Human model of primary carnitine deficiency cardiomyopathy reveals ferroptosis as a novel disease mechanism

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Human model of primary carnitine deficiency cardiomyopathy reveals ferroptosis as a novel disease mechanism

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Abstract

Primary carnitine deficiency (PCD) is an autosomal recessive monogenic disorder caused by mutations in SLC22A5. This gene encodes for OCTN2 which transports the essential metabolite carnitine into the cell. PCD patients suffer from muscular weakness and dilated cardiomyopathy. Detailed molecular disease mechanisms remain unclear. Two OCTN2-defective human induced pluripotent stem cell lines were generated from a healthy control line, carrying a full OCTN2-knockout and a homozygous OCTN2 (N32S) loss of function mutation. OCTN2-defective genotypes showed lower cardiac differentiation efficiency, lower force development, and resting length in engineered heart tissue format compared to isogenic control. Force was sensitive to fatty acid-based media and associated with lipid accumulation, mitochondrial alteration, higher glucose uptake, and metabolic remodelling, replicating findings in animal models. Importantly, genome wide analysis and pharmacological inhibitor experiments identified ferroptosis, an iron- and lipid-dependent cell death pathway linked to fibroblast activation as a novel PCD cardiomyopathy disease mechanism.
Primary carnitine deficiency (PCD) is an autosomal recessive disorder resulting in insufficient cellular carnitine \((\beta\text{-hydroxy-\gamma\text{-trimethylammonium butyrate})}\) uptake and low cytoplasmic concentrations \(^{1}\). PCD is caused by pathogenic variants in the \(SLC22A5\) gene, leading to loss of function of the encoded organic cation transporter novel family member 2 (OCTN2). OCTN2 is strongly expressed in the myocardium, skeletal muscle, fibroblasts, renal tubules, placental tissue and intestine \(^{2}\). OCTN2 transports carnitine in a sodium-dependent manner and maintains intracellular carnitine concentrations 20-50-fold higher than in the extracellular space \(^{3,4}\). In the cytoplasm, carnitine palmitoyltransferase 1 (CPT1) catalyzes the formation of acylcarnitine from carnitine and long chain acyl-CoA. The carnitine-acylcarnitine translocase (CACT) transports acylcarnitine across the mitochondrial membrane into the mitochondria, where carnitine palmitoyltransferase 2 (CPT2) reconverts acylcarnitine to acyl-CoA that subsequently enters beta-oxidation. Low cytoplasmic carnitine concentration impairs fatty acid beta-oxidation leading to insufficient ATP generation under high workload conditions and cytoplasmic lipid accumulation. This results in glucose-dependency as energy metabolism, inhibited gluconeogenesis (due to inhibition of pyruvate carboxylase) and diminished ketogenesis (due to a lack of acetyl-CoA derived from beta-oxidation) \(^{1}\). The strong reliance of cardiomyocytes on fatty acids as energy substrate makes the heart particularly susceptible to PCD pathomechanisms. Apart from the mitochondrial fatty acid transfer, carnitine conjugation has a crucial role to reduce the number of coenzyme A (CoA) molecules attached to acyl residues. Excessive accumulation of cytoplasmic acyl-CoA results in the formation of ceramides, TAG and cholesteryl-ester that were reported to induce inflammation and apoptosis in different organs \(^{5}\).

Typical clinical PCD symptoms are hypoglycemia and hypoketonemia under fasting conditions, liver dysfunction, muscular weakness and dilated cardiomyopathy (DCM) \(^{6,7}\). Symptomatic patients are typically diagnosed in the first five years of life and receive a lifetime treatment with high dose of carnitine (100–200 mg/kg/day)\(^{6}\). Despite inhibited cellular uptake, PCD patients have low plasma carnitine concentrations (0-5 µmol/l, physiological: 25-50 µmol/l \(^{8}\)) because OCTN2-mediated carnitine reabsorption in renal proximal tubules system is impaired \(^{9}\). Relevant side effects of carnitine supplementation include nausea, vomiting, abdominal cramps, diarrhea, a fishy body odor, and accumulation of atherogenic trimethylamine N-oxide (TMAO) \(^{10-12}\). Moreover, numerous untreated PCD patients reach
adulthood and remain asymptomatic but still have an increased risk for sudden cardiac death. Detailed mechanisms of the PCD DCM remain poorly understood. PCD is a rare disease. Prevalence range from 1:20,000–1:70,000 (United States), 1:120,000 (Australia) to 1:8000 - 1:17,000 (China). The Faroe Island, an isolated archipelago in the Northern Atlantic, has by far the highest prevalence with 1:300. The overall approximate allelic frequency of SLC22A5 pathogenic variants in the population is 0.5-1%. SLC22A5 was recently reported to be frequently associated with autosomal recessive mitochondrial disorders in gnomAD. Pathogenic variants were found in all SLC22A5 exonic coding regions but are often located in the first exon of the SLC22A5 gene. The SLC22A5 c.95A>G (N32S) mutation is the characteristic PCD mutation on Faroe Island.

Juvenile visceral steatosis (JVS) mice are an animal model of carnitine deficiency. This strain was discovered by coincidence to have an OCTN2 p.L352R missense mutation. JVS mice demonstrate high renal carnitine excretion and tissue lipid accumulation in the first week and hyperammonaemia, hypoglycemia, hepatic microvesicular steatosis, and growth retardation three weeks after birth. JVS mice develop cardiac hypertrophy 10 days after birth associated with cardiac steatosis, accumulation of diacylglycerols (DAG) and triglycerides (TAG), but no ceramide, lower myocardial ATP content, and high expression of the pyruvate dehydrogenase (PDH) inhibitor PDH kinase 4 (PDK4). A pharmacological carnitine deficiency animal model was established by administering the competitive OCTN2- and BBOX1 (γ butyrobetaine hydroxylase) inhibitor N-trimethyl-hydrazine-3-propionate (THP) to wildtype rats for three weeks. The rats revealed increased renal carnitine excretion and hepatic steatosis but no cardiac or skeletal phenotype. Compensatory upregulation of proteins involved in the carnitine shuttle system, such as CPT1 has been previously described. No human induced pluripotent stem cell (hiPSC) model of PCD has been published so far.

The aim of this study was the development of a predictive hiPSC model of PCD DCM. Two hiPSC lines derived from an established control hiPSC line (OCTN2 (+/+)) were generated by CRISPR/Cas9 technology. The lines carried either a full OCTN2-knockout (OCTN2 (-/-)) or the homozygous missense founder mutation (OCTN2 (N32S), SLC22A5 c.95A>G) characteristic for PCD patients from the Faroe Islands. Cardiomyocytes were differentiated, and the PCD disease phenotype was analyzed in genome-wide, molecular, functional (contractility), and morphological assays. These experiments show that this hiPSC PCD model replicates a wide
range of PCD DCM characteristics found in patients and animal models and moreover reveals ferroptosis linked to fibroblast activation as a novel disease mechanism.
Results

CRISPR/Cas9

An established control hiPSC line (OCTN2 (+/+)) was used for the CRISPR/Cas9 engineering approach and served as the isogenic control. The CRISPR/Cas9 strategy is presented in Supplementary Figure 1A, B. A representative Sanger sequencing trace of successfully edited clones is depicted in Supplementary Figure 2A and shows the OCTN2 wildtype sequence (upper lane) and the heterozygous silent point mutation (c.277C>T) in the isogenic control hiPSC line. The middle lane shows the homozygous introduction of the OCTN2 (N32S) c.95A>G founder point mutation in exon 1 of the SLC22A5 gene. Due to the large deletion, Sanger sequencing trace of OCTN2 (-/-) in the lower lane could not be aligned. PCR products derived from internal and flanking primers of both CRISPR cutting sites in the OCTN2 (+/+) hiPSC line are shown in Supplementary Figure 2B-E. The successful knockout was confirmed by quantitative reverse transcription PCR (qPCR) of the SLC22A5 transcript (Supplementary Figure 2C). Southern blots validated the integrity of the edited locus. Supplementary Figure 3A displays the predicted cutting sites for the two restriction endonucleases HindIII and EcoRI of the SLC22A5 gene. Predicted fragment size and Southern blot results are shown in Supplementary Figure 3B and C. Nanostring nCounter human karyotype assay revealed normal karyotype for all lines (Supplementary Figure 4).

Functional analysis

Cardiomyocytes were successfully differentiated from all 3 hiPSC lines. Supplementary Figure 5A shows no difference in the percentage of cardiac troponin T (cTnT) positive cells for the cardiac differentiation experiments between the three lines (OCTN2 (+/+)) 87.5±2.5%, n= 10 differentiations; OCTN2 (N32S): 84.1±5.0%, n= 10 differentiations; OCTN2 (-/-): 86.2±8.2%, n= 9 differentiations). Cardiac differentiation efficiency (output-hiPSC-CM / input-hiPSC cells) revealed significantly lower differentiation efficiency for OCTN2 (N32S) (23.5±10.5%) and OCTN2 (-/-) (19.4±2.8%) compared to OCTN2 (+/+)) (79.0±10.0%) (Supplementary Figure 5B).

