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

Malte Loos

University Medical Center Hamburg-Eppendorf

Birgit Klampe

University Medical Center Hamburg-Eppendorf

Thomas Schulze

University Medical Center Hamburg-Eppendorf

Xiaoke Yin

King's College London   https://orcid.org/0000-0002-5172-0935

Konstantinos Theofilatos

KCL   https://orcid.org/0000-0001-6799-0553

Bärbel Ulmer

University Medical Center Hamburg-Eppendorf

Carl Schulz

University Medical Center Hamburg-Eppendorf

Charlotta Behrens

University Medical Center Hamburg-Eppendorf

Tessa Van Bergen

Max Delbrück Center for Molecular Medicine in the Helmholtz Association

Eleonora Adami

Max-Delbruck-Center for Molecular Medicine (MDC)

Henrike Maatz

Centre for Molecular Medicine- MDC

Michaela Schweizer

Center for Molecular Neurobiology Hamburg (ZMNH), University Medical Center Hamburg-Eppendorf, Hamburg

Susanne Brodesser

CECAD Research Center   https://orcid.org/0000-0001-5631-0663

Boris Skryabin

Muenster University Hospital

Timofey Rozhdestvensky

University of Muenster   https://orcid.org/0000-0002-3384-925X

Sara Bodbin

University of Nottingham

Torsten Christ

University Medical Center Hamburg-Eppendorf   https://orcid.org/0000-0002-8935-5304

Chris Denning

University of Nottingham

Norbert Huebner

Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC)

https://orcid.org/0000-0002-1218-6223

Manuel Mayr

King's College London British Heart Foundation Centre, School of Cardiovascular Medicine and Sciences, London   https://orcid.org/0000-0002-0597-829X

Thomas Eschenhagen

University of Hamburg   https://orcid.org/0000-0003-1750-1170

Arne Hansen (✉ ar.hansen@uke.de)

University Medical Center Hamburg-Eppendorf

Article

Keywords:

---

1 **Titel**

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

4 **Authors**

5 Malte Loos[1, 2], Birgit Klampe[1], Thomas Schulze[1], Xiaoke Yin[3], Konstantinos Theofilatos[3], Bärbel Maria
6 Ulmer[1, 2], Carl Schulz[1, 2], Charlotta Behrens[1, 2], Tessa Diana van Bergen[4], Eleonora Adami[4], Henrike
7 Maatz[4], Michaela Schweizer[5], Susanne Brodesser[6], Boris V. Skryabin[7], Timofey S. Rozhdestvensky[7],
8 Sara Bodbin[8], Torsten Christ[1, 2], Chris Denning[8], Norbert Hübner[4, 9, 10], Manuel Mayr[3], Thomas
9 Eschenhagen[1, 2], Arne Hansen[1, 2]

10 **Affiliations**

11 [1]University Medical Center Hamburg-Eppendorf, Department of Experimental Pharmacology and
12 Toxicology, 20246 Hamburg, Germany

13 [2]German Center for Heart Research (DZHK), Partner site Hamburg/Lübeck/Kiel, Germany

14 [3]King's British Heart Foundation Centre of Research Excellence, King's College London, United Kingdom

15 [4]Cardiovascular and Metabolic Sciences, Max Delbrück Center for Molecular Medicine in the
16 Helmholtz Association (MDC), 13125 Berlin, Germany

17 [5]Electron Microscopy Unit, Center for Molecular Neurobiology Hamburg, University Medical Center
18 Hamburg-Eppendorf, 20251 Hamburg, Germany

19 [6]Cluster of Excellence Cellular Stress Responses in Aging-associated Diseases (CECAD), Faculty of
20 Medicine and University Hospital of Cologne, 50931 Cologne, Germany

21 [7] Transgenic animal and genetic engineering Models (TRAM), Faculty of Medicine of the Westfalian
22 Wilhelms-University, 48149 Muenster, Germany

23 [8]Division of Cancer & Stem Cells, Biodiscovery Institute, University of Nottingham, NG7 2RD, United
24 Kingdom.

25 [9]DZHK (German Centre for Cardiovascular Research), Partner Site Berlin, 13347 Berlin, Germany

26 [10]Charité -Universitätsmedizin, 10117 Berlin, Germany

27 **Contact**

28 Malte Loos (m.loos@uke.de)

29 Arne Hansen (ar.hansen@uke.de)

30   <u>Abstract</u>

31   Primary carnitine deficiency (PCD) is an autosomal recessive monogenic disorder caused by

32   mutations in *SLC22A5*. This gene encodes for OCTN2 which transports the essential metabolite

33   carnitine into the cell. PCD patients suffer from muscular weakness and dilated

34   cardiomyopathy. Detailed molecular disease mechanisms remain unclear. Two OCTN2-

35   defective human induced pluripotent stem cell lines were generated from a healthy control

36   line, carrying a full OCTN2-knockout and a homozygous OCTN2 (N32S) loss of function

37   mutation. OCTN2-defective genotypes showed lower cardiac differentiation efficiency, lower

38   force development, and resting length in engineered heart tissue format compared to isogenic

39   control. Force was sensitive to fatty acid-based media and associated with lipid accumulation,

40   mitochondrial alteration, higher glucose uptake, and metabolic remodelling, replicating

41   findings in animal models. Importantly, genome wide analysis and pharmacological inhibitor

42   experiments identified ferroptosis, an iron- and lipid-dependent cell death pathway linked to

43   fibroblast activation as a novel PCD cardiomyopathy disease mechanism.

<u>Introduction</u>

45   Primary carnitine deficiency (PCD) is an autosomal recessive disorder resulting in insufficient

46   cellular carnitine (β-hydroxy-γ-trimethylammonium butyrate) uptake and low cytoplasmic

47   concentrations [1]. PCD is caused by pathogenic variants in the *SLC22A5* gene, leading to loss of

48   function of the encoded organic cation transporter novel family member 2 (OCTN2). OCTN2 is

49   strongly expressed in the myocardium, skeletal muscle, fibroblasts, renal tubules, placental

50   tissue and intestine [2]. OCTN2 transports carnitine in a sodium-dependent manner and

51   maintains intracellular carnitine concentrations 20-50-fold higher than in the extracellular

52   space [3,4]. In the cytoplasm, carnitine palmitoyltransferase 1 (CPT1) catalyzes the formation of

53   acylcarnitine from carnitine and long chain acyl-CoA . The carnitine-acylcarnitine translocase

54   (CACT) transports acylcarnitine across the mitochondrial membrane into the mitochondria,

55   where carnitine palmitoyltransferase 2 (CPT2) reconverts acylcarnitine to acyl-CoA that

56   subsequently enters beta-oxidation. Low cytoplasmic carnitine concentration impairs fatty

57   acid beta-oxidation leading to insufficient ATP generation under high workload conditions and

58   cytoplasmic lipid accumulation. This results in glucose-dependency as energy metabolism,

59   inhibited gluconeogenesis (due to inhibition of pyruvate carboxylase) and diminished

60   ketogenesis (due to a lack of acetyl-CoA derived from beta-oxidation) [1]. The strong reliance of

61   cardiomyocytes on fatty acids as energy substrate makes the heart particularly susceptible to

62   PCD pathomechanisms. Apart from the mitochondrial fatty acid transfer, carnitine

63   conjugation has a crucial role to reduce the number of coenzyme A (CoA) molecules attached

64   to acyl residues. Excessive accumulation of cytoplasmic acyl-CoA results in the formation of

65   ceramides, TAG and cholesteryl-ester that were reported to induce inflammation and

66   apoptosis in different organs [5].

67   Typical clinical PCD symptoms are hypoglycemia and hypoketonemia under fasting conditions,

68   liver dysfunction, muscular weakness and dilated cardiomyopathy (DCM) [6,7]. Symptomatic

69   patients are typically diagnosed in the first five years of life and receive a lifetime treatment

70   with high dose of carnitine (100–200 mg/kg/day)[6]. Despite inhibited cellular uptake, PCD

71   patients have low plasma carnitine concentrations (0-5 µmol/l, physiological: 25-50 µmol/l [8])

72   because OCTN2-mediated carnitine reabsorption in renal proximal tubules system is

73   impaired [9]. Relevant side effects of carnitine supplementation include nausea, vomiting,

74   abdominal cramps, diarrhea, a fishy body odor, and accumulation of atherogenic

75   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 [13,14]. 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) [7], 1:120,000 (Australia) [15] to 1:8000 - 1:17,000 (China) [16,17]. The Faroe Island, an isolated archipelago in the Northern Atlantic, has by far the highest prevalence with 1:300 [9]. 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 [18]. Pathogenic variants were found in all *SLC22A5* exonic coding regions but are often located in the first exon of the *SLC22A5* gene [19]. The *SLC22A5* c.95A>G (N32S) mutation is the characteristic PCD mutation on Faroe Island [9].

Juvenile visceral steatosis (JVS) mice are an animal model of carnitine deficiency [20]. This strain was discovered by coincidence to have an OCTN2 p.L352R missense mutation [21]. 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 [22]. JVS mice develop cardiac hypertrophy 10 days after birth associated with cardiac steatosis, accumulation of diacylglycerols (DAG) and triglycerides (TAG), but no ceramide [23], lower myocardial ATP content [24,] and high expression of the pyruvate dehydrogenase (PDH) inhibitor PDH kinase 4 (*PDK4*) [25]. 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 [26]. Compensatory upregulation of proteins involved in the carnitine shuttle system, such as CPT1 has been previously described [27,28]. 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 [9,29]. 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

107   range of PCD DCM characteristics found in patients and animal models and moreover reveals

108   ferroptosis linked to fibroblast activation as a novel disease mechanism.

Results

110   CRISPR/Cas9

111   An established control hiPSC line (OCTN2 (+/+)) was used for the CRISPR/Cas9 engineering

112   approach and served as the isogenic control. The CRISPR/Cas9 strategy is presented in

113   Supplementary Figure 1A, B. A representative Sanger sequencing trace of successfully edited

114   clones is depicted in Supplementary Figure 2A and shows the OCTN2 wildtype sequence

115   (upper lane) and the heterozygous silent point mutation (c.277C>T) in the isogenic control

116   hiPSC line. The middle lane shows the homozygous introduction of the OCTN2 (N32S) c.95A>G

117   founder point mutation in exon 1 of the *SLC22A5* gene. Due to the large deletion, Sanger

118   sequencing trace of OCTN2 (-/-) in the lower lane could not be aligned. PCR products derived

119   from internal and flanking primers of both CRISPR cutting sites in the OCTN2 (+/+) hiPSC line

120   are shown in Supplementary Figure 2B-E. The successful knockout was confirmed by

121   quantitative reverse transcription PCR (qPCR) of the *SLC22A5* transcript (Supplementary

122   Figure 2C). Southern blots validated the integrity of the edited locus. Supplementary Figure

123   3A displays the predicted cutting sites for the two restriction endonucleases HindIII and EcoRI

124   of the *SLC22A5* gene. Predicted fragment size and Southern blot results are shown in

125   Supplementary Figure 3B and C. Nanostring nCounter human karyotype assay revealed

126   normal karyotype for all lines (Supplementary Figure 4).

