were then fixed and permeabilized for cleaved caspase-3. Cardiomyocytes were co-stained with cleaved caspase-3 (Cell Signaling) and cardiac troponin T (Thermo Fisher Scientific), followed by a second antibody staining with Alexa 488 (Invitrogen) and Alexa Fluor 555. Images were acquired with a Leica microscope. Cardiac troponin T protein stains green, cleaved caspase-3 stains red, and nuclei stains blue.

Hypoxia and Reoxygenation Stress Assay

iPSC-derived cardiomyocytes were maintained in serum-free medium with or without palmitate followed by exposure to hypoxia (94% N_2, 5% CO_2, and 1% O_2) for 18 hr. Cells were then moved back to an environment with 20% O_2 (5% CO_2) for reoxygenation. After 24 hr, cells were fixed for analysis of TUNEL or cleaved caspase-3 (Portal et al., 2013).

ELISA

Levels of albumin (Bethyl Laboratories) and apoB (Mabtech) in the medium were determined using commercial ELISA kits.

Statistical Analysis

Data were analyzed for statistical significance using a two-tailed unpaired Student's t test (GraphPad Prism). p values less than 0.05 were considered statistically significant. All quantitative data are presented as mean ± SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.04.064.

AUTHOR CONTRIBUTIONS

E.E.M. and D.J.R. conceived and designed the study. Y.L., D.M.C., and X.B. designed and performed the experiments and analyzed the data. Y.L., D.J.R., and E.E.M. wrote the manuscript. Other authors also directly participated in the planning, execution, or analysis of the study. All authors read and approved the final version of the submitted manuscript.

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