Engineered Heart Tissue (EHT) samples were subjected to video-optical force analysis under spontaneous beating conditions starting on day 7 (Supplementary Figure 5C-H). Contractile parameters changed in EHTs from all three cell lines and reached a plateau after day 21. OCTN2 (-/-) showed a lower force, higher contraction time and shorter resting length for the
entire culture time. Beating frequency was higher for OCTN2 (N32S) and OCTN2 (-/-) during the initial phase of development, but not thereafter. Relaxation time was longer only for OCTN2 (-/-) for the last 2 weeks of development. No difference could be detected for the RR scatter as a surrogate for arrhythmic beating. Contractile values of day 21 were compared (Figure 1A-F) and showed lower force and a shorter resting length for OCTN2 (-/-) compared to OCTN2 (+/+). OCTN2 (+/+): 0.194 ± 0.004 mN, n = 153 EHTs; OCTN2 (N32S): 0.16 ± 0.01 mN, n = 108 EHTs; OCTN2 (-/-): 0.11 ± 0.01 mN, n = 91 EHTs. Both OCTN2-defective genotypes exhibited a higher contraction time, and OCTN2 (-/-) a higher relaxation time. Figure 2A, B depict representative average contraction peaks and video-optical EHT images. A representative video of EHTs from all three genotypes can be found in the supplement (Supplementary Video 1-3). The contractile phenotype was associated with a shorter APD90 of 211.0 ± 13.6 (SEM, n=7) in OCTN2 (N32S) versus 288.2 ± 15.5 (SEM, n=9) in OCTN2 (+/+). Importantly, force and resting length of both OCTN2-defective genotypes showed a positive correlation with the cardiomyocyte purity of the input cell population (Figure 3A, B), implying an important role of non-cardiomyocytes. To study the ability to metabolize long-chain fatty acids (LCFA), EHTs were switched to a medium containing only LCFA plus carnitine [50 µM]. Force remained stable in OCTN2 (+/+ EHTs but declined in OCTN2-defective EHTs (Figure 3C), indicating a reduced ability to metabolize LCFA.

Delta glucose and lactate values were higher for OCTN2 (N32S), but not OCTN2 (-/-) compared to OCTN2 (+/+). The delta lactate/delta glucose ratio as a surrogate for anaerobic glucose metabolism showed no difference (Supplementary Figure 6A-C). Higher glucose consumption for OCTN2 (N32S) and OCTN2 (-/-) became evident when normalized to workload (force x beating frequency) (OCTN2 (+/+): 0.23 ± 0.01 mM/bpm × mN; OCTN2 (N32S): 0.3 ± 0.1 mM; OCTN2 (-/-): 0.4 ± 0.1 mM) (Figure 3D).

Proteomics, Seahorse

3,425 proteins were detected by tandem mass tag (TMT)-based proteomic analysis, of which 1,772 proteins differed significantly between OCTN2 (+/+) and OCTN2 (N32S) and 2050 differed significantly between OCTN2 (+/+) and OCTN2 (-/-), respectively (p<0.05). A detailed summary of detected proteins is shown in Supplementary Table 1. Principal component analysis revealed separate clustering of OCTN2 (+/+) from OCTN2 (N32S), and OCTN2 (-/-)
Volcano plot depiction highlights a higher abundance of fibrosis-related- and extracellular matrix proteins like caldesmon1 (CALD1), collagen type I alpha 1 chain (COL1A1), transgelin 2 (TAGLN2), fibronectin 1 (FN1) and vitronectin (VTN) in OCTN2 (N32S) EHTs (Figure 4B). Moreover, ceramide transfer protein (CERT) was among the 10 most abundant proteins in OCTN2 (N32S). In contrast, the fatty acid transporters cluster of differentiation 36 (CD36), fatty acid-binding protein 5 (FABP5) and cardiomyogenesis transcriptional regulator GATA binding protein 4 (GATA4) were among the top 10 lower abundant proteins in OCTN2 (N32S).

Detailed grouping of proteins related to their participation in pathways, such as cardiac physiology, lipid metabolism, glycolysis, carnitine shuttle, electron transport chain, TCA cycle, and beta-oxidation revealed a concordant expression pattern for both, OCTN2 (N32S) and OCTN2 (-/-) versus OCTN2 (+/+). KEGG pathway overrepresentation analysis revealed enrichment of N-glycan- and O-glycan biosynthesis, ferroptosis, and cholesterol metabolism in OCTN2 (N32S) high abundant proteins. Conversely, enrichment analysis of lower abundant proteins in OCTN2 (N32S) revealed the KEGG pathways pyruvate- and propanoate metabolism, glycolysis, pentose phosphate pathway glyoxylate and dicarboxylate metabolism, and different pathways associated with amino acid metabolism. A detailed summary of specific proteins in the enriched pathways is depicted in Supplementary Table 2 and 3. Quantification of mitochondrial DNA revealed a lower level in OCTN2 (N32S), indicating lower abundance of mitochondria. Both OCTN2-defective lines showed lower oxygen consumption rate and baseline, oligomycin, FCCP and rotenone conditions in Seahorse experiments (Supplementary Figure 7A, B).

Carnitine supplementation: Acylcarnitine and ceramide content, force, lipid mass spectrometry, TEM

EHT media was supplemented with carnitine (2 mM) for the entire culture time. This was accompanied by a reduction of glucose consumption and lactate production for all genotypes (Supplementary Figure 8A-C). Notably, glucose consumption normalized to cardiac workload was reduced only for the two OCTN2-defective cell lines (Figure 5A). This was associated with a small increase in force for all cell lines (Figure 5B, C) and a substantial increase in relaxation time (Supplementary Figure 8D-G). Notably, transcript levels of PDK4, the inhibitor of PDH and important metabolic regulator, were higher in OCTN2 (N32S) versus OCTN2 (+/+) and attenuated to isogenic control level by carnitine supplementation (Supplementary Figure 8H).
Liquid chromatography–mass spectrometry (LC-MS) revealed 5-fold lower content for C16:1-, C18:0-, C18:1- and C18:2 acylcarnitines in OCTN2 (N32S) compared to OCTN2 (+/+). Carnitine supplementation resulted in a higher content of C16:0-, C16:1-, C18:1- and C18:2 acylcarnitines for OCTN2 (+/+ ) and C18:1-, C18:2 acylcarnitines for OCTN2 (N32S). Ceramides are one metabolite of accumulated cytoplasmic acyl-CoA. Quantification of ceramide content (Cer16:0, Cer18:0, Cer22:0, Cer24:0, Cer24:1) revealed no difference between the genotypes and no effect of carnitine supplementation (Figure 5D, E).

Transmission electron microscopy (TEM, Figure 6A-F) showed elongated myofilaments and structured mitochondria in OCTN2 (+/+) EHTs. OCTN2 (N32S) displayed a lower abundance of mitochondria and structural mitochondrial defects, and a high frequency of large lipid droplets in close association with mitochondria and sarcomeres. OCTN2 (-/-) also exhibited mitochondria with degraded structure and increased membrane density but no pronounced aggregation of lipid droplets. Carnitine supplementation appeared to increase mitochondria frequency for all genotypes and to reduce the frequency of lipid droplets for the OCTN2-defective genotypes.

Single nuclear RNA sequencing

A pool of 4 EHTs per genotype was subjected to single nuclear RNA sequencing (snRNA seq). OCTN2 (+/+), (N32S) and (-/-) samples were sequenced with an average sequencing depth of 39,324, 28,771 and 26,374 read pairs per nucleus. Following quality control filtering, snRNA seq data of all three genotypes were pooled to a total number of 11,225 nuclei ((OCTN2 (+/+)) = 3,135, OCTN2 (N32S) = 3,761, OCTN2 (-/-) = 4,329 cells). Uniform manifold approximation and projection (UMAP) and leiden clustering revealed 5 main cell clusters. Marker genes for these clusters delineated: cardiomyocytes, proliferating cardiomyocytes, fibroblasts, endothelial and myeloid cells (Figure 7A). In OCTN2 (+/+), cardiomyocytes represented 94% of all cells with 14% of these cells showing markers of proliferation (Figure 7B, C). Sub-clustering of cardiomyocytes revealed 10 subclusters (Supplementary Figure 9). Subcluster CM4 was dominant in OCTN2 (+/+), while subclusters 1 and 2 were more prominent in OCTN2-defective lines. Interestingly, KEGG analysis revealed enrichment of the GPR40 pathway in CM4, describing free fatty acid receptor 1 signalling (Supplementary Figure 9C). A lower representation of CM4 in the OCTN2-defective lines is compatible with the lower abundance of fatty acid transporters in the proteomics analysis. In OCTN2 defective lines, cardiomyocytes
represented a smaller fraction of all cells (OCTN2 (N32S): 85%, OCTN2 (-/-): 67%). Reversely, these lines showed a higher fraction of fibroblasts (OCTN2 (+/+): 4%, OCTN2 (N32S): 10%, OCTN2 (-/-): 23%), (Figure 7B, C). Sub-clustering of fibroblasts identified 4 states. Fibroblasts states with markers of TGF-beta signaling, proliferation and secretion (FB1, FB3, FB4), were more prominent in OCTN2-defective lines (Figure 7D, E, F, Supplementary Figure 10A). Genotype-specific analysis of significant KEGG pathway enrichment in all fibroblast subcluster revealed relaxin-, ECM- and focal adhesion-related pathways (Supplementary Figure 10B).

Endothelial and myeloid cells were almost absent in OCTN2 (+/+) and represented 5% and 1% in OCTN2 (N32S) and 8% and 1% in OCTN2 (-/-) respectively (Figure 7B). Endothelial cells expressed PDE3A, CASC15 and the typical marker gene MECOM, myeloid cells expressed PTPRC (CD45), CD163 and AOAH (Figure 7C). Comparative analysis of significant KEGG pathway enrichment was not possible because significant pathways could only be detected for OCTN2 (-/-) endothelial cells (Supplementary Figure 10B).