127   Functional analysis

128   Cardiomyocytes were successfully differentiated from all 3 hiPSC lines. Supplementary Figure

129   5A shows no difference in the percentage of cardiac troponin T (cTnT) positive cells for the

130   cardiac differentiation experiments between the three lines (OCTN2 (+/+) 87.5±2.5%, n= 10

131   differentiations; OCTN2 (N32S): 84.1±5.0%, n= 10 differentiations; OCTN2 (-/-): 86.2±8.2%, n=

132   9 differentiations). Cardiac differentiation efficiency (output-hiPSC-CM / input-hiPSC cells)

133   revealed significantly lower differentiation efficiency for OCTN2 (N32S) (23.5±10.5%) and

134   OCTN2 (-/-) (19.4±2.8%) compared to OCTN2 (+/+) (79.0±10.0%) (Supplementary Figure 5B).

135   Engineered Heart Tissue (EHT) samples were subjected to video-optical force analysis under

136   spontaneous beating conditions starting on day 7 (Supplementary Figure 5C-H). Contractile

137   parameters changed in EHTs from all three cell lines and reached a plateau after day 21.

138   OCTN2 (-/-) showed a lower force, higher contraction time and shorter resting length for the

139   entire culture time. Beating frequency was higher for OCTN2 (N32S) and OCTN2 (-/-) during

140   the initial phase of development, but not thereafter. Relaxation time was longer only for

141   OCTN2 (-/-) for the last 2 weeks of development. No difference could be detected for the RR

142   scatter as a surrogate for arrhythmic beating. Contractile values of day 21 were compared

143   (Figure 1A - F) and showed lower force and a shorter resting length for OCTN2 (-/-) compared

144   to OCTN2 (+/+) (OCTN2 (+/+): 0.194±0.004 mN, n= 153 EHTs; OCTN2 (N32S): 0.16±0.01 mN,

145   n= 108 EHTs; OCTN2 (-/-): 0.11±0.01 mN, n= 91 EHTs). Both OCTN2-defective genotypes

146   exhibited a higher contraction time, and OCTN2 (-/-) a higher relaxation time. Figure 2A, B

147   depict representative average contraction peaks and video-optical EHT images. A

148   representative video of EHTs from all three genotypes can be found in the supplement

149   (Supplementary Video 1-3). The contractile phenotype was associated with a shorter APD_{90} of

150   211.0±13.6 (SEM, n=7) in OCTN2 (N32S) versus 288.2±15.5 (SEM, n=9) in OCTN2 (+/+) as

151   measured by sharp microelectrode protocol (Figure 2C, D). Importantly, force and resting

152   length of both OCTN2-defective genotypes showed a positive correlation with the

153   cardiomyocyte purity of the input cell population (Figure 3A, B), implying an important role of

154   non-cardiomyocytes. To study the ability to metabolize long-chain fatty acids (LCFA), EHTs

155   were switched to a medium containing only LCFA plus carnitine [50 µM]. Force remained

156   stable in OCTN2 (+/+) EHTs but declined in OCTN2-defective EHTs (Figure 3C), indicating a

157   reduced ability to metabolize LCFA.

158   Delta glucose and lactate values were higher for OCTN2 (N32S), but not OCTN2 (-/-) compared

159   to OCTN2 (+/+). The delta lactate/delta glucose ratio as a surrogate for anaerobic glucose

160   metabolism showed no difference (Supplementary Figure 6A-C). Higher glucose consumption

161   for OCTN2 (N32S) and OCTN2 (-/-) became evident when normalised to workload (force x

162   beating frequency) (OCTN2 (+/+): 0.23±0.01 mM/bpm × mN; OCTN2 (N32S): 0.3±0.1 mM;

163   OCTN2 (-/-): 0.4±0.1 mM) (Figure 3D).

164   Proteomics, Seahorse

165   3,425 proteins were detected by tandem mass tag (TMT)-based proteomic analysis, of which

166   1,772 proteins differed significantly between OCTN2 (+/+) and OCTN2 (N32S) and 2050

167   differed significantly between OCTN2 (+/+) and OCTN2 (-/-), respectively (p<0.05). A detailed

168   summary of detected proteins is shown in Supplementary Table 1. Principal component

169   analysis revealed separate clustering of OCTN2 (+/+) from OCTN2 (N32S), and OCTN2 (-/-)

170 (Figure 4A). Volcano plot depiction highlights a higher abundance of fibrosis-related- and
171 extracellular matrix proteins like caldesmon1 (CALD1), collagen type I alpha 1 chain (COL1A1),
172 transgelin 2 (TAGLN2), fibronectin 1 (FN1) and vitronectin (VTN) in OCTN2 (N32S) EHTs (Figure
173 4B). Moreover, ceramide transfer protein (CERT) was among the 10 most abundant proteins
174 in OCTN2 (N32S). In contrast, the fatty acid transporters cluster of differentiation 36 (CD36),
175 fatty acid-binding protein 5 (FABP5) and cardiomyogenesis transcriptional regulator GATA
176 binding protein 4 (GATA4) were among the top 10 lower abundant proteins in OCTN2 (N32S).
177 Detailed grouping of proteins related to their participation in pathways, such as cardiac
178 physiology, lipid metabolism, glycolysis, carnitine shuttle, electron transport chain, TCA cycle,
179 and beta-oxidation revealed a concordant expression pattern for both, OCTN2 (N32S) and
180 OCTN2 (-/-) versus OCTN2 (+/+) (Figure 4C). KEGG pathway overrepresentation analysis
181 revealed enrichment of N-glycan- and O-glycan biosynthesis, ferroptosis, and cholesterol
182 metabolism in OCTN2 (N32S) high abundant proteins. Conversely, enrichment analysis of
183 lower abundant proteins in OCTN2 (N32S) revealed the KEGG pathways pyruvate- and
184 propanoate metabolism, glycolysis, pentose phosphate pathway glyoxylate and dicarboxylate
185 metabolism, and different pathways associated with amino acid metabolism (Figure 4D). A
186 detailed summary of specific proteins in the enriched pathways is depicted in Supplementary
187 Table 2 and 3. Quantification of mitochondrial DNA revealed a lower level in OCTN2 (N32S),
188 indicating lower abundance of mitochondria. Both OCTN2-defective lines showed lower
189 oxygen consumption rate und baseline, oligomycin, FCCP and rotenone conditions in Seahorse
190 experiments (Supplementary Figure 7A, B).

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

193 EHT media was supplemented with carnitine (2 mM) for the entire culture time. This was
194 accompanied by a reduction of glucose consumption and lactate production for all genotypes
195 (Supplementary Figure 8A-C). Notably, glucose consumption normalized to cardiac workload
196 was reduced only for the two OCTN2-defective cell lines (Figure 5A). This was associated with
197 a small increase in force for all cell lines (Figure 5B, C) and a substantial increase in relaxation
198 time (Supplementary Figure 8D-G). Notably, transcript levels of PDK4, the inhibitor of PDH and
199 important metabolic regulator, were higher in OCTN2 (N32S) versus OCTN2 (+/+) and
200 attenuated to isogenic control level by carnitine supplementation (Supplementary Figure 8H).

201 Liquid chromatography–mass spectrometry (LC-MS) revealed 5-fold lower content for C16:1-
202 , C18:0-, C18:1- and C18:2 acylcarnitines in OCTN2 (N32S) compared to OCTN2 (+/+). Carnitine
203 supplementation resulted in a higher content of C16:0-, C16:1-, C18:1- and C18:2
204 acylcarnitines for OCTN2 (+/+) and C18:1-, C18:2 acylcarnitines for OCTN2 (N32S). Ceramides
205 are one metabolite of accumulated cytoplasmic acyl-CoA. Quantification of ceramide content
206 (Cer16:0, Cer18:0, Cer22:0, Cer24:0, Cer24:1) revealed no difference between the genotypes
207 and no effect of carnitine supplementation (Figure 5D, E).

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

216 Single nuclear RNA sequencing

217 A pool of 4 EHTs per genotype was subjected to single nuclear RNA sequencing (snRNA seq).
218 OCTN2 (+/+), (N32S) and (-/-) samples were sequenced with an average sequencing depth of
219 39,324, 28,771 and 26,374 read pairs per nucleus. Following quality control filtering, snRNA
220 seq data of all three genotypes were pooled to a total number of 11,225 nuclei ((OCTN2 (+/+)
221 = 3,135, OCTN2 (N32S) = 3,761, OCTN2 (-/-) = 4,329 cells). Uniform manifold approximation
222 and projection (UMAP) and leiden clustering revealed 5 main cell clusters. Marker genes for
223 these clusters delineated: cardiomyocytes, proliferating cardiomyocytes, fibroblasts,
224 endothelial and myeloid cells (Figure 7A). In OCTN2 (+/+), cardiomyocytes represented 94% of
225 all cells with 14% of these cells showing markers of proliferation (Figure 7B, C). Sub-clustering
226 of cardiomyocytes revealed 10 subclusters (Supplementary Figure 9). Subcluster CM4 was
227 dominant in OCTN2 (+/+), while subclusters 1 and 2 were more prominent in OCTN2-defective
228 lines. Interestingly, KEGG analysis revealed enrichment of the GPR40 pathway in CM4,
229 describing free fatty acid receptor 1 signalling (Supplementary Figure 9C). A lower
230 representation of CM4 in the OCTN2-defective lines is compatible with the lower abundance
231 of fatty acid transporters in the proteomics analysis. In OCTN2 defective lines, cardiomyocytes

232 represented a smaller fraction of all cells (OCTN2 (N32S): 85%, OCTN2 (-/-): 67%). Reversely,

233 these lines showed a higher fraction of fibroblasts (OCTN2 (+/+): 4%, OCTN2 (N32S): 10%,

234 OCTN2 (-/-): 23%), (Figure 7B, C). Sub-clustering of fibroblasts identified 4 states. Fibroblasts

235 states with markers of TGF-beta signaling, proliferation and secretion (FB1, FB3, FB4), were

236 more prominent in OCTN2-defective lines (Figure 7D, E, F, Supplementary Figure 10A).

237 Genotype-specific analysis of significant KEGG pathway enrichment in all fibroblast subcluster

238 revealed relaxin-, ECM- and focal adhesion-related pathways (Supplementary Figure 10B).