Ferroptosis, fibroblast activation

Proteomics analysis revealed enrichment of proteins related to the KEGG pathway ferroptosis, an iron-dependent lipid peroxidation-mediated cell death mechanism. Extraction of an extended list of pro- and anti-ferroptotic proteins from the proteomics data set identified a strikingly uniform regulation with a higher abundance of pro- and a lower abundance of anti-ferroptotic proteins in the OCTN2-defective genotypes (Figure 8A). Noteworthy among these were also the key regulators ACSL4 and LPCAT3. These two proteins synergistically drive the accumulation of iron-dependent lethal lipid peroxides (LPO). On the other hand, pro- and anti-ferroptosis transcripts did not show a differential expression in snRNA seq, suggesting a post-transcriptional regulation (Figure 8B). Evidence for ferroptosis and fibroblast activation in this study and previous reports demonstrating a mechanistic link between these two pathways were the reason to analyse the effect of the potent ferroptosis inhibitor liproxstatin on fibrosis markers. OCTN2 (N32S) EHTs revealed higher transcript levels of ACTA2, COL1A1, POSTN, TGFB, FN, and CCN2 than OCTN2 (+/+). Liproxstatin induced a significant attenuation of fibrosis transcript levels in OCTN2 (N32S), which was associated with a moderate increase in force development (Figure 8C-D).
Discussion

This study aimed to establish a human PCD DCM \textit{in vitro} model. The main results of this work are 1: successful genetic engineering of two experimental hiPSC lines, a homozygous OCTN2 (N32S) and an OCTN2 (-/-) knockout hiPSC line; 2: Replication of the PCD DCM phenotype in the hiPSC PCD model by low acylcarnitine tissue content and low force development, complex metabolic remodeling, and ultrastructural alteration; 3: Validation of the of OCTN2 (N32S) loss-of-function disease phenotype by high-level concordance with the OCTN2 (-/-) knockout hiPSC line across various assays; 4: Discovery of ferroptosis activation linked to fibroblast activation as novel PCD DCM mechanism.

The role of \textit{SLC22A5} in hiPSC biology is not yet understood. RNA sequencing data from Liu et al. \cite{35} demonstrated a continuous expression of \textit{SLC22A5} during all stages of hiPSC cardiomyocyte differentiation, suggesting the relevance of carnitine metabolism throughout this process. OCTN2-defective hiPSC lines might therefore be compromised during cardiac differentiation. Compatible with this, the OCTN2 (+/+ ) control hiPSC line outperformed both OCTN2-defective hiPSC lines with respect to differentiation efficiency (cardiomyocyte output in relation to the input of hiPSC). The approach to engineer the human-relevant OCTN2 (N32S) loss of function point mutation in parallel with a complete OCTN2 knockout turned out to be very insightful since the concordant changes of several parameters (e.g. contractile parameters, protein expression of metabolic markers, clustering of (non)-cardiomyocyte subpopulations) in both defective lines validated the implication of OCTN2.

Typical features of PCD DCM in both patients and established animal models are markedly reduced tissue contents of different carnitine derivates, functional impairment of glucose and lipid metabolism, myocardial steatosis, severe hyperglycemia\textsuperscript{22,24} and short QT syndrome\textsuperscript{36,37}. Several key aspects could be replicated in this hiPSC-CM model: OCTN2-defective EHTs revealed lower force and higher sensitivity of force to fatty acid-based media, reduced acylcarnitine tissue content intracellular lipid droplet accumulation, and shorter action potential duration. Genome-wide analysis revealed complex metabolic remodeling and mitochondrial dysfunction. These alterations are compatible with (acyl)-carnitine deprivation and have previously been described in PCD-animal models\textsuperscript{24,37}. Upregulation of carnitine shuttle proteins likely represents a compensatory effect in response to carnitine deprivation, similar to findings in secondary carnitine deficiency animal models\textsuperscript{38,39}. Moreover, this model
showed a lower abundance of glycolytic proteins but higher glucose uptake. This is compatible with the poor correlation between glycolytic enzyme abundance and glycolysis in previous studies.\(^{41}\)

One consequence of low cytoplasmic carnitine concentration is a decrease of acylcarnitine formation and subsequent beta-oxidation.\(^8\) Indeed, mass spectrometry analysis revealed lower tissue content of several long-chain acylcarnitines in OCTN2 (N32S) EHTs. In addition, declining force in LCFA media suggested a defect in LCFA metabolism. Surprisingly, mass spectrometry did not reveal a difference in ceramide content between the genotypes. However, ceramides do not represent the final product but a metabolic intermediate that can be processed to sphingolipid derivates such as glucosylceramides and sphingomyelin.\(^{42}\) For this conversion, ceramides are transported from the endoplasmic reticulum (ER) into the trans-Golgi apparatus by the ceramide transporter CERT.\(^{43}\) Remarkably, CERT was among the 10 most significantly higher abundant proteins in OCTN2 (N32S) EHTs, suggesting that it belongs to the compensatory mechanisms and prevents the accumulation of toxic ceramides.

Carnitine supplementation (2 mM) had a strong effect on metabolic aspects of the PCD disease phenotype like acylcarnitine tissue content, glucose consumption per cardiac work, PDK4 transcript level and lipid droplet accumulation. Nevertheless, the force restoration was minor and the carnitine-induced increase in force was in the same range for all three genotypes, suggesting a non-specific effect potentially related to induction of sodium current and subsequent inhibition of Na\(^+/K^+\)-ATPase as previously described.\(^{44,45}\) The discrepancy between strong metabolic and small force effects of carnitine supplementation suggests additional mechanisms to be relevant. The shorter EHT resting length, the positive correlation between cardiomyocyte percentage of the input cell population with force and resting length and the enrichment of extracellular matrix KEGG pathway the OCTN2-defective genotypes suggest fibrosis to be relevant. In support of this, snRNA seq revealed a more prominent fibroblast cluster in the OCTN2 –defective genotypes, which expressed markers indicative of activated and secretory state. Of note, markers of activated fibroblasts in this study (POSTN, FN1, FAP, NOX4) overlap substantially with the fibroblast signatures in two failing heart snRNA seq DCM studies.\(^{46,47}\) Interestingly, the central role of fibroblasts in this hiPSC-CM PCD model is paralleled by clinical findings of strong myocardial fibrosis in PCD patients.\(^6,48,49\).
Proteomic analysis provided evidence for ferroptosis, an iron-dependent cell death mechanism related to lipid peroxidation, to be relevant in this model. Ferroptosis is well compatible with PCD DCM, since it is driven by the accumulation of polyunsaturated fatty acids (PUFAs) linked to coenzyme A (CoA) in cell membranes. PUFA accumulation is relevant for PCD as cytosolic carnitine deficiency impairs PUFA metabolism to acylcarnitine. ACSL4 catalyzes the esterification of long-chain PUFA to acyl-CoA and represents a central pro-ferroptotic regulator. This marker showed a higher expression and protein abundance in OCTN2-defective lines in contrast to other ferroptotic proteins. Notably, glutathione is an important cofactor for GPX4 activity and proteins involved in glutathione metabolism such as Glutamate-Cysteine Ligase (GCLC), Glutathione Synthetase (GSS) and glutathione-disulfide reductase (GSR) were lower abundant in OCTN2-defective lines.

Importantly, ferroptosis was recently shown to be linked to fibrosis development and cardiomyopathy. Mechanistically, profibrotic factors released from ferroptotic cells were identified to drive fibroblast activation. A similar mechanistic link is likely relevant in this model since the ferroptosis inhibitor liproxstatin induced a reduction of fibrosis transcript levels and higher force. Overall, the discovery of ferroptosis activation reveals novel insight into the development of PCD-associated metabolic cardiomyopathy and is paving the way to the development of specific antifibrotic treatment strategies.
Experimental procedures

HiPSC cell culture conditions

An established hiPSC control cell line (hiPSCreg code UKEi001-A) derived from a healthy individual served as the starting point for the genetic engineering approach and as the isogenic control for the engineered hiPSC lines. This hiPSC line was generated by reprogramming dermal fibroblast from a skin biopsy using the CytoTune (Life Technologies) 2.0 Sendai Reprogramming Kit under feeder-free conditions. All basic stem cell culture work was performed as recently described. In brief, hiPSC culture was based on the expansion of a master cell bank (MCB) at passage 25-35 on Geltrex-coated cell culture flasks in FTDA-medium (Supplementary Table 4) under hypoxic conditions (5% O₂). Standard passaging was performed twice a week (3-4 day passaging interval) with Accutase solution (Sigma-Aldrich). Plating density was 4.5-7.0×10⁴ hiPSC/cm². Maximal expansion was for 40 passages with regular screening for mycoplasma contamination by PCR amplification. SSEA3 surface marker served as a pluripotency marker and was analyzed by flow cytometry. All procedures involving the generation and analysis of hiPSC lines were approved by the local ethics committee in Hamburg (Az PV4798, 28.10.2014).

CRISPR/Cas9-mediated gene editing

OCTN2 (N32S) missense mutation

The SLC22A5 gene locus was Sanger sequenced in the hiPSC OCTN2 (+/) control line. CRISPR/Cas9 technology was used to engineer the c.95A>G (N32S) mutation into the SLC22A5 wild type. IDT Custom Alt-R CRISPR-Cas9 gRNA software and CRISPOR were used to identify potential gRNA binding sites at the gene locus. The OCTN2 NCBI Reference (NG_008982.2) was provided as a target sequence. Targets for gRNA’s were chosen based on the lowest cut-to-mutation distance under consideration of a high on-target potential and low off-target risk. A single-stranded oligodeoxynucleotide (ssODN) served as an exogenous donor template, containing the OCTN2 c.95A>G, p.N32S mutation. Additionally, a silent mutation was introduced in the PAM sequence to prevent CRISPR/Cas9 re-cutting after successful genomic integration of the template by HDR. Edited clones were identified by PCR amplification and subsequent Sanger sequencing. A schematic overview of the HDR strategy is depicted in Supplementary Figure 1A. SsODN- and gRNA sequences are shown in Supplementary Table 5.
OCTN2 (-/-) knockout

A combinatorial CRISPR strategy was used to engineer a knockout of the SLC22A5 gene in the isogenic control hiPSC OCTN2 (+/+). Two gRNA's were designed to introduce a deletion of 17.3 kb spanning from the promoter region to exon 5 (NG_008982.2). For deletion validation primer pairs were designed to amplify products inside the deletion region and the gRNA target sites. Also, primers flanking the two cutting sites were designed. Edited clones were identified by PCR amplification and subsequent Sanger sequencing. A schematic overview of the knockout strategy is displayed in Supplementary Figure 1B. To distinguish between unedited, heterozygous and homozygous edited clones, the PCR products were separated by agarose gel electrophoresis (1% (w/v)), followed by Midori green staining. The target gRNA sequences are shown in Supplementary Table 5.