239 Endothelial and myeloid cells were almost absent in OCTN2 (+/+) and represented 5% and 1%

240 in OCTN2 (N32S) and 8% and 1% in OCTN2 (-/-) respectively (Figure 7B). Endothelial cells

241 expressed PDE3A, CASC15 and the typical marker gene MECOM, myeloid cells expressed

242 PTPRC (CD45), CD163 and AOAH (Figure 7C). Comparative analysis of significant KEGG

243 pathway enrichment was not possible because significant pathways could only be detected

244 for OCTN2 (-/-) endothelial cells (Supplementary Figure 10B).

245 Ferroptosis, fibroblast activation

246 Proteomics analysis revealed enrichment of proteins related to the KEGG pathway ferroptosis,

247 an iron-dependent lipid peroxidation-mediated cell death mechanism. Extraction of an

248 extended list of pro- and anti-ferroptotic proteins [30] from the proteomics data set identified a

249 strikingly uniform regulation with a higher abundance of pro- and a lower abundance of anti-

250 ferroptotic proteins in the OCTN2-defective genotypes (Figure 8A). Noteworthy among these

251 were also the key regulators ACSL4 [31] and LPCAT3. These two proteins synergistically drive the

252 accumulation of iron-dependent lethal lipid peroxides (LPO) [32]. On the other hand, pro- and anti-

253 ferroptosis transcripts did not show a differential expression in snRNA seq, suggesting a post-

254 transcriptional regulation (Figure 8B). Evidence for ferroptosis and fibroblast activation in this

255 study and previous reports demonstrating a mechanistic link between these two pathways

256 [33,34] were the reason to analyse the effect of the potent ferroptosis inhibitor liproxstatin on

257 fibrosis markers. OCTN2 (N32S) EHTs revealed higher transcript levels of *ACTA2*, *COL1A1*,

258 *POSTN*, *TGFB*, *FN,* and *CCN2* than OCTN2 (+/+). Liproxstatin induced a significant attenuation

259 of fibrosis transcript levels in OCTN2 (N32S), which was associated with a moderate increase

260 in force development (Figure 8C-D).

Discussion

This study aimed to establish a human PCD DCM *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 *SLC22A5* in hiPSC biology is not yet understood. RNA sequencing data from Liu et al. [35] demonstrated a continuous expression of *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 [22,24] and short QT syndrome [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 [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 [38,39]. Moreover, this model

292 showed a lower abundance of glycolytic proteins but higher glucose uptake. This is compatible

293 with the poor correlation between glycolytic enzyme abundance and glycolysis in previous

294 studies [41].

295 One consequence of low cytoplasmic carnitine concentration is a decrease of acylcarnitine

296 formation and subsequent beta-oxidation [8]. Indeed, mass spectrometry analysis revealed

297 lower tissue content of several long-chain acylcarnitines in OCTN2 (N32S) EHTs. In addition,

298 declining force in LCFA media suggested a defect in LCFA metabolism. Surprisingly, mass

299 spectrometry did not reveal a difference in ceramide content between the genotypes.

300 However, ceramides do not represent the final product but a metabolic intermediate that can

301 be processed to sphingolipid derivates such as glucosylceramides and sphingomyelin [42]. For

302 this conversion, ceramides are transported from the endoplasmic reticulum (ER) into the

303 trans-Golgi apparatus by the ceramide transporter CERT [43]. Remarkably, CERT was among the

304 10 most significantly higher abundant proteins in OCTN2 (N32S) EHTs, suggesting that it

305 belongs to the compensatory mechanisms and prevents the accumulation of toxic ceramides.

306 Carnitine supplementation (2 mM) had a strong effect on metabolic aspects of the PCD disease

307 phenotype like acylcarnitine tissue content, glucose consumption per cardiac work, PDK4

308 transcript level and lipid droplet accumulation. Nevertheless, the force restoration was minor

309 and the carnitine-induced increase in force was in the same range for all three genotypes,

310 suggesting a non-specific effect potentially related to induction of sodium current and

311 subsequent inhibition of Na^+/K^+-ATPase as previously described [44,45]. The discrepancy

312 between strong metabolic and small force effects of carnitine supplementation suggests

313 additional mechanisms to be relevant. The shorter EHT resting length, the positive correlation

314 between cardiomyocyte percentage of the input cell population with force and resting length

315 and the enrichment of extracellular matrix KEGG pathway the OCTN2-defective genotypes

316 suggest fibrosis to be relevant. In support of this, snRNA seq revealed a more prominent

317 fibroblast cluster in the OCTN2 –defective genotypes, which expressed markers indicative of

318 activated and secretory state. Of note, markers of activated fibroblasts in this study (*POSTN*,

319 *FN1*, *FAP*, *NOX4*) overlap substantially with the fibroblast signatures in two failing heart snRNA

320 seq DCM studies [46,47]. Interestingly, the central role of fibroblasts in this hiPSC-CM PCD model

321 is paralleled by clinical findings of strong myocardial fibrosis in PCD patients [6,48,49].

322   Proteomic analysis provided evidence for ferroptosis, an iron-dependent cell death

323   mechanism related to lipid peroxidation, to be relevant in this model. Ferroptosis is well

324   compatible with PCD DCM, since it is driven by the accumulation of polyunsaturated fatty

325   acids (PUFAs) linked to coenzyme A (CoA) in cell membranes [50]. PUFA accumulation is relevant

326   for PCD as cytosolic carnitine deficiency impairs PUFA metabolization to acylcarnitine. ACSL4

327   catalyzes the esterification of long-chain PUFA to acyl-CoA and represents a central pro-

328   ferroptotic regulator [51]. This marker showed a higher expression and protein abundance in

329   this model. The abundance of the central anti-ferroptotic enzyme GPX4 was not lower in

330   OCTN2-defective lines in contrast to other ferroptotic proteins. Notably, glutathione is an

331   important cofactor for GPX4 activity and proteins involved in glutathione metabolism such as

332   Glutamate-Cysteine Ligase (GCLC), Glutathione Synthetase (GSS) and glutathione-disulfide

333   reductase (GSR) were lower abundant in OCTN2-defective lines.

334   Importantly, ferroptosis was recently shown to be linked to fibrosis development and

335   cardiomyopathy [33,34]. Mechanistically, profibrotic factors released from ferroptotic cells were

336   identified to drive fibroblast activation [33]. A similar mechanistic link is likely relevant in this

337   model since the ferroptosis inhibitor liproxstatin induced a reduction of fibrosis transcript

338   levels and higher force. Overall, the discovery of ferroptosis activation reveals novel insight

339   into the development of PCD-associated metabolic cardiomyopathy and is paving the way to

340   the development of specific antifibrotic treatment strategies.

341

342   <u>Experimental procedures</u>

343   HiPSC cell culture conditions

344   An established hiPSC control cell line (hiPSCreg code UKEi001-A) derived from a healthy

345   individual served as the starting point for the genetic engineering approach and as the isogenic

346   control for the engineered hiPSC lines. This hiPSC line was generated by reprogramming

347   dermal fibroblast from a skin biopsy using the CytoTune (Life Technologies) 2.0 Sendai

348   Reprogramming Kit under feeder-free conditions. All basic stem cell culture work was

349   performed as recently described [52]. In brief, hiPSC culture was based on the expansion of a

350   master cell bank (MCB) at passage 25-35 on Geltrex-coated cell culture flasks in FTDA-medium

351   (Supplementary Table 4) under hypoxic conditions (5% O_2). Standard passaging was

352   performed twice a week (3-4 day passaging interval) with Accutase solution (Sigma-Aldrich).

353   Plating density was $4.5\text{-}7.0\times10^4$ hiPSC/cm^2. Maximal expansion was for 40 passages with

354   regular screening for mycoplasma contamination by PCR amplification. SSEA3 surface marker

355   served as a pluripotency marker and was analyzed by flow cytometry. All procedures involving

356   the generation and analysis of hiPSC lines were approved by the local ethics committee in

357   Hamburg (Az PV4798, 28.10.2014).

358   CRISPR/Cas9-mediated gene editing

359   OCTN2 (N32S) missense mutation

360   The *SLC22A5* gene locus was Sanger sequenced in the hiPSC OCTN2 (+/+) control line.

361   CRISPR/Cas9 technology was used to engineer the c.95A>G (N32S) mutation into the *SLC22A5*

362   wild type. IDT Custom Alt-R CRISPR-Cas9 gRNA software and CRISPOR [53] were used to identify

363   potential gRNA binding sites at the gene locus. The OCTN2 NCBI Reference (NG_008982.2)

364   was provided as a target sequence. Targets for gRNA's were chosen based on the lowest cut-

365   to-mutation distance under consideration of a high on-target potential and low off-target risk.

366   A single-stranded oligodeoxynucleotide (ssODN) served as an exogenous donor template,

367   containing the OCTN2 c.95A>G, p.N32S mutation. Additionally, a silent mutation was

368   introduced in the PAM sequence to prevent CRISPR/Cas9 re-cutting after successful genomic

369   integration of the template by HDR. Edited clones were identified by PCR amplification and

370   subsequent Sanger sequencing. A schematic overview of the HDR strategy is depicted in

371   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 promotor 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.

403 Single-stranded DNA oligonucleotide (ssODN) repair template oligos were resuspended in

404 IDTE Buffer (IDT) to a stock concentration of 100 µM. 1 µL of ssODN repair template (100 µM)

405 and 4 µL of RNP-complex were mixed with the hiPSC solution by gently pipetting, incubated

406 for 5 min at room temperature and transferred to the nucleofection cuvette. Additionally, 1

407 µL Alt-R Cas9 Enhancer (100 µM, IDT) was added to the solution to promote transfection

408 efficiency. The nucleofection cuvette was placed in the 4D-Nucleofector (Lonza) and hiPSCs

409 were nucleofected by using the program CA137. After nucleofection, the cuvette was

410 incubated for 5 min under cell culture conditions. Subsequently, hiPSCs were seeded in

411 conditioned medium supplemented with Y-27632 (10 µM) and bFGF (30 ng/ml)

412 (Supplementary Table 4) on a Matrigel-coated 24-well plate for 72 hours at 37 °C.