Nucleofection

The AmaxaTM P3 Primary Cell 4D-Nucleofector X Kit L (Lonza) was used for delivery of the CRISPR/Cas9 ribonucleoprotein (RNP) complex into hiPSCs. A working cell bank aliquot of the control hiPSC (passage 25-30) was cultured for at least 2 passages on a 6-well plate to reach 60-70% confluency on the day of nucleofection. HiPSCs were incubated with the apoptosis inhibitor Y-27632 (10 µM) two hours prior to nucleofection. The fluorescence-labeled tracrRNA-ATTO 550 (IDT) was used to monitor the electroporation efficiency. The tracrRNA oligos and the CRISPR-Cas9 crRNA (IDT) oligos were resuspended in RNAse-free IDTE Buffer (IDT) to a final stock concentration of 100 µM. For gRNA duplex formation, 5 µL of crRNA (100 µM) were annealed with 5 µL tracrRNA (100 µM), incubated for 5 min at 95 °C and cooled down to room temperature. For formation of the RNP- complex, 5 µL of the gRNA duplex were mixed with 5 µL Cas9 protein (61 µM, IDT) and incubated for 1.5 hours at room temperature under light protection. For the knockout approach 5 µL of the second gRNA duplex were added to the suspension in addition. To prepare the nucleofector solution, 82 µL P3 reagent and 18 µL supplement reagent (Lonza) were mixed per reaction according to the instruction of the AmaxaTM P3 Primary Cell 4D-Nucleofector X Kit L (Lonza). HiPSCs were washed twice with PBS buffer and dissociated into single cells with 1 mL accutase solution (Sigma) per well at 37 °C. The dissociation was stopped by adding 1 mL FTDA medium per 6-well. The hiPSCs were resuspended in the media by gentle pipetting and centrifuged for 2 min at 200xg. 8x10^5 hiPSCs were used in 100 µL nucleofector solution per electroporation reaction.
Single-stranded DNA oligonucleotide (ssODN) repair template oligos were resuspended in IDTE Buffer (IDT) to a stock concentration of 100 µM. 1 µL of ssODN repair template (100 µM) and 4 µL of RNP-complex were mixed with the hiPSC solution by gently pipetting, incubated for 5 min at room temperature and transferred to the nucleofection cuvette. Additionally, 1 µL Alt-R Cas9 Enhancer (100 µM, IDT) was added to the solution to promote transfection efficiency. The nucleofection cuvette was placed in the 4D-Nucleofector (Lonza) and hiPSCs were nucleofected by using the program CA137. After nucleofection, the cuvette was incubated for 5 min under cell culture conditions. Subsequently, hiPSCs were seeded in conditioned medium supplemented with Y-27632 (10 µM) and bFGF (30 ng/ml) (Supplementary Table 4) on a Matrigel-coated 24-well plate for 72 hours at 37 °C.

Subcloning and off-target analysis

72 hours after nucleofection, hiPSC were dissociated with Accutase and seeded in conditioned medium with Y-27632 (10 µM) and bFGF (30 ng/mL) at low seeding densities of 100, 250, 750, 1000 hiPSCs per well (10 cm²) in a Matrigel-coated 6-well plate. In addition, the remaining nucleofected hiPSCs were seeded at a higher density of 5x10⁵ cells per well (10 cm²) in a Matrigel-coated 6-well plate. Low-density hiPSC seedings were expanded for 9 to 10 days under daily conditioned medium change until clonal hiPSC colonies reached a size appropriate to pick. HiPSC cultures were incubated with conditioned medium with Y-27632 (10 µM) for 2 hours and sterile 100 µL-pipette tips were used to carefully scrape individual colonies from the 6-well plate and transfer them to Matrigel-coated 48-well plates into individual wells. 30 to 50 clones were picked per transfection approach and were sub-cultivated for 3-4 more days before they reached confluency and were splitted with a ratio of 1:2 into two 48-well copy plates. Colonies were again expanded with daily medium change until they reached confluency. One of the copy plates was used for cryo-preservation, while the second plate was used for DNA isolation. Cryopreservation was performed in 90% FBS and 10% DMSO.

QIAcube HT System (Qiagen) and QIAamp 96 DNA QIAcube HT kit (Qiagen) were used for DNA isolation according to the manufacturer’s instructions. Cryotubes of successfully edited hiPSC clones were thawed and expanded for master cell bank and working cell bank. Ten most likely off-targets were predicted (*in silico* tool IDT, Custom Alt R CRISPR-Cas9 gRNA software and CRISPOR software). Corresponding PCR primers were designed and PCR products were
analysed by 1% (w/v) agarose electrophoresis and Sanger sequencing. Off-target primer sequences can be found in Supplementary Table 6.

Karyotyping

Karyotype analysis was performed using the nCounter Human Karyotype Panel (Nanostring Technologies) according to the manufacturer’s instructions with 250 µg DNA as starting material. The nCounter CNV Collector Tool software (Nanostring) was used for analysis.

Cardiac differentiation

HiPSC were differentiated into cardiomyocytes with an embryoid body (EB)- and growth factor-based three-stage protocol which was recently described \(^{54}\). In brief, hiPSC were expanded on Geltrex-coated T80-flasks to a confluency of 90-100% and detached with EDTA. The formation of EBs was induced in 500 mL spinner flasks with a density of 30-35x10^6 hiPSCs per 100 mL of EB formation medium (Supplementary Table 4). HiPSC suspension was cultivated overnight at 40 rpm glass ball impeller rotation speed. Mesoderm induction was induced in mesoderm induction medium (Supplementary Table 4) with a volume of 200-300 µL EB per pluronic-coated T175-flask for three days under hypoxic conditions (5% O₂) with 50% media exchange daily. After washing the EBs again, cardiac differentiation was induced in cardiac differentiation medium 1 (Supplementary Table 4) with a volume of 250-300 µL EBs per pluronic-coated T175-flask with 50% media exchange daily for three days under normoxic conditions (21% O₂). Then, media was completely removed and exchanged for cardiac differentiation medium 2 (Supplementary Table 4). After a daily 50% medium change for four days, culturing medium was exchanged with cardiac differentiation medium 3 (Supplementary Table 4). After washing EBs in HBBS-solution buffer, beating cardiomyocytes were dissociated with collagenase II solution (200 units/mL; Worthington) containing myosin II ATPase inhibitor N-benzyl-p-toluene sulphonamide (BTS) for 2-3 hours until dispersing single cells could be observed. Dissociated hiPSC-CM were frozen in freezing media containing 90% FBS and 10% DMSO or resuspended in EHT casting medium for subsequent EHT generation. Differentiation efficiency (% cTNT-positive cells) was determined by fluorescent-labeled cardiac troponin T (cTNT)- antibody (Miltenyi Biotech) by flow cytometer FACSCanto II (BD). Adjustment of gates adjusted according to the isotype control and performed with FACSDiva software (BD).
Differentiation runs with at least 75% cTNT-positive cells were used for further functional experiments. FACS reagents are shown in Supplementary Table 7.

Engineered heart tissues (EHT)

Engineered heart tissues (EHT) were generated as recently described. In brief, dissociated hiPSC-CMs were centrifuged (100xg, 10 minutes) and resuspended in EHT casting medium containing DMEM, horse serum, and glutamine (Supplementary Table 4). Polytetrafluoroethylene (PTFE) spacers (EHT Technologies) were placed in a warm 2% (w/v) agarose/PBS solution in a 24-well plate. Agarose solidification at room temperature led to the formation of agarose molds. PTFE spacers were removed from the 24 well plates and flexible polydimethylsiloxane (PDMS) posts were placed on the 24-well plates so that pairs of elastic PDMS posts reached into each casting mold. 100 μL of the mastermix (Supplementary Table 4) containing 2x DMEM, Y-27632, fibrinogen and 1.0x10^6 hiPSC-CMs was resuspended in EHT casting medium, rapidly mixed with 3 μL thrombin and pipetted into one agarose casting mold. Afterwards the preparation was placed in an incubator for 1.5 hours at 37 °C until a fibrin gel formed in the agarose molds around the PDMS posts. 200-300 μL of pre-warmed EHT culture medium was added dropwise into each well to ameliorate the detachment of the fibrin gel from the agarose mold. After additional 15-30 minutes of incubation, racks with fibrin gels attached to the PDMS posts were transferred into a new 24-well plate, filled with pre-warmed EHT culture medium, and incubated at 40% O\textsubscript{2}, 37 °C, 7% CO\textsubscript{2}, and 98% humidity. EHTs were cultured for 28-42 days with medium changes 3 times per week. After 5-7 days of culture, EHTs started to develop spontaneous macroscopic contractions.

Video-optical contraction analysis

EHT contractile analysis was performed as previously described. EHT contraction parameters, e.g. force, frequency, and contraction kinetics, were monitored over time of EHT development 2 hours after each medium change. EHTs were electrically stimulated as previously described by Hirt et al. PDMS racks with EHTs were mounted onto custom-made graphite pacing units and stimulated by using Grass S88X Dual Output Square stimulator (Natus Neurology Incorporated). The pacing frequency was adjusted to a value of 1.5 to 2-fold of the spontaneous beating frequency of the EHT batch with an output voltage of 2 V in
biphasic pulses of 4 ms. EHTs that were not able to follow the pacing frequency, were excluded from the analysis. Average contraction peaks were calculated with an average of 10-15 peaks.

Glucose- and lactate measurement

Glucose- and lactate concentrations were measured in EHT cell culture media by blood gas analysis. Samples were collected at baseline and after 24 hours of incubation in EHT medium on day 21 of culture and stored at -20°C. The blood gas analysis instrument ABL90 FLEX Analysator (Radiometer) was used to determine the metabolite concentrations by injecting the supernatant (0.5 mL) into the instrument with a 1 mL syringe.

Fatty acid- and carnitine supplementation

EHTs were cultured in serum-containing EHT culture medium until force values reached their plateau phase approximately at day 21-28 after generation. Then, EHTs were transferred to a serum- and glucose-free DMEM medium containing linoleic acid- and oleic acid-albumin (Sigma). The detailed serum-free fatty acid medium composition is shown in Supplementary Table 4. Contraction analysis was done daily after 2 hours of medium incubation for 4 days. Supplementation with carnitine was conducted by adding 2 mM L-carnitine hydrochloride (Sigma) to the EHT medium over the entire time of tissue cultivation.

Transmission electron microscopy

EHTs were transferred into a 24-well plate containing Tyrode’s solution (Supplementary Table 4) with 1.8 mM Ca²⁺ and 30 mM butanedione monoxide (BDM) for 3-4 hours until EHTs completely stopped contraction. For fixation, EHTs were shifted into 4% paraformaldehyde (PFA, Thermo Scientific) in 0.1 M phosphate buffer containing 1% glutaraldehyde (Science Services) overnight at 4 °C. Samples were rinsed three times in 0.1 M sodium cacodylate buffer (pH 7.2–7.4) and osmicated using 1% osmium tetroxide in cacodylate buffer. Following osmication, the samples were dehydrated using ascending ethanol concentrations, followed by two rinses in propylene oxide. Infiltration of the embedding medium was performed by immersion in a 1:1 mixture of propylene oxide and Epon (Science Services, Germany), followed by neat Epon and hardening at 60 °C for 48 hours. For light microscopy, semi-thin sections (0.5 μm) with longitudinal orientation were mounted on glass slides and stained for 1 minute with 1% toluidine blue. For electron microscopy, ultra-thin sections (60 nm) were cut and mounted
on copper grids and stained using uranyl acetate and lead citrate. Sections were examined and photographed using an EM902 (Zeiss) electron microscope equipped with a TRS 2K digital camera (A. Tröndle, Moorenweis, Germany).

Analysis of acylcarnitines and ceramides

EHT cells were homogenized in Milli-Q water (approximately $1.0 \times 10^6$ hiPSC-CM per 100 µl) using the Precellys 24 Homogenisator (Peqlab). The protein content of the homogenate was routinely determined using bicinchoninic acid. Levels of acylcarnitines and ceramides in the cell homogenates were determined by Liquid Chromatography coupled to Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS).

Acylcarnitines

Acylcarnitines were derivatized to butyl esters and using a procedure previously described with several modifications: To 100 µL of cell homogenate 750 µL of extraction solution (methanol (containing 0.005% 3,5-di-tert.-4-butylhydroxytoluol)/Milli-Q water/chloroform 4:1:1 (v/v/v)) and 20 µL of an internal standard mixture, containing deuterated acylcarnitines (Lyophilized Internal Standard MassChrom Amino Acids and Acylcarnitines from Dried Blood, Chromsystems, reconstituted in 2.5 ml, then 1:5 diluted), were added. After thorough mixing and centrifugation (16,100 RCF, 10 min, 4 °C), the supernatant was transferred to a new tube. The residue was re-extracted with 750 µL of extraction solution. The supernatants were pooled and dried under a stream of nitrogen. The evaporated extracts were treated with 200 µL of freshly prepared derivatization solution (n-butanol/acetyl chloride 95:5 (v/v)). After incubation for 20 min at 60 °C in a ThermoMixer (Eppendorf) at 800 rpm, the samples were again dried under nitrogen. After the addition of 100 µL of methanol/water 3:1 (v/v) and centrifugation (16,100 RCF, 10 min, 4 °C), 80 µL of supernatant were transferred to autoinjector vials. LC-ESI-MS/MS analysis was performed as previously described. The LC chromatogram peaks of butyl esters of endogenous acylcarnitines and internal standards were integrated using the MultiQuant 3.0.2 software (SCIEX). Endogenous acylcarnitine species were quantified by normalizing their peak areas to the peak area of the internal standards. These normalized peak areas were normalized to the protein content of the sample.
Ceramides

To 50 µL of cell homogenate 50 µL of Milli-Q water, 750 µL of methanol/chloroform 2:1 (v/v), and internal standard (127 pmol ceramide 12:0, Avanti Polar Lipids) were added. Lipid extraction and LC-ESI-MS/MS analysis were performed as previously described. The LC chromatogram peaks of endogenous ceramide species and the internal standard ceramide 12:0 were integrated using the MultiQuant 3.0.2 software (SCIEX). Endogenous ceramide species were quantified by normalizing their peak areas to the peak area of the internal standard ceramide 12:0. These normalized peak areas were normalized to the protein content of the sample.

Actions potential

Actions potential (AP) measurements in EHT were performed with standard sharp microelectrode as described previously. The EHTs were transferred from the 24-well EHT culture plate into the AP measuring chamber by cutting the silicone posts and were fixed with needles in recording chamber. All measurements were done with tissues continuously superfused with Tyrode's solution (NaCl 127 mM, KCl 5.4 mM, MgCl$_2$ 1.05 mM, CaCl$_2$ 1.8 mM, glucose 10 mM, NaHCO$_3$ 22 mM, NaHPO$_4$ 0.42 mM, balanced with O$_2$-CO$_2$ [95:5] at 36 °C, pH 7.4). Microelectrodes had a resistance between 25 - 55 MΩ when filled with 2 M KCl. The signals were amplified by a BA-1s npi amplifier (npi electronic GmbH, Tamm, Germany). APs were recorded and analyzed using the Lab-Chart software (version 5, AD Instruments Pty Ltd., Castle Hill NSW, Australia). Take-of potential (TOP) was defined as the diastolic membrane potential directly before the upstroke.

Analysis of mitochondrial respiration

The Seahorse™ XF96 extracellular flux analyzer was used to assess mitochondrial respiration as previously described (Mosqueira et al, 2019), using the Mito Stress Kit (Agilent Technologies). Briefly, cryopreserved isogenic sets of hiPSC-CMs were seeded into Matrigel™-coated (BD #356235) XF96 well plates at a density of approximately 5000 cells/mm$^2$. HiPSC-CMs were cultured for 2 days in RPMI1640 (USBiological Life Sciences #R9010-01) supplemented with B-27 with insulin (LifeTechnologies #0080085-SA), 2 mM L-glutamine (Life Technologies #25030-081), 10% Fetal Bovine Serum (Gibco #16000044) and 0.6 mM CaCl$_2$. After 2 weeks, medium was exchanged for XF basal medium (Agilent Technologies #102353).
supplemented with 10 mM glucose (Sigma #G7528), 1 mM sodium pyruvate (Sigma #S8636) and 2 mM L-glutamine (Life Technologies #25030-081) 1h before the conduction of the assay. Selective inhibitors were sequentially injected during the measurements (1.5 μM oligomycin, 0.4 μM FCCP, 1 μM rotenone; Agilent Technologies), following the manufacturer’s instructions. The measured Oxygen Consumption Rate (OCR) values were normalized to the number of cells in each well, quantified by 1:400 Hoechst33342 staining (Sigma #B2261) in PBS (Gibco #14190-094) using fluorescence at 355 nm excitation and 460 nm emission in an automated imaging platform (CellaVista, Synentec).

Tandem Mass Tag (TMT)-based quantitative proteomic analysis

EHT harvesting for quantitative mass spectrometry

EHTs from hiPSC-CMs were cultured in EHT medium for 28 days before proteome analysis. EHTs were washed twice in warm PBS buffer and incubated with collagenase II solution (collagenase II (200 units per mL), HBSS minus Ca\(^{2+}\)/Mg\(^{2+}\), HEPES (10 mM), Y-27632 (10 µM), and BTS (30 µM)) in a falcon tube for 3 hours. Dissociated hiPSC-CMs were gently titrated with a 1000 µL-pipette (Eppendorf) until the last cluster of cells was disaggregated, spun down for 5 min at 200xg and supernatant was removed. The pellet was frozen in liquid nitrogen and stored at -80 °C before subjection to quantitative proteome analysis.

Tissue protein extraction and digestion for mass spectrometry

Cell pellets were lysed in 50 mM Tris, 0.1% SDS, pH=8.8, with protease inhibitors. After centrifugation at 4 °C at 16,000xg for 10 min, the supernatant was transferred to new 1.5 mL tubes and protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific). For each sample, 23 µg of proteins were denatured by the addition of 9 M urea, 3 M thiourea (final conc. 6 M urea, 2 M thiourea) and reduced by the addition of 100 mM DTT (final conc. 10 mM) followed by incubation at 37 °C for 1 hour, 240 rpm. The samples were then alkylated by the addition of 500 mM iodoacetamide (final conc. 50 mM) followed by incubation in the dark for 1h at room temperature. Pre-chilled (-20 °C) acetone (1:9 volume ratio) was used to precipitate the samples overnight at -20 °C. Samples were centrifuged at 16,000xg for 30 min at 4 °C and the supernatant was subsequently discarded. Protein pellets were dried using a vacuum centrifuge (Thermo Fisher Scientific, Savant SPD131DDA), re-suspended in 0.1M TEAB buffer, pH 8.2 (Sigma T7408), and 0.6 µg trypsin was added. The
digestion was performed overnight at 37 °C, 240 rpm, and stopped by adding 10% TFA (final conc. 1%). C18 clean-up was performed using Agilent Bravo AssayMAP and the eluted peptides were dried using a vacuum centrifuge.