413 Subcloning and off-target analysis

414 72 hours after nucleofection, hiPSC were dissociated with Accutase and seeded in conditioned

415 medium with Y-27632 (10 µM) and bFGF (30 ng/mL) at low seeding densities of 100, 250, 750,

416 1000 hiPSCs per well (10 cm^2) in a Matrigel-coated 6-well plate. In addition, the remaining

417 nucleofected hiPSCs were seeded at a higher density of 5x10^5 cells per well (10 cm^2) in a

418 Matrigel-coated 6-well plate. Low-density hiPSC seedings were expanded for 9 to 10 days

419 under daily conditioned medium change until clonal hiPSC colonies reached a size appropriate

420 to pick. HiPSC cultures were incubated with conditioned medium with Y-27632 (10 µM) for 2

421 hours and sterile 100 µL-pipette tips were used to carefully scrape individual colonies from

422 the 6-well plate and transfer them to Matrigel-coated 48-well plates into individual wells. 30

423 to 50 clones were picked per transfection approach and were sub-cultivated for 3-4 more days

424 before they reached confluency and were splitted with a ratio of 1:2 into two 48-well copy

425 plates. Colonies were again expanded with daily medium change until they reached

426 confluency. One of the copy plates was used for cryo-preservation, while the second plate was

427 used for DNA isolation. Cryopreservation was performed in 90% FBS and 10% DMSO.

428 QIAcube HT System (Qiagen) and QIAamp 96 DNA QIAcube HT kit (Qiagen) were used for DNA

429 isolation according to the manufacturer's instructions. Cryotubes of successfully edited hiPSC

430 clones were thawed and expanded for master cell bank and working cell bank. Ten most likely

431 off-targets were predicted (*in silico* tool IDT, Custom Alt R CRISPR-Cas9 gRNA software and

432 CRISPOR software). Corresponding PCR primers were designed and PCR products were

433   analysed by 1% (w/v) agarose electrophoresis and Sanger sequencing. Off-target primer

434   sequences can be found in Supplementary Table 6.

435   Karyotyping

436   Karyotype analysis was performed using the nCounter Human Karyotype Panel (Nanostring

437   Technologies) according to the manufacturer's instructions with 250 µg DNA as starting

438   material. The nCounter CNV Collector Tool software (Nanostring) was used for analysis.

439   Cardiac differentiation

440   HiPSC were differentiated into cardiomyocytes with an embryoid body (EB)- and growth

441   factor-based three-stage protocol which was recently described [54]. In brief, hiPSC were

442   expanded on Geltrex-coated T80-flasks to a confluency of 90-100% and detached with EDTA.

443   The formation of EBs was induced in 500 mL spinner flasks with a density of 30-35x10^6 hiPSCs

444   per 100 mL of EB formation medium (Supplementary Table 4). HiPSC suspension was

445   cultivated overnight at 40 rpm glass ball impeller rotation speed. Mesoderm induction was

446   induced in mesoderm induction medium (Supplementary Table 4) with a volume of 200-300

447   µL EB per pluronic-coated T175-flask for three days under hypoxic conditions (5% O_2) with

448   50% media exchange daily. After washing the EBs again, cardiac differentiation was induced

449   in cardiac differentiation medium 1 (Supplementary Table 4) with a volume of 250-300 µL EBs

450   per pluronic-coated T175-flask with 50% media exchange daily for three days under normoxic

451   conditions (21% O_2). Then, media was completely removed and exchanged for cardiac

452   differentiation medium 2 (Supplementary Table 4). After a daily 50% medium change for four

453   days, culturing medium was exchanged with cardiac differentiation medium 3 (Supplementary

454   Table 4). After washing EBs in HBBS-solution buffer, beating cardiomyocytes were dissociated

455   with collagenase II solution (200 units/mL; Worthington) containing myosin II ATPase inhibitor

456   N-benzyl-p-toluene sulphonamide (BTS) for 2-3 hours until dispersing single cells could be

457   observed. Dissociated hiPSC-CM were frozen in freezing media containing 90% FBS and 10%

458   DMSO or resuspended in EHT casting medium for subsequent EHT generation. Differentiation

459   efficiency (% cTNT-positive cells) was determined by fluorescent-labeled cardiac troponin T

460   (cTNT)- antibody (Miltenyi Biotech) by flow cytometer FACSCanto II (BD). Adjustment of gates

461   adjusted according to the isotype control and performed with FACSDiva software (BD).

462   Differentiation runs with at least 75% cTNT-positive cells were used for further functional

463   experiments. FACS reagents are shown in Supplementary Table 7.

464   Engineered heart tissues (EHT)

465   Engineered heart tissues (EHT) were generated as recently described [55]. In brief, dissociated

466   hiPSC-CM were centrifuged (100xg, 10 minutes) and resuspended in EHT casting medium

467   containing DMEM, horse serum, and glutamine (Supplementary Table 4).

468   Polytetrafluorethylene (PTFE) spacers (EHT Technologies) were placed in a warm 2% (w/v)

469   agarose/PBS solution in a 24-well plate. Agarose solidification at room temperature led to the

470   formation of agarose molds. PTFE spacers were removed from the 24 well plates and flexible

471   polydimethylsiloxane (PDMS) posts were placed on the 24-well plates so that pairs of elastic

472   PDMS posts reached into each casting mold. 100 µL of the mastermix (Supplementary Table

473   4) containing 2x DMEM, Y-27632, fibrinogen and $1.0x10^6$ hiPSC-CMs was resuspended in EHT

474   casting medium, rapidly mixed with 3 µL thrombin and pipetted into one agarose casting mold.

475   Afterwards the preparation was placed in an incubator for 1.5 hours at 37 °C until a fibrin gel

476   formed in the agarose molds around the PDMS posts. 200-300 µL of pre-warmed EHT culture

477   medium was added dropwise into each well to ameliorate the detachment of the fibrin gel

478   from the agarose mold. After additional 15-30 minutes of incubation, racks with fibrin gels

479   attached to the PDMS posts were transferred into a new 24-well plate, filled with pre-warmed

480   EHT culture medium, and incubated at 40% O_2, 37 °C, 7% CO_2, and 98% humidity. EHTs were

481   cultured for 28-42 days with medium changes 3 times per week. After 5-7 days of culture,

482   EHTs started to develop spontaneous macroscopic contractions.

483   Video-optical contraction analysis

484   EHT contractile analysis was performed as previously described [54,55]. EHT contraction

485   parameters, e.g. force, frequency, and contraction kinetics, were monitored over time of EHT

486   development 2 hours after each medium change. EHTs were electrically stimulated as

487   previously described by Hirt et al. [56]. PDMS racks with EHTs were mounted onto custom-made

488   graphite pacing units and stimulated by using Grass S88X Dual Output Square stimulator

489   (Natus Neurology Incorporated). The pacing frequency was adjusted to a value of 1.5 to 2-fold

490   of the spontaneous beating frequency of the EHT batch with an output voltage of 2 V in

491   biphasic pulses of 4 ms. EHTs that were not able to follow the pacing frequency, were excluded

492   from the analysis. Average contraction peaks were calculated with an average of 10-15 peaks.

493   Glucose- and lactate measurement

494   Glucose- and lactate concentrations were measured in EHT cell culture media by blood gas

495   analysis. Samples were collected at baseline and after 24 hours of incubation in EHT medium

496   on day 21 of culture and stored at -20°C. The blood gas analysis instrument ABL90 FLEX

497   Analysator (Radiometer) was used to determine the metabolite concentrations by injecting

498   the supernatant (0.5 mL) into the instrument with a 1 mL syringe.

499   Fatty acid- and carnitine supplementation

500   EHTs were cultured in serum-containing EHT culture medium until force values reached their

501   plateau phase approximately at day 21-28 after generation. Then, EHTs were transferred to a

502   serum- and glucose-free DMEM medium containing linoleic acid- and oleic acid-albumin

503   (Sigma). The detailed serum-free fatty acid medium composition is shown in Supplementary

504   Table 4. Contraction analysis was done daily after 2 hours of medium incubation for 4 days.

505   Supplementation with carnitine was conducted by adding 2 mM L-carnitine hydrochloride

506   (Sigma) to the EHT medium over the entire time of tissue cultivation.

507   Transmission electron microscopy

508   EHTs were transferred into a 24-well plate containing Tyrode's solution (Supplementary

509   Table 4) with 1.8 mM Ca^{2+} and 30 mM butanedione monoxime (BDM) for 3-4 hours until EHTs

510   completely stopped contraction. For fixation, EHTs were shifted into 4% paraformaldehyde

511   (PFA, Thermo Scientific) in 0.1 M phosphate buffer containing 1% glutaraldehyde (Science

512   Services) overnight at 4 °C. Samples were rinsed three times in 0.1 M sodium cacodylate buffer

513   (pH 7.2–7.4) and osmicated using 1% osmium tetroxide in cacodylate buffer. Following

514   osmication, the samples were dehydrated using ascending ethanol concentrations, followed

515   by two rinses in propylene oxide. Infiltration of the embedding medium was performed by

516   immersion in a 1:1 mixture of propylene oxide and Epon (Science Services, Germany), followed

517   by neat Epon and hardening at 60 °C for 48 hours. For light microscopy, semi-thin sections (0.5

518   μm) with longitudinal orientation were mounted on glass slides and stained for 1 minute with

519   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 1.0×10^6 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 [57]

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 [58]. 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.

549   Ceramides

550   To 50 µL of cell homogenate 50 µL of Milli-Q water, 750 µL of methanol/chloroform 2:1 (v/v),

551   and internal standard (127 pmol ceramide 12:0, Avanti Polar Lipids) were added. Lipid

552   extraction and LC-ESI-MS/MS analysis were performed as previously described [59]. The LC

553   chromatogram peaks of endogenous ceramide species and the internal standard ceramide

554   12:0 were integrated using the MultiQuant 3.0.2 software (SCIEX). Endogenous ceramide

555   species were quantified by normalizing their peak areas to the peak area of the internal

556   standard ceramide 12:0. These normalized peak areas were normalized to the protein content

557   of the sample.

558   Actions potential

559   Actions potential (AP) measurements in EHT were performed with standard sharp

560   microelectrode as described previously [60]. The EHTs were transferred from the 24-well EHT

561   culture plate into the AP measuring chamber by cutting the silicone posts and were fixed with

562   needles in recording chamber. All measurements were done with tissues continuously

563   superfused with Tyrode´s solution (NaCl 127 mM, KCl 5.4 mM, $MgCl_2$ 1.05 mM, $CaCl_2$ 1.8 mM,

564   glucose 10 mM, $NaHCO_3$ 22 mM, $NaHPO_4$ 0.42 mM, balanced with O_2-CO_2 [95:5] at 36 °C, pH

565   7.4). Microelectrodes had a resistance between 25 - 55 MΩ when filled with 2 M KCl. The

566   signals were amplified by a BA-1s npi amplifier (npi electronic GmbH, Tamm, Germany). APs

567   were recorded and analyzed using the Lab-Chart software (version 5, AD Instruments Pty Ltd.,

568   Castle Hill NSW, Australia). Take-of potential (TOP) was defined as the diastolic membrane

569   potential directly before the upstroke.

570   Analysis of mitochondrial respiration

571   The Seahorse™ XF96 extracellular flux analyzer was used to assess mitochondrial respiration

572   as previously described (Mosqueira et al, 2019), using the Mito Stress Kit (Agilent

573   Technologies). Briefly, cryopreserved isogenic sets of hiPSC-CMs were seeded into Matrigel™-

574   coated (BD #356235) XF96 well plates at a density of approximately 5000 cells/mm². HiPSC-

575   CMs were cultured for 2 days in RPMI1640 (USBiological Life Sciences #R9010-01)

576   supplemented with B-27 with insulin (LifeTechnologies #0080085-SA), 2 mM L-glutamine (Life

577   Technologies #25030-081), 10% Fetal Bovine Serum (Gibco #16000044) and 0.6 mM CaCl2.

578   After 2 weeks, medium was exchanged for XF basal medium (Agilent Technologies #102353),

579 supplemented with 10 mM glucose (Sigma #G7528), 1 mM sodium pyruvate (Sigma #S8636)

580 and 2 mM L-glutamine (Life Technologies #25030-081) 1h before the conduction of the assay.

581 Selective inhibitors were sequentially injected during the measurements (1.5 µM oligomycin,

582 0.4 µM FCCP, 1 µM rotenone; Agilent Technologies), following the manufacturer's

583 instructions. The measured Oxygen Consumption Rate (OCR) values were normalized to the

584 number of cells in each well, quantified by 1:400 Hoechst33342 staining (Sigma #B2261) in

585 PBS (Gibco #14190-094) using fluorescence at 355 nm excitation and 460 nm emission in an

586 automated imaging platform (CellaVista, Synentec).

587 Tandem Mass Tag (TMT)-based quantitative proteomic analysis

588 EHT harvesting for quantitative mass spectrometry

589 EHTs from hiPSC-CMs were cultured in EHT medium for 28 days before proteome analysis.

590 EHTs were washed twice in warm PBS buffer and incubated with collagenase II solution

591 (collagenase II (200 units per mL), HBSS minus Ca^{2+}/Mg^{2+}, HEPES (10 mM), Y-27632 (10 µM),

592 and BTS (30 µM)) in a falcon tube for 3 hours. Dissociated hiPSC-CMs were gently titrated with

593 a 1000 µL-pipette (Eppendorf) until the last cluster of cells was disaggregated, spun down for

594 5 min at 200xg and supernatant was removed. The pellet was frozen in liquid nitrogen and

595 stored at -80 °C before subjection to quantitative proteome analysis.

596 Tissue protein extraction and digestion for mass spectrometry

597 Cell pellets were lysed in 50 mM Tris, 0.1% SDS, pH=8.8, with protease inhibitors. After

598 centrifugation at 4 °C at 16,000xg for 10 min, the supernatant was transferred to new 1.5 mL

599 tubes and protein concentration was measured using a BCA protein assay kit (Thermo Fisher

600 Scientific). For each sample, 23 µg of proteins were denatured by the addition of 9 M urea,

601 3 M thiourea (final conc. 6 M urea, 2 M thiourea) and reduced by the addition of 100 mM DTT

602 (final conc. 10 mM) followed by incubation at 37 °C for 1 hour, 240 rpm. The samples were

603 then alkylated by the addition of 500 mM iodoacetamide (final conc. 50 mM) followed by

604 incubation in the dark for 1h at room temperature. Pre-chilled (-20 °C) acetone (1:9 volume

605 ratio) was used to precipitate the samples overnight at -20 °C. Samples were centrifuged at

606 16,000xg for 30 min at 4 °C and the supernatant was subsequently discarded. Protein pellets

607 were dried using a vacuum centrifuge (Thermo Fisher Scientific, Savant SPD131DDA), re-

608 suspended in 0.1M TEAB buffer, pH 8.2 (Sigma T7408), and 0.6 µg trypsin was added. The

609 digestion was performed overnight at 37 °C, 240 rpm, and stopped by adding 10% TFA (final

610 conc. 1%). C18 clean-up was performed using Agilent Bravo AssayMAP and the eluted peptides

611 were dried using a vacuum centrifuge.

612 Sample labeling for mass spectrometry

613 The samples were resuspended in 0.1 M TEAB and a pooled sample was made by taking the

614 same amount of proteins from each individual sample. Samples were labeled with Tandem

615 Mass Tag (TMT) 11-plex reagent (Thermo Fisher Scientific) following the manufacturer's

616 instructions. The pooled sample labeled with TMT-126 was used as an internal standard. The

617 samples labeled with different tags of the 11-plex TMT were combined, dried, and

618 resuspended in 300 µl of 0.1% TEA. Samples were further fractionated using high pH RP HPLC

619 (Agilent 300Extend-C18 3.5um 4.6x150mm P/N 763973-902) and 16 fractions were collected

620 for each TMT mixture. All fractions were dried and resuspended in LC solution (2% acetonitrile

621 (ACN), 0.05% TFA).

622 Mass spectrometry analysis

623 Samples were injected and separated by a nanoflow HPLC (Ultimate 3000 RSLCnano, Thermo

624 Fisher Scientific) on an EASY-Spray column (C18, 75 µm x 50 cm, 2 µm) using 2 hour LC

625 gradient: 0-10 min, 4%-10%B; 10-75 min, 10%-30%B; 75-80 min, 30%-40%B; 80-85 min, 40%-

626 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

627 H2O. The flow rate was 250 nl/min and column temperature was set at 45°C. The separated

628 peptides were directly injected to an Orbitrap Fusion Lumos MS (Thermo Fisher Scientific) and

629 analyzed using a synchronous precursor selection (SPS)-based MS3 method for TMT-labeled

630 sample. Full MS spectra were collected on the Orbitrap with a resolution of 120,000 and scan

631 range 375-1500 m/z. The most abundant ions were fragmented using CID and MS2 spectra

632 were collected on a linear ion trap, with dynamic exclusion enabled. The 5 most abundant ions

633 from every MS2 spectrum were selected and fragmented at the same time using HCD with

634 collision energy 65% and MS3 spectra were collected on the Orbitrap with a resolution of

635 60,000 and a scan range of 110-500 m/z to measure the TMT reporter ions. The cycle time

636 was set at 3 seconds.

637 Raw data were analyzed using Proteome Discoverer 2.4. The 16 fractions of each TMT

638 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 [61] 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.

668   Gene expression analysis, mitochondrial DNA quantification

669   Total RNA was extracted from samples and complementary DNA (cDNA) was generated by

670   reverse transcription using the TRIzol and high-capacity cDNA reverse transcription kit

671   (Applied Biosystems) according to the manufacturer's instructions. All experiments were

672   performed using technical triplicates. The ΔΔCt method was used for calculation of relative

673   transcript expression levels. Primer sequences are listed in Supplementary Table 8. Gene

674   expression of target genes was normalized to the reference transcripts of the housekeeping

675   gene glucuronidase-beta (GUSB). The qPCR experiments were conducted with the AbiPrism

676   7900HT Fast Real-Time PCR System (Applied Biosystems) using HOT FIREPol EvaGreen qPCR

677   Mix Plus (Solis BioDyne).

678   Quantification of mitochondrial DNA (mtDNA) was performed according to a qPCR protocol

679   recently described by Ulmer et al. [62]. In brief, genomic DNA (including mitochondrial DNA) was

680   isolated by TRIzol extraction according to the manufacturer's instruction. DNA concentrations

681   of each sample was adjusted to 16.5 µg/µL prior to the experiment by dilution. The mt-DNA

682   content was quantified by normalizing gene expression values of the mitochondrially encoded

683   NADH dehydrogenase- 1 (mt-ND1) and -2 (mt-ND2) to the nuclear-encoded globular actin (g-

684   actin). Primer sequences were used as described [62,63] and are listed in Supplementary table 8.

685   Southern blot

686   Southern blot probe design.

687   The 5'HR probe was cloned using PCR primer pairs SLC_5HR1 (Supplementary Table 9), and

688   PCR amplified using pairs SLC_5HR2. The 3'HR probe was cloned using PCR primer pairs

689   SLC_3HR1, and PCR amplified using pairs SLC_3HR2 (Supplementary Table 9).

690   The Southern blot procedure was performed according to Skryabin et al [64]. HiPSC were

691   thawed from an MCB aliquot and expanded to T25 flask format with 100% confluency. HiPSCs

692   were washed with 5 mL PBS per flask and lysed in 1 mL standard lysis buffer containing 100

693   mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 g/mL Proteinase K (Roche)

694   incubated overnight at 37 °C. DNA was extracted by phenol, phenol/chloroform extraction,

695   precipitated in isopropanol and washed in 80% ethanol. DNA samples were dissolved in TE

696   buffer. Approximately 10-15 µg of genomic DNA was digested with *Eco*RI, and *Hind*III

697   restriction endonucleases, fractionated on 0.8% agarose gels and transferred to GeneScreen

698   nylon membranes (NEN DuPont, USA). The membranes were hybridized with a ^{32}P-labeled

699   0,5-kb 5'HR probe containing sequences 5' to the deleted region (5'HR probe, Supplementary

700   Figure 3) and washed with SSPE buffer (0.09 M NaCl, 5 mM NaH$_2$PO4, and 0.5 mM EDTA [pH

701   7.7]) and 0.5% sodium dodecyl sulfate at 65°C. Similarly, membranes were hybridized and

702   washed with a ^{32}P-labeled 1,2-kb 3'HR probe containing sequences 3' to the deleted region

703   (3'HR probe, Supplementary Figure 3).

704   Single-nucleus RNA sequencing

705   EHTs were washed with PBS, detached from the PDMS posts, frozen in liquid nitrogen, and

706   stored at -150 °C. Single-nucleus RNA sequencing (snRNA seq) was performed according to

707   Litviňuková et al. [65]. In brief, single nuclei were isolated from frozen EHTs and purified by

708   fluorescent-activated cell sorting (FACS). Nuclei were further processed using the Chromium

709   Controller (10X Genomics) according to the manufacturer's protocol with a targeted nuclei

710   recovery of 5,000 per reaction. 3' gene expression libraries were prepared according to the

711   manufacturer's instructions of v3 Chromium Single Cell Reagent Kits (10X Genomics).

712   Sequencing data analysis

713   Bcl files were converted to Fastq files by using bcl2fastq. Each sample was mapped to the

714   human reference genome GRCh38 (release Ens84) using the CellRanger suite (v.3.0.1).

715   Mapping quality was assessed using the cellranger summary statistics; Empty droplets were

716   identified by Emptydrops, implemented in the CellRanger workflow, and subsequently

717   removed, while doublets were identified and filtered using Solo [66].