Sample labeling for mass spectrometry

The samples were resuspended in 0.1 M TEAB and a pooled sample was made by taking the same amount of proteins from each individual sample. Samples were labeled with Tandem Mass Tag (TMT) 11-plex reagent (Thermo Fisher Scientific) following the manufacturer’s instructions. The pooled sample labeled with TMT-126 was used as an internal standard. The samples labeled with different tags of the 11-plex TMT were combined, dried, and resuspended in 300 µl of 0.1% TEA. Samples were further fractionated using high pH RP HPLC (Agilent 300Extend-C18 3.5µm 4.6x150mm P/N 763973-902) and 16 fractions were collected for each TMT mixture. All fractions were dried and resuspended in LC solution (2% acetonitrile (ACN), 0.05% TFA).

Mass spectrometry analysis

Samples were injected and separated by a nanoflow HPLC (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) on an EASY-Spray column (C18, 75 µm x 50 cm, 2 µm) using 2 hour LC gradient: 0-10 min, 4%-10%B; 10-75 min, 10%-30%B; 75-80 min, 30%-40%B; 80-85 min, 40%-99%B; 85-90 min, 99%B; 90-120 min, 4%B; A=0.1% FA in H2O and B= 0.1% FA, 80% ACN in H2O. The flow rate was 250 nl/min and column temperature was set at 45°C. The separated peptides were directly injected to an Orbitrap Fusion Lumos MS (Thermo Fisher Scientific) and analyzed using a synchronous precursor selection (SPS)-based MS3 method for TMT-labeled sample. Full MS spectra were collected on the Orbitrap with a resolution of 120,000 and scan range 375-1500 m/z. The most abundant ions were fragmented using CID and MS2 spectra were collected on a linear ion trap, with dynamic exclusion enabled. The 5 most abundant ions from every MS2 spectrum were selected and fragmented at the same time using HCD with collision energy 65% and MS3 spectra were collected on the Orbitrap with a resolution of 60,000 and a scan range of 110-500 m/z to measure the TMT reporter ions. The cycle time was set at 3 seconds.

Raw data were analyzed using Proteome Discoverer 2.4. The 16 fractions of each TMT mixtures were loaded as fractions and analyzed together. Each TMT tag was assigned to the
correct sample and group. Data were searched against the human UniProt/SwissProt database (version 2020_01, 20365 protein entries). Trypsin was used as an enzyme and maximum 2 missed cleavage was allowed. The precursor mass tolerance was set at 10 ppm and fragment mass tolerance was set at 0.8 Da. Carbamidomethylation on cysteine and TMT 6plex on N-terminal and lysine were used as static modifications. Oxidation on methionine was used as a dynamic modification. Reporter ions S/N were used for quantification. The quantification values were normalized to total peptide amount and scaled on controls (pooled sample labeled with TMT-126). The scaled abundance was exported for further analysis.

The dataset was first imputed to replace missing relative quantities to zeros when these were consistent among any of the examined phenotypes. In specific, when the percent of missing values in one examined phenotype exceeded 90% and the percentage of missing values for the other phenotypes was below 10% then the missing values of the examined phenotype were imputed to zeros. The relative quantities of the proteins were then scaled using log2 transformation. Next, the dataset was filtered to keep only proteins with less than 30% missing values, without considering the previously imputed missing values as missing. The remaining missing values were imputed using KNN-Impute method with k equal to 3. The limma package has been used to compare different phenotypes using the Ebayes algorithm and correcting for selected covariates. The initial p-values were adjusted for multiple testing using Benjamini-Hochberg method and a threshold of 0.05 was used for the adjusted p-values to infer statistically significant changes.

Pathway enrichment analysis of significant proteins

The bioinformatic webtool Webgestalt was used for pathway enrichment analysis of KEGG terms. KEGG pathways of significantly enriched proteins (fold change ≥ 1.4) were inferred by Benjamini-Hochberg testing with a p-value threshold of 0.05 and a maximum number of 150 proteins per category. To visualize the samples based on their proteomic profiles, principal component analysis (PCA) was conducted, and samples were projected in a 2D space based on their 2 most significant principal components. Samples were color-coded based on their phenotype. Volcano plots for the visualization of differentially expressed proteins were generated in GraphPad PRISM.
Gene expression analysis, mitochondrial DNA quantification

Total RNA was extracted from samples and complementary DNA (cDNA) was generated by reverse transcription using the TRIzol and high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. All experiments were performed using technical triplicates. The ΔΔCt method was used for calculation of relative transcript expression levels. Primer sequences are listed in Supplementary Table 8. Gene expression of target genes was normalized to the reference transcripts of the housekeeping gene glucuronidase-beta (GUSB). The qPCR experiments were conducted with the AbiPrism 7900HT Fast Real-Time PCR System (Applied Biosystems) using HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne).

Quantification of mitochondrial DNA (mtDNA) was performed according to a qPCR protocol recently described by Ulmer et al. In brief, genomic DNA (including mitochondrial DNA) was isolated by TRIzol extraction according to the manufacturer’s instruction. DNA concentrations of each sample was adjusted to 16.5 µg/µL prior to the experiment by dilution. The mt-DNA content was quantified by normalizing gene expression values of the mitochondrially encoded NADH dehydrogenase-1 (mt-ND1) and -2 (mt-ND2) to the nuclear-encoded globular actin (g-actin). Primer sequences were used as described and are listed in Supplementary table 8.

Southern blot

The 5’HR probe was cloned using PCR primer pairs SLC_5HR1 (Supplementary Table 9), and PCR amplified using pairs SLC_5HR2. The 3’HR probe was cloned using PCR primer pairs SLC_3HR1, and PCR amplified using pairs SLC_3HR2 (Supplementary Table 9).

The Southern blot procedure was performed according to Skryabin et al. HiPSC were thawed from an MCB aliquot and expanded to T25 flask format with 100% confluency. HiPSCs were washed with 5 mL PBS per flask and lysed in 1 mL standard lysis buffer containing 100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 g/mL Proteinase K (Roche) incubated overnight at 37 °C. DNA was extracted by phenol, phenol/chloroform extraction, precipitated in isopropanol and washed in 80% ethanol. DNA samples were dissolved in TE buffer. Approximately 10-15 µg of genomic DNA was digested with EcoRI, and HindIII
restriction endonucleases, fractionated on 0.8% agarose gels and transferred to GeneScreen nylon membranes (NEN DuPont, USA). The membranes were hybridized with a $^{32}$P-labeled 0.5-kb 5′HR probe containing sequences 5′ to the deleted region (5′HR probe, Supplementary Figure 3) and washed with SSPE buffer (0.09 M NaCl, 5 mM NaH$_2$PO$_4$, and 0.5 mM EDTA [pH 7.7]) and 0.5% sodium dodecyl sulfate at 65°C. Similarly, membranes were hybridized and washed with a $^{32}$P-labeled 1.2-kb 3′HR probe containing sequences 3′ to the deleted region (3′HR probe, Supplementary Figure 3).

Single-nucleus RNA sequencing

EHTs were washed with PBS, detached from the PDMS posts, frozen in liquid nitrogen, and stored at -150 °C. Single-nucleus RNA sequencing (snRNA seq) was performed according to Litviňuková et al. 65. In brief, single nuclei were isolated from frozen EHTs and purified by fluorescent-activated cell sorting (FACS). Nuclei were further processed using the Chromium Controller (10X Genomics) according to the manufacturer’s protocol with a targeted nuclei recovery of 5,000 per reaction. 3′ gene expression libraries were prepared according to the manufacturer’s instructions of v3 Chromium Single Cell Reagent Kits (10X Genomics).

Sequencing data analysis

Bcl files were converted to Fastq files by using bcl2fastq. Each sample was mapped to the human reference genome GRCh38 (release Ens84) using the CellRanger suite (v.3.0.1). Mapping quality was assessed using the cellranger summary statistics; Empty droplets were identified by Emptydrops, implemented in the CellRanger workflow, and subsequently removed, while doublets were identified and filtered using Solo 66.