718   Downstream analysis was performed using the Python Scanpy v1.5.1 toolkit. Single nuclei

719   were filtered for counts (300 ≤ n_counts ≤20,000), genes (500 ≤ n_genes ≤5,500),

720   mitochondrial genes (percent_mito ≤1%), ribosomal genes (percent_ribo ≤1%), and soft max

721   score detected by Solo (solo_softmax_scores ≤0.5). After read count normalization and log-

722   transformation, highly variable genes were selected. Principal components were computed,

723   and elbow plots were used to define the appropriate number of principal components for

724   neighbor graph construction. Prior to manifold construction using UMAP, selected principal

725   components were harmonized by using Python Harmonypy. Finally, nuclei were clustered

726   using the network-based Leiden algorithm.

727   Statistical analysis

728   GraphPad Prism software 8.4.3 was used to perform statistical analysis. All data was depicted

729   as mean±SEM either as scatterplots or bar graphs. Where possible, data sets were tested for

730   normal distribution and the appropriate statistical test was chosen accordingly. Either the

731   unpaired or nested Student's t-test, a nested-, a classical one-way ANOVA or a two-way

732   ANOVA (plus Bonferroni's post-test) was used to determine whether the difference between

733   groups was statistically significant. A p-value lower than 0.05 was statistically significant.

734 References

735 1. Longo, N., Frigeni, M. & Pasquali, M. Carnitine transport and fatty acid oxidation.
736 *Biochim. Biophys. Acta - Mol. Cell Res.* **1863**, 2422–2435 (2016).

737 2. Wagner, C. A. *et al.* Functional and pharmacological characterization of human Na+-
738 carnitine cotransporter hOCTN2. *Am. J. Physiol. - Ren. Physiol.* **279**, (2000).

739 3. Wu, X., Prasad, P. D., Leibach, F. H. & Ganapathy, V. cDNA Sequence, Transport
740 Function, and Genomic Organization of Human OCTN2, a New Member of the Organic
741 Cation Transporter Family. *Biochem. Biophys. Res. Commun.* **246**, 589–595 (1998).

742 4. Tamai, I. *et al.* Molecular and functional identification of sodium ion-dependent, high
743 affinity human carnitine transporter OCTN2. *J. Biol. Chem.* **273**, 20378–20382 (1998).

744 5. Cooper, D. E., Young, P. A., Klett, E. L. & Coleman, R. A. Physiological consequences of
745 compartmentalized acyl-CoA metabolism. *J. Biol. Chem.* **290**, 20023–20031 (2015).

746 6. Wang, S. S., Rao, J., Li, Y. F., Zhang, Z. W. & Zeng, G. H. Primary carnitine deficiency
747 cardiomyopathy. *Int. J. Cardiol.* **174**, 171–173 (2014).

748 7. Magoulas, P. L. & El-Hattab, A. W. Systemic primary carnitine deficiency: An overview
749 of clinical manifestations, diagnosis, and management. *Orphanet Journal of Rare*
750 *Diseases* vol. 7 (2012).

751 8. Longo, N., Amat Di San Filippo, C., Pasquali, M., Filippo, C. A. di S. & Pasquali, M.
752 Disorders of carnitine transport and the carnitine cycle. *Am. J. Med. Genet. - Semin.*
753 *Med. Genet.* **142 C**, 77–85 (2006).

754 9. Rasmussen, J. *et al.* Carnitine levels in 26,462 individuals from the nationwide
755 screening program for primary carnitine deficiency in the Faroe Islands. *J. Inherit.*
756 *Metab. Dis.* **37**, 215–222 (2014).

757 10. Alesci, S. *et al.* Carnitine: Lessons from one hundred years of research. *Ann. N. Y.*
758 *Acad. Sci.* **1033**, (2004).

759 11. Koeth, R. A. *et al.* Intestinal microbiota metabolism of l-carnitine, a nutrient in red
760 meat, promotes atherosclerosis. *Nat. Med.* **19**, 576–585 (2013).

761 12. Rebouche, C. J. Kinetics, pharmacokinetics, and regulation of L-carnitine and acetyl-L-

762   carnitine metabolism. *Ann. N. Y. Acad. Sci.* **1033**, 30–41 (2004).

763   13.   Spiekerkoetter, U. *et al.* Silent and symptomatic primary carnitine deficiency within
764   the same family due to identical mutations in the organic cation/carnitine transporter
765   OCTN2. *J. Inherit. Metab. Dis.* **26**, 613–615 (2003).

766   14.   Rasmussen, J. *et al.* Increased risk of sudden death in untreated primary carnitine
767   deficiency. *J. Inherit. Metab. Dis.* **43**, 290–296 (2020).

768   15.   Wilcken, B., Wiley, V., Hammond, J. & Carpenter, K. Screening Newborns for Inborn
769   Errors of Metabolism by Tandem Mass Spectrometry. *N. Engl. J. Med.* **348**, 2304–2312
770   (2003).

771   16.   Lin, Y. *et al.* Increased detection of primary carnitine deficiency through second-tier
772   newborn genetic screening. *Orphanet J. Rare Dis.* **16**, 1–7 (2021).

773   17.   Yang, X. *et al.* Newborn Screening and Genetic Analysis Identify Six Novel Genetic
774   Variants for Primary Carnitine Deficiency in Ningbo Area, China. *Front. Genet.* **12**, 1–8
775   (2021).

776   18.   Tan, J. *et al.* Lifetime risk of autosomal recessive mitochondrial disorders calculated
777   from genetic databases. *EBioMedicine* **54**, 102730 (2020).

778   19.   Li, F. Y. *et al.* Molecular spectrum of SLC22A5 (OCTN2) gene mutations detected in 143
779   subjects evaluated for systemic carnitine deficiency. *Hum. Mutat.* **31**, (2010).

780   20.   Tomomura, M. *et al.* Abnormal expression of urea cycle enzyme genes in juvenile
781   visceral steatosis (jvs) mice. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1138**, 167–171
782   (1992).

783   21.   Koizumi, T., Nikaidoi, H., Nonomura, A. & Yoneda, T. Infantile disease with
784   microvesicular fatty infiltration of viscera spontaneously occurring in the C3H-H-2°
785   strain of mouse with similarities to Reye's syndrome. *Lab. Anim.* **22**, 83–87 (1988).

786   22.   Horiuchi, M. *et al.* Cardiac hypertrophy in juvenile visceral steatosis (jvs) mice with
787   systemic carnitine deficiency. *FEBS Lett.* **326**, 267–271 (1993).

788   23.   Saburi, Y. *et al.* Changes in distinct species of 1,2-diacylglycerol in cardiac hypertrophy
789   due to energy metabolic disorder. *Cardiovasc. Res.* **57**, 92–100 (2003).

790 24. Asai, T. *et al.* Combined therapy with PPARα agonist and l-carnitine rescues lipotoxic
791   cardiomyopathy due to systemic carnitine deficiency. *Cardiovasc. Res.* **70**, 566–577
792   (2006).

793 25. Horiuchi, M., Kobayashi, K., Masuda, M., Terazono, H. & Saheki, T. Pyruvate
794   dehydrogenase kinase 4 mRNA is increased in the hypertrophied ventricles of
795   carnitine-deficient juvenile visceral steatosis (JVS) mice. *BioFactors* **10**, 301–309
796   (1999).

797 26. Spaniol, M. *et al.* Development and characterization of an animal model of carnitine
798   deficiency. *Eur. J. Biochem* **268**, 1876–1887 (2001).

799 27. Liepinsh, E. *et al.* Effects of Long-Term Mildronate Treatment on Cardiac and Liver
800   Functions in Rats. *Basic Clin. Pharmacol. Toxicol.* **105**, 387–394 (2009).

801 28. Degrace, P. *et al.* Fatty acid oxidation and related gene expression in heart depleted of
802   carnitine by mildronate treatment in the rat. *Mol. Cell. Biochem.* **258**, 171–182 (2004).

803 29. Steuerwald, U. *et al.* Neonatal screening for primary carnitine deficiency: Lessons
804   learned from the Faroe Islands. *Int. J. Neonatal Screen.* **3**, 1–10 (2017).

805 30. Chen, X., Kang, R., Kroemer, G. & Tang, D. Broadening horizons: the role of ferroptosis
806   in cancer. *Nat. Rev. Clin. Oncol.* **18**, 280–296 (2021).

807 31. Doll, S. *et al.* ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid
808   composition. *Nat. Chem. Biol.* **13**, 91–98 (2017).

809 32. Li, D. & Li, Y. The interaction between ferroptosis and lipid metabolism in cancer.
810   *Signal Transduct. Target. Ther.* **5**, 1–10 (2020).

811 33. Zhang, B. *et al.* Liproxstatin-1 attenuates unilateral ureteral obstruction-induced renal
812   fibrosis by inhibiting renal tubular epithelial cells ferroptosis. *Cell Death Dis.* **12**, 1–10
813   (2021).

814 34. Gong, Y., Wang, N., Liu, N. & Dong, H. Lipid Peroxidation and GPX4 Inhibition Are
815   Common Causes for Myofibroblast Differentiation and Ferroptosis. *DNA Cell Biol.* **38**,
816   725–733 (2019).

817 35. Liu, Q. *et al.* Genome-Wide Temporal Profiling of Transcriptome and Open Chromatin

of Early Cardiomyocyte Differentiation Derived from hiPSCs and hESCs. *Circ. Res.* **121**, 376–391 (2017).

36. Gélinas, R., Leach, E., Horvath, G. & Laksman, Z. Molecular Autopsy Implicates Primary Carnitine Deficiency in Sudden Unexplained Death and Reversible Short QT Syndrome. *Can. J. Cardiol.* **35**, 1256.e1-1256.e2 (2019).

37. Roussel, J. *et al.* Carnitine deficiency induces a short QT syndrome. *Hear. Rhythm* **13**, 165–174 (2016).

38. Jalil, M. A. *et al.* Attenuation of cardiac hypertrophy in carnitine-deficient juvenile visceral steatosis (JVS) mice achieved by lowering dietary lipid. *J. Biochem.* **139**, 263–270 (2006).

39. Liepinsh, E. *et al.* Mildronate decreases carnitine availability and up-regulates glucose uptake and related gene expression in the mouse heart. *Life Sci.* **83**, 613–619 (2008).

40. Li, J. M. *et al.* Systemic regulation of L-carnitine in nutritional metabolism in zebrafish, Danio rerio. *Sci. Rep.* **7**, (2017).

41. Kolwicz, S. C. & Tian, R. Glucose metabolism and cardiac hypertrophy. *Cardiovasc. Res.* **90**, 194–201 (2011).

42. MacEyka, M. & Spiegel, S. Sphingolipid metabolites in inflammatory disease. *Nature* **510**, 58–67 (2014).

43. Bandet, C. L. & Hajduch, E. CERT-Dependent Ceramide Transport, A Crucial Process in Cells. *J. Diabetes Clin. Res.* **3**, 40–45 (2021).