Downstream analysis was performed using the Python Scanpy v1.5.1 toolkit. Single nuclei were filtered for counts (300 ≤ n_counts ≤20,000), genes (500 ≤ n_genes ≤5,500), mitochondrial genes (percent_mito ≤1%), ribosomal genes (percent_ribo ≤1%), and softmax score detected by Solo (solo_softmax_scores ≤0.5). After read count normalization and log-transformation, highly variable genes were selected. Principal components were computed, and elbow plots were used to define the appropriate number of principal components for neighbor graph construction. Prior to manifold construction using UMAP, selected principal components were harmonized by using Python Harmonypy. Finally, nuclei were clustered using the network-based Leiden algorithm.
GraphPad Prism software 8.4.3 was used to perform statistical analysis. All data was depicted as mean±SEM either as scatterplots or bar graphs. Where possible, data sets were tested for normal distribution and the appropriate statistical test was chosen accordingly. Either the unpaired or nested Student’s t-test, a nested-, a classical one-way ANOVA or a two-way ANOVA (plus Bonferroni’s post-test) was used to determine whether the difference between groups was statistically significant. A p-value lower than 0.05 was statistically significant.
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T.E. is a member of the DiNAQOR Scientific Advisory Board and holds shares in DiNAQOR.
Figure 1. A-F: Effect of OCTN2 genotype on contractile parameters of spontaneous beating EHTs on day 21. OCTN2 (+/+): n=153 EHTs from 9 batches, OCTN2 (N32S): n=108 EHTs from 7 batches, OCTN2 (-/-): n=91 EHTs from 5 batches. Nested 1-way ANOVA followed by Bonferroni’s post-test for multiple comparisons, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Each data point represents one EHT. Each color represents one independent differentiation batch. Data are expressed as mean±SEM.
Figure 2. EHT contraction analysis and action potential measurement. A: Representative average EHT contraction peaks of OCTN2 (+/+), OCTN2 (N32S), OCTN2 (-/-). EHTs were electrically paced at 1.5 Hz in standard EHT medium, n=9-14 EHTs from one batch. B: Representative video-optical EHT images, scale bar: 1 mm. C: Representative action potential for OCTN2 (+/+) and OCTN2 (N32S). D: Action potential duration (APD90) of OCTN2 (+/+) and OCTN2 (N32S) by sharp microelectrode measurement at 1.5 Hz. Student’s t-test vs OCTN2 (+/+), *p<0.05. Data are expressed as mean±SEM. Each data point represents one EHT.
Figure 3. Pearson correlation of A: Force- and B: Resting length of EHTs with percentage of cTNT-positive input cells for EHT generation. OCTN2 (+/+) : n= 10; OCTN2 (N32S): n= 7 and OCTN2 (-/-): n= 7 differentiation batches. Each replicate represents the mean value of 7 to 20 EHTs for the specific differentiation batch. C: EHT force development in fatty acid medium. Serum-free cell culture medium was supplemented with 50 µM carnitine, linoleic acid- and oleic acid-albumin. Data are normalized to baseline force. OCNT2 (+/+) : n=11 EHTs from 2 batches, OCTN2 (N32S): n=11 EHTs from 2 batches, OCTN2 (-/-): n=12 EHTs from 2 batches. 2-way ANOVA vs OCNT2 (+/+) followed by Bonferroni’s post-test for multiple comparisons, *p<0.05. Data are expressed as mean±SEM. D: Difference in ∆glucose media concentration divided by product of individual spontaneous beating frequency x force. (∆Glucose = Glucose concentration at baseline minus glucose concentration after 24 h of incubation) OCNT2 (+/+) : n=59 EHTs from 5 batches, OCTN2 (N32S): n=51 EHTs from 4 batches, OCTN2 (-/-): n=28 EHTs from 4 batches. 1-way ANOVA followed by Bonferroni’s post-test for multiple comparisons, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. One data point represents one EHT. Data are expressed as mean±SEM.
Figure 4. TMT-based quantitative proteomic analysis of EHTs. **A:** Principal component analysis (PCA) of OCNT2 (+/+)(black, n=10) OCTN2 (N32S)(red, n=10) and OCTN2 (-/-)(blue, n=10) EHTs based on their proteomic profiles. Each dot represents one EHT. **B:** Volcano plot of log2 fold changes of OCTN2 (N32S) vs OCTN2 (+/+). **C:** Volcano plot of log2 fold changes of OCTN2 (N32S) vs OCTN2 (+/+) and log10 of the p values with color-coded significance levels (p>0.05) and fold change >1.4. **C:** Clustering analysis of proteins related to metabolic pathways. Heatmaps display the relative abundance of proteins involved in glycolysis, carnitine shuttle, electron transport chain (ETC), beta-oxidation and TCA cycle, the myocardium, lipid metabolism. OCNT2 (+/+): mean of 10 EHTs from 1 batch; OCTN2 (N32S): mean of 10 EHTs from 1 batch; OCTN2 (-/-): mean of 10 EHTs from 1 batch. Protein levels are depicted as a color code ranging from blue (low abundance) to red (high abundance). **D:** Pathway enrichment analysis of proteins identified by proteomic analysis. Depicted are KEGG pathways of significantly enriched proteins that were significantly higher (red) or lower (blue) abundant in OCTN2 (N32S) vs OCTN2 (+/+), p<0.05, fold change >1.4.
Figure 5. A: Effect of carnitine supplementation \( \Delta \text{glucose} \) per workload (\( \Delta \text{glucose} = \text{glucose} \text{concentration at baseline minus glucose concentration after 24 hours of incubation; workload} \) = force \times \text{frequency). Nested t-test vs CON, **p<0.01, ***p<0.001, ****p<0.0001. OCNT2 (+/+)
control: n=27 EHTs from 3 batches. OCNT2 (+/+)+ carnitine (2 mM): n=28 EHTs from 3 batches. OCNT2 (N32S) control: n=23 EHTs from 3 batches. OCNT2 (N32S) + carnitine (2 mM): n=23 EHTs from 3 batches, OCTN2 (-/-) control: n=13 EHTs from 3 batches, OCTN2 (-/-)+ carnitine (2 mM): n=16 EHTs from 3 batches. Data are expressed as mean±SEM. B: Effect of carnitine supplementation on force of spontaneous beating EHTs at the last day of treatment (Day 33-42). Values were normalized to last day of treatment of untreated control. Student’s t-test vs CON, **p<0.01, ***p<0.001, ****p<0.0001. OCNT2 (+/) control: n=54 EHTs from 4 batches. OCTN2 (N32S) control: n=36 EHTs from 3 batches, OCTN2 (N32S)+ carnitine (2 mM): n=33 EHTs from 3 batches, OCTN2 (-/-) control: n=9 EHTs from 1 batch, OCTN2 (-/-)+ carnitine (2 mM): n=9 EHTs from 1 batch. Data are expressed as mean±SEM. C: Effect of carnitine supplementation on average contraction peaks. Depicted are representative average EHT contraction peaks of OCTN2 (+/+), OCTN2 (N32S), OCTN2 (-/-). EHTs were electrically paced at 1.5 Hz in standard EHT medium ± carnitine (2 mM). Values were normalized to untreated control. n=9-16 EHTs per condition from 1 batch. D, E: LC-MS- analysis of acylcarnitines and ceramides. Effect of carnitine supplementation on D: Acylcarnitine- and E: ceramide content of OCNT2 (+/) and OCTN2 (N32S) EHTs after 33 days of culture and supplementation. 2-way ANOVA followed by Bonferroni’s post-test for multiple comparisons, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are expressed as mean±SEM. n=4 EHT pools (containing 3 EHTs each) per genotype and carnitine supplementation from 1 batch.
Figure 6. Transmission electron microscopy of OCTN2 EHTs. A + B: OCTN2 (+/+), C + D: OCTN2 (N32S), E + F: OCTN2 (-/-). A, C and E untreated, B, D and F supplemented with carnitine (2 mM). mf: myofilaments, z: z-line, m: mitochondria, L: lipid droplet. Scale bar = 1 µm.
**Figure 7. Cellular heterogeneity in OCTN2 genotypes in EHTs.**

A: Representative UMAP plot after single-nucleus RNA sequencing of all samples and individual genotypes, n=1 EHT pool (4 EHTs) per genotype; OCTN2 (+/+) (3674 cells), OCTN2 (N32S) (4525 cells) OCTN2 (-/-) (5108 cells). 5 distinct cell clusters were identified: cardiomyocytes, cardiomyocytes (proliferating), endothelial cells, fibroblasts and myeloid cells.

B: Percentage of cell types per genotype. C: Dot plot graph showing the relative expression of cell-specific marker genes. Expression levels are depicted as a color code ranging from light red (low expression) to dark red (high expression) as mean of log2 fold of expression.

D: Representative fibroblast subcluster FB1-4 UMAP plot of all samples and individual genotypes. E: Percentage of fibroblast states per genotype. For each genotype, the total percentage of fibroblast states equals the percentage of fibroblast abundance identified in B.

F: Dot plot graph showing the relative expression of fibroblast-specific marker genes in fibroblast states. Scaled expression levels are depicted as a color code ranging from light red (low expression) to dark red (high expression) as mean of log2 fold of expression.
Figure 8. Evidence for ferroptosis pathway activation in TMT-based quantitative proteomic analysis and pharmacological inhibitor experiments. 

A: Proteomic analysis heatmaps display the relative abundance of pro- and anti-ferroptotic proteins of all genotypes. OCTN2 (+/+): mean of 10 EHTs from 1 batch; OCTN2 (N32S): mean of 10 EHTs from 1 batch; OCTN2 (-/-): mean of 10 EHTs from 1 batch. Protein levels are depicted as a color code ranging from blue (low abundance) to red (high abundance).

B: Single-nucleus RNA sequencing dot plot graph showing the scaled relative expression of pro- and anti-ferroptotic markers across all genotypes for all cells.

C: Effect of ferroptosis inhibitor liproxstatin: Quantitative PCR analysis of gene expression genes related to fibroblast activation. Gene expression was normalized to GUSB over OCTN2 (+/+) control. OCTN2 (+/+): n=8 EHTs from 2 batches, OCTN2 (N32S): n=8 EHTs from 2 batches; OCTN2 (-/-): n=6-8 EHTs from 1 batches. 1-way ANOVA followed by Bonferroni’s post-test for multiple comparisons, *p<0.05, **p<0.01, ****p<0.0001. Data are expressed as mean±SEM.