44. Wu, J. & Corr, P. B. Palmitoyl transient carnitine modifies sodium currents and induces inward current in ventricular myocytes. *Am. J. Physiol.* **266**, 1034–1046. (1994).

45. Wu, J. & Corr, P. B. Palmitoylcarnitine transient inward increases [Na +] i and initiates current in adult ventricular myocytes. *Am. J. Physiol.* **268**, 2405–2417 (1995).

46. Chaffin, M. *et al.* Single-nucleus profiling of human dilated and hypertrophic cardiomyopathy. *Nature* (2022) doi:10.1038/s41586-022-04817-8.

47. Nicin, L. *et al.* Single Nuclei Sequencing Reveals Novel Insights into the Regulation of Cellular Signatures in Children with Dilated Cardiomyopathy. *Circulation* 1704–1719

846   (2021) doi:10.1161/CIRCULATIONAHA.120.051391.

847   48.   Tomlinson, S., Atherton, J. & Prasad, S. Primary Carnitine Deficiency: A Rare,

848   Reversible Metabolic Cardiomyopathy. *Case Reports Cardiol.* **2018**, 1–3 (2018).

849   49.   Grünert, S. C. *et al.* Primary carnitine deficiency-diagnosis after heart transplantation:

850   Better late than never! *Orphanet J. Rare Dis.* **15**, 4–9 (2020).

851   50.   Yang, W. S. *et al.* Peroxidation of polyunsaturated fatty acids by lipoxygenases drives

852   ferroptosis. *Proc. Natl. Acad. Sci. U. S. A.* **113**, E4966–E4975 (2016).

853   51.   Doll, S. *et al.* ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid

854   composition. *Nat. Chem. Biol.* **13**, 91–98 (2017).

855   52.   Shibamiya, A. *et al.* Cell Banking of hiPSCs: A Practical Guide to Cryopreservation and

856   Quality Control in Basic Research. *Curr. Protoc. Stem Cell Biol.* **55**, 1–26 (2020).

857   53.   Concordet, J. P. & Haeussler, M. CRISPOR: Intuitive guide selection for CRISPR/Cas9

858   genome editing experiments and screens. *Nucleic Acids Res.* **46**, W242–W245 (2018).

859   54.   Breckwoldt, K. *et al.* Differentiation of cardiomyocytes and generation of human

860   engineered heart tissue. *Nat. Protoc.* **12**, 1177–1197 (2017).

861   55.   Mannhardt, I. *et al.* Human Engineered Heart Tissue: Analysis of Contractile Force.

862   *Stem Cell Reports* **7**, 29–42 (2016).

863   56.   Hirt, M. N. *et al.* Functional improvement and maturation of rat and human

864   engineered heart tissue by chronic electrical stimulation. *J. Mol. Cell. Cardiol.* **74**, 151–

865   161 (2014).

866   57.   Giesbertz, P., Ecker, J., Haag, A., Spanier, B. & Daniel, H. An LC-MS/MS method to

867   quantify acylcarnitine species including isomeric and odd-numbered forms in plasma

868   and tissues. *J. Lipid Res.* **56**, 2029–2039 (2015).

869   58.   Aravamudhan, S. *et al.* Phosphoproteomics of the developing heart identifies PERM1 -

870   An outer mitochondrial membrane protein. *J. Mol. Cell. Cardiol.* **154**, 41–59 (2021).

871   59.   Schwamb, J. *et al.* B-cell receptor triggers drug sensitivity of primary CLL cells by

872   controlling glucosylation of ceramides. *Blood* **120**, 3978–3985 (2012).

873   60.   Lemoine, M. D. *et al.* Human Induced Pluripotent Stem Cell-Derived Engineered Heart

874 Tissue as a Sensitive Test System for QT Prolongation and Arrhythmic Triggers. *Circ.*

875 *Arrhythmia Electrophysiol.* **11**, 1–15 (2018).

876 61. Liao, Y., Wang, J., Jaehnig, E. J., Shi, Z. & Zhang, B. WebGestalt 2019: gene set analysis

877 toolkit with revamped UIs and APIs. *Nucleic Acids Res.* **47**, W199–W205 (2019).

878 62. Ulmer, B. M. *et al.* Contractile Work Contributes to Maturation of Energy Metabolism

879 in hiPSC-Derived Cardiomyocytes. *Stem Cell Reports* **10**, 834–847 (2018).

880 63. Burkart, A. M. *et al.* Insulin Resistance in Human iPS Cells Reduces Mitochondrial Size

881 and Function. *Sci. Rep.* **6**, 1–12 (2016).

882 64. Skryabin, B. V. *et al.* Pervasive head-to-tail insertions of DNA templates mask desired

883 CRISPR-Cas9-mediated genome editing events. *Sci. Adv.* **6**, (2020).

884 65. Litviňuková, M. *et al.* Cells of the adult human heart. *Nature* **588**, 466–472 (2020).

885 66. Bernstein, N. J. *et al.* Solo: Doublet Identification in Single-Cell RNA-Seq via Semi-

886 Supervised Deep Learning. *Cell Syst.* **11**, 95-101.e5 (2020).

887

888

898   Author contribution

899   CRISPR/Cas9 (M.L., C.B., B.M.U.), Southern blot (B.V.S., T.S.R.), PCR (M.L.), cardiac
900   differentiation (M.L., B.K., T.S.), EHT generation and maintenance (M.L., B.K., T.S.), force
901   measurement (M.L.), action potential (C.S., T.C.), proteomics/bioinformatics (M.M., K.T., X.Y.,
902   M.L.), single nuclear RNA sequencing (TD.v.B., E.A., H.M., N.H.), lipid analysis (S.B.), mt DNA-,
903   quantitative PCR (M.L.), TEM (M.S.), Seahorse (S.B., C.D.), concept (A.H. M.L., T.E.), funding
904   (T.E., A.H.), writing (M.L., T.E., A.H.)