D: Effect of liproxstatin (200nM) on contractile force in OCTN2 (N32S) EHTs. Data are expressed as mean±SEM, *p<0.05, unpaired t-test.
Supplementary Figure 1. A: Schematic overview of CRISPR/Cas9 strategy for OCTN2 (N32S) generation. A ssODN containing the desired point mutation was co-transfected with CRISPR components to introduce the mutation c.95A>G, p.N32S in exon1 of the SLC22A5 gene. Depicted is the gRNA target site and the predictive DNA sequence after successful integration of the repair template.

B: Schematic overview over CRISPR/Cas9 strategy for OCTN2 (-/-) generation. Two gRNA were co-transfected to induce simultaneous cutting and a large deletion in the SLC22A5 gene. Depicted are the gRNA target sites and the predictive DNA sequence after successful editing. Red arrows indicate the predicted Cas9 cutting sites. Black arrows indicate the primer binding sites for PCR validation. P1: Primer target region gRNA1; P2: Primer target region gRNA2; PI: Primer internal; PE: Primer external; Pink: PAM1; Green: PAM2.
Supplementary Figure 2. Genotype characterization of CRISPR/Cas9-edited hiPSCs. A: Sanger sequencing traces of OCTN2 (+/+) and derived single cell clones for OCTN2 (N32S) and OCTN2 (-/-) genotypes. The red boxes indicate the silent PAM- and c.95A>G mutation and the heterozygous silent c277C>T mutation in the OCTN2 (N32S) clone. B: Schematic overview of primer localization in relation to the deleted fragment in the SLC22A5 gene. C: qPCR analysis of SLC22A5 transcripts for all genotypes. D: Gel electrophoresis of PCR products of OCTN2 (+/+) and OCTN2 (-/-) hiPSC clones; M = 1 kb DNA standard marker. E: Sanger sequencing trace of OCTN2 (-/-) PCR product amplified with external primers. Depicted are the binding sites of both gRNA.
Supplementary Figure 3. Southern blot analysis of genomic DNA from OCTN2 (+/+) and OCTN2 (-/-) hiPSC. A: Schematic presentation of Southern blot probes hybridization position. HindIII and EcoRI enzymes were used for enzymatic digestion of genomic DNA samples. B: Fragment size prediction of 5' HR and 3'HR hybridization after HindIII or EcoRI restriction enzyme digest. C: Southern blot analysis of OCTN2 (+/+), OCTN2 (-/) and OCTN2 (-/-) hiPSC.
Supplementary Figure 4. Nanostring karyotype analysis of hiPSC master cell bank samples. A: OCTN2 (+/+) B: OCTN2 (N32S) and C: OCTN2 (-/-).
Supplementary Figure 5. Characterization of cardiomyocyte differentiations and contractile parameters. A: FACS analysis of cardiac troponin T-positive cells in the differentiated cell population. The dashed line (75%) indicates threshold value for EHT generation. B: Differentiation efficiency calculated as the ratio of number of differentiated cells divided by the number of input hiPSCs. OCNT2 (+/+) n=10, OCNT2 (N32S): n=10, OCNT2 (-/-): n=9 differentiation batches, data are expressed as mean±SEM, 1-way ANOVA followed by Bonferroni’s post-test for multiple comparisons, ****p<0.0001. C-H: Effect of OCTN2 genotype on force, frequency, contraction time (TTP80), relaxation time (RT80), resting length and R-R Scatter (parameter of irregularity) of spontaneous beating EHTs between day 7 and day 26. OCNT2 (+/+): n=152 EHTs from 9 batches, OCNT2 (N32S): n=108 EHTs from 7 batches, OCNT2 (-/-): n=91 EHTs from 5 batches, data are expressed as EHT batch mean±SEM.
Supplementary Figure 6. Glucose- and lactate measurements. Difference in A: Glucose- and B: Lactate concentration of EHT culture media (Δglucose = glucose concentration at baseline minus glucose concentration after 24 h of incubation, Δlactate = lactate concentration at baseline minus lactate concentration after 24 h of incubation). C: ΔLactate of EHT culture media divided by Δglucose. OCTN2 (+/+): n=59 EHTs from 5 batches, OCTN2 (N32S): n=51 EHTs from 4 batches, OCTN2 (-/-): n=28 EHTs from 4 batches. 1-way ANOVA followed by Bonferroni’s post-test for multiple comparisons, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. One data point represents one independent EHT. Data are expressed as mean±SEM.
Supplementary Figure 7. A: Effect of OCTN2 genotype on mitochondrial DNA analyzed by quantitative PCR. MtDNA was normalized to nuclear-encoded globular actin (g-actin). OCTN2 (+/+) n=5 EHTs from 1 batch, OCTN2 (N32S): n=7 EHTs from 1 batch, OCTN2 (-/-): n=7 EHTs from 1 batch. 1-way ANOVA followed by Bonferroni’s post-test for multiple comparisons, *p<0.05. Data are expressed as mean±SEM.

B: Oxygen consumption rate in OCTN2 (+/+), (N32S) and (-/-). Mean ± SEM, n=2 biological replicates (each biological replicate represents the average of 12 wells of a 96-well Seahorse plate), Mann-Whitney U test, *p<0.05.
Supplementary Figure 8. A-C: Effect of carnitine supplementation on glucose consumption and lactate production. A: ∆Glucose (∆glucose= glucose concentration at baseline minus glucose concentration after 24 hours of incubation); B: ∆Lactate (∆lactate= lactate concentration after 24 hours of incubation minus lactate concentration at baseline). Nested t-test vs CON, **p<0.01, ***p<0.001, ****p<0.0001. OCNT2 (+/+) control: n=27 EHTs from 3 batches. OCNT2 (+/+)+ carnitine (2 mM): n=28 EHTs from 3 batches. OCTN2 (N32S) control: n=23 EHTs from 3 batches. OCTN2 (N32S)+ carnitine (2 mM): n=23 EHTs from 3 batches, OCTN2 (-/-) control: n=13 EHTs from 3 batches, OCTN2 (-/-)+ carnitine (2 mM): n=16 EHTs from 3 batches. Data are expressed as mean±SEM.

D-G: Effect of carnitine supplementation on spontaneous beating EHTs at the last day of treatment (Day 33-42). D: Frequency, E: Time to peak, F: Relaxation time and G: Resting length. Values were normalized to last day of treatment of untreated control. Student’s t-test vs CON, **p<0.01, ***p<0.001, ****p<0.001. OCNT2 (+/+ ) control: n=54 EHTs from 4 batches. OCNT2 (+/+ ) + carnitine (2 mM): n=49 EHTs from 4 batches. OCTN2 (N32S) control: n=36 EHTs from 3 batches. OCTN2 (N32S)+ carnitine (2 mM): n=33 EHTs from 3 batches, OCTN2 (-/-) control: n=9 EHTs from 1 batch, OCTN2 (-/-)+ carnitine (2 mM): n=9 EHTs from 1 batch. Data are expressed as mean±SEM. H: Effect of carnitine supplementation on PDK4 mRNA expression. OCNT2 (+/+ ) and OCTN2 (N32S) EHTs were treated over the entire culture time harvested on day 42. Gene expression was normalized to GUSB over OCTN2 (+/+) control. n=7 EHTs per genotype and carnitine treatment from 1 batch. 2-way ANOVA followed by Bonferroni’s post-test for multiple comparisons, *p<0.05. Data are expressed as mean±SEM.
Supplementary Figure 9. Cardiomyocyte and fibroblast subcluster analysis in OCTN2 genotypes. A: Representative UMAP plot after single-nucleus RNA sequencing of all samples and individual genotypes. B: Percentage of cardiomyocyte subcluster per genotype. C: Dot plot graph showing the relative expression of upregulated genes per cell cluster. Expression levels are depicted as a color code ranging from light red (low expression) to dark red (high expression) as mean of log2 fold of expression. D: Enrichment analysis of significantly upregulated genes from cardiomyocyte subclusters CM1, CM2, CM4.
Supplementary Figure 10. Fibroblast and endothelial cell subcluster analysis in OCTN2 genotypes. A: Dot plot graph showing the relative expression of specific marker genes related to fibroblast activation in fibroblast states. Unscaled expression levels are depicted as a color code ranging from light red (low expression) to dark red (high expression) as mean of log2 fold of expression. B: Enrichment analysis of significantly upregulated genes from fibroblast and endothelial cell subclusters. OCTN2 (+/+) and OCTN2 (N32S) endothelial cell and all myeloid subclusters did not reveal significant enriched pathways.
Supplementary Tables

Supplementary Table 1. Detected proteins proteomic analysis

Supplementary Table 2. KEGG Enrichment analysis of proteins with significant higher abundance in OCTN2 (N32) vs OCTN2 (++)

Supplementary Table 3. KEGG Enrichment analysis of proteins with significant lower abundance in OCTN2 (N32) vs OCTN2 (++)

Supplementary Table 4. Composition of stem cell related culture media

Supplementary Table 5. SsODN- and gRNA sequences

Supplementary Table 6. Primer pairs for PCR and Sanger sequencing

Supplementary Table 7. Primary antibodies used for flow cytometry/FACS

Supplementary Table 8. Primer pairs for qPCR

Supplementary Table 9. Primer pairs for 5’HR probe and 3’ HR probe design

Supplementary Videos

Supplementary Video 1. Spontaneously beating OCTN2 (+/+) EHT

Supplementary Video 2. Spontaneously beating OCTN2 (N32S) EHT

Supplementary Video 3. Spontaneously beating OCTN2 (-/-) EHT
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.Detectedproteinsproteomicanalysis.xlsx
- SupplementaryTable2KEGGEnrichmentanalysisofproteinswithsignificanthigherabundanceinOCTN2N32vsOCTN2.xlsx
- SupplementaryTable3KEGGEnrichmentanalysisofproteinswithsignificantlowerabundanceinOCTN2N32vsOCTN2.xlsx
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