905   Competing interests

906   T.E. is a member of the DiNAQOR Scientific Advisory Board and holds shares in DiNAQOR.
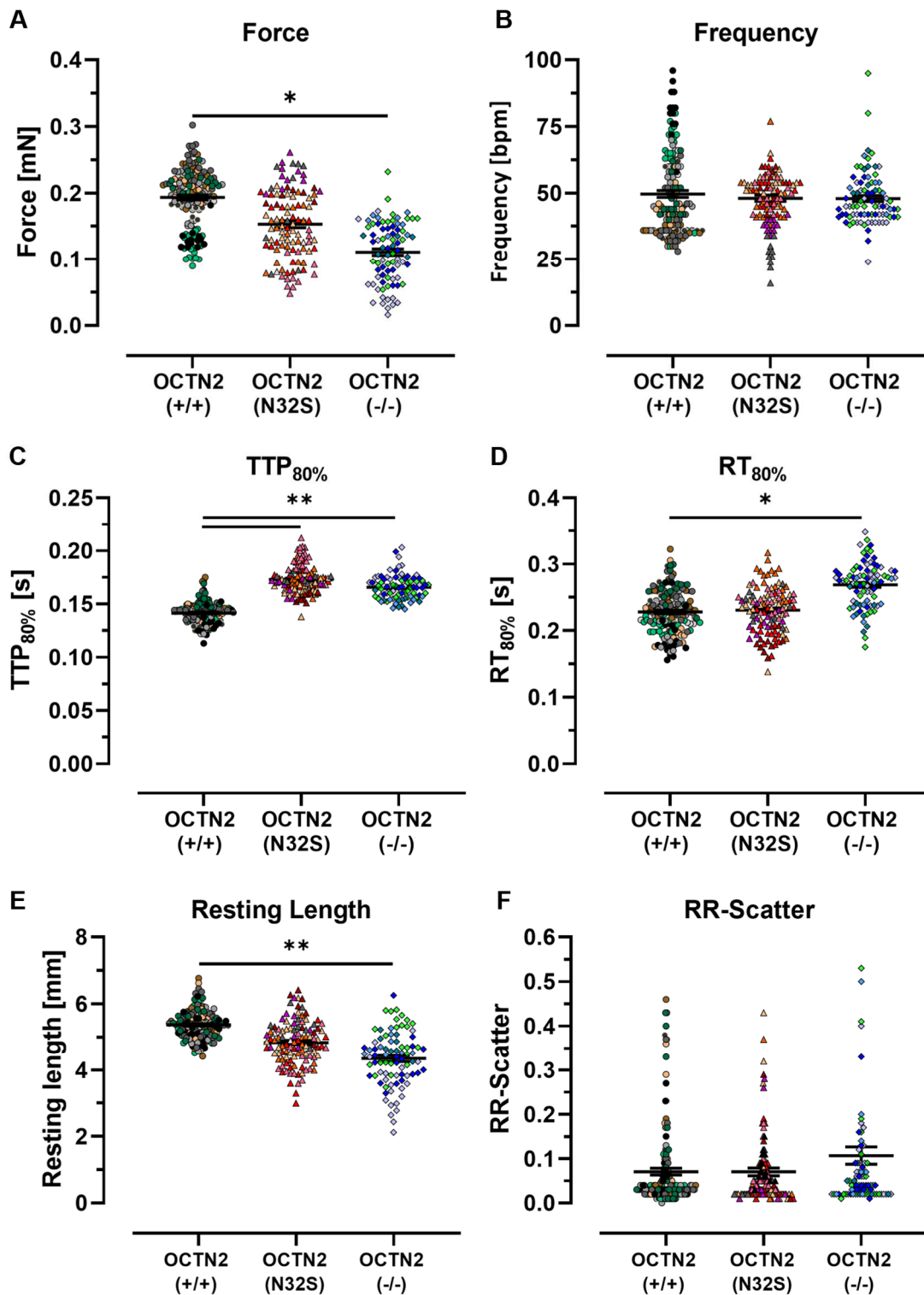
Figure 1. A-F: Effect of OCTN2 genotype on contractile parameters of spontaneous beating EHTs on day 21. OCNT2 (+/+): n=153 EHTs from 9 batches, OCTN2 (N32S): n=108 EHTs from 7 batches, OCTN2 (-/-): n=91 EHTs from 5 batches. Nested 1-way ANOVA followed by Bonferroni's post-test for multiple comparisons, *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.
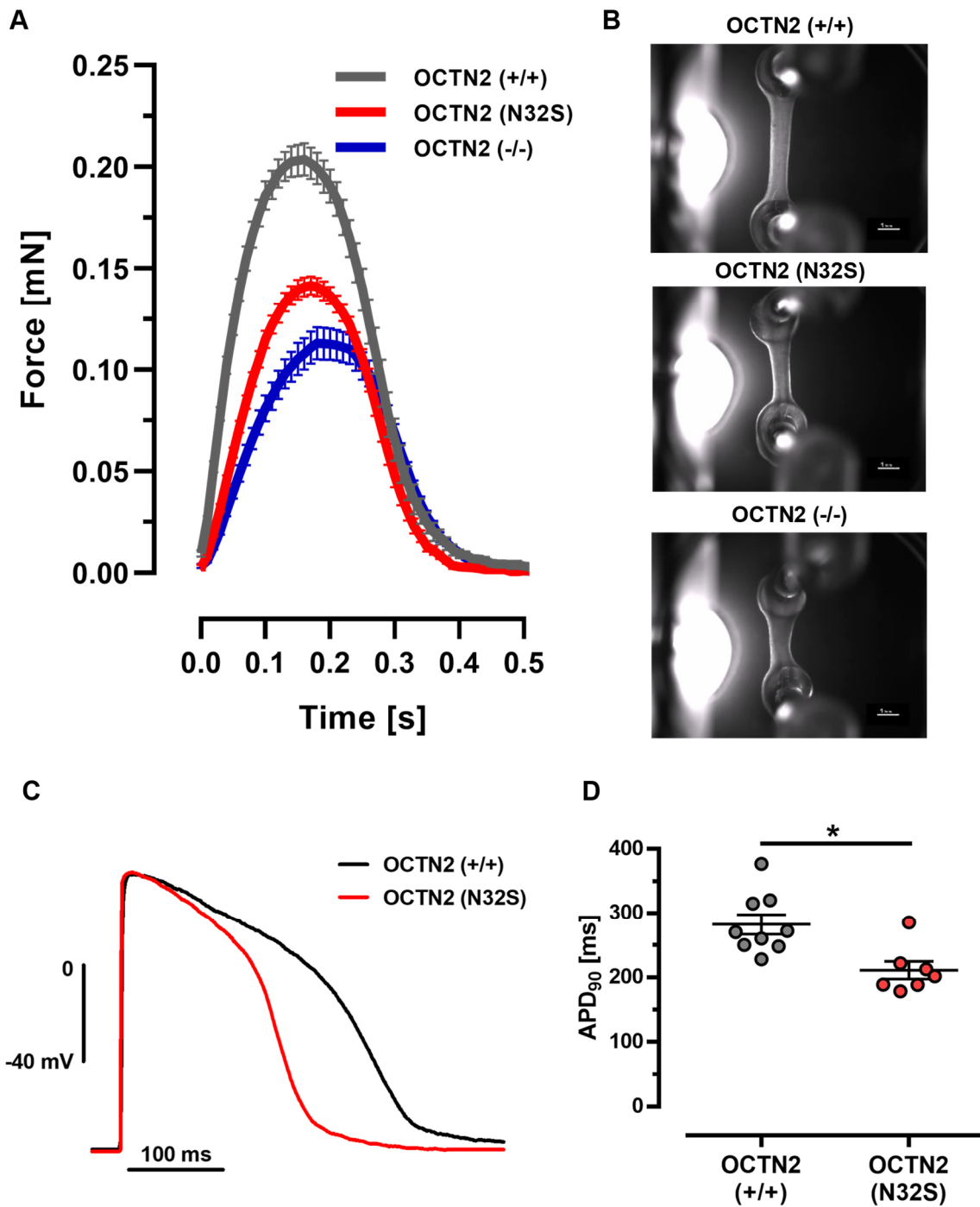
915
Figure 2. EHT contraction analysis and action potential measurement. A: Representative average EHT contraction peaks of OCTN2 (+/+), OCTN2 (N32S), OCTN2 (-/-). EHTs were electrically paced at 1.5 Hz in standard EHT medium, n=9-14 EHTs from one batch. **B**: Representative video-optical EHT images, 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.
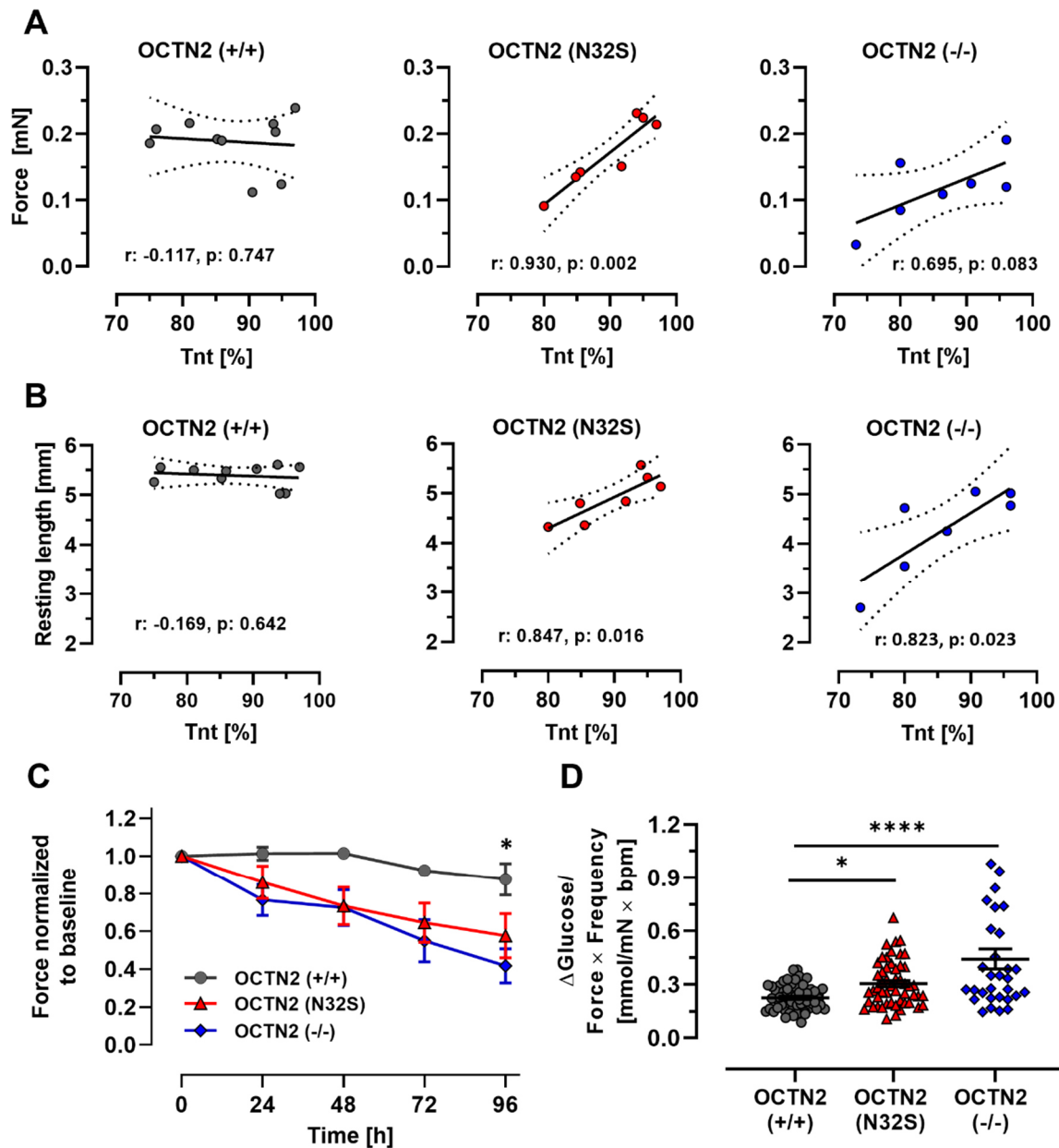
Figure 3. Pearson correlation of A: Force- and B: Resting length of EHTs with percentage of cTNT-positive input cells for EHT generation. OCTN2 (+/+): n= 10; OCTN2 (N32S): n= 7 and OCTN2 (-/-): n= 7 differentiation batches. Each replicate represents the mean value of 7 to 20 EHTs for the specific differentiation 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 Δglucose media concentration divided by product of individual spontaneous beating frequency x force. (ΔGlucose = 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.

A

OCNT2(+/+) OCNT2(N32S) OCNT2 (-/-)

Component 2 (16.82%)

Component 1 (47.42%)

B

Fatty acid transporter | Ferroptosis | ECM/ Fibrosis | Lipid metabolism

Down in N32S (n=203)   Up in N32S (n=338)

LOG₁₀(P-value)
OCTN2 (N32S) vs. OCTN2 (+/+)

LOG₂ fold change
(OCTN2 (N32S) vs. OCTN2 (+/+))

SGCD, LGALS3, CD36, FABP5, GATA4, ACAT2, ARG2, PPP1R1A, NPPA, STAT3

DES, ACTB, BMP1, NEFH, APOL2, TAPBP, CALD1, MRC2, EPCAM, TAGLN, ANXA1, TAGLN2, MTTP, CERT, PLIN2, SLC3A2, COL6A1, TPM4, MB, FN1, THY1, COL1A1, APOE, MARCKS, APOA1, SLC25A20, VTN, LPCAT3, ACSL4, VDAC3, CPT1B, FITM1, CP

C

Glycolysis: ALDOC, ALDOA, LDHB, PGAM2, PGAM1, ADH5, LDHA, ALDH7A1, PFKM, PKM, ALDH9A1, GAPDH, PGM1, TPT1, ENO2, DLAT, ENO3, HK2, HK1

Carnitine Shuttle: SLC27A6, SLC27A1, CPT2, ACADVL, SLC25A20, CPT1B, CPT1A

Cardiac proteins: MYL7, MYL3, CKM, MYL2, TNNC1, MYL4, RYR2, TNNT2, MYH6, CKMT2, PLN, ATP2A2, MYH7, FHL2, MYBPC3, ACTN2, ACTN1

ETC: NDUFA4, NDUFA11, NDUFA8, NDUFA10, ATP5S, ATP5H, ATP5O, COX6C, COX7C, COX7A2, COX7A1

Beta oxidation: DECR1, ACADM, ACAD8, ECI2, ACADSB, ECI1, ECHS1, ACADS, ACAD10, HADHB, ECH1, HADHA, ACADVL, ACAD9

TCA-Cycle: ACAT2, MDH1, MDH2, ACO1, SDHA, ACAT1, IDH3A, IDH3B, OGDH, CS, PDK3

Lipid metabolism: CD36, FABP5, APOE, SLC27A6, APOA1, FABP3, FITM1, ACSL4, HMOX2, VLDLR, ACBD6, LRP1, LDLR, NCEH1, HSD17B12, MTTP, LPCAT3

OCTN2 (+/+) OCTN2 (N32S) OCTN2 (-/-)

D

Ribosome
Other types of O-glycan biosynthesis
N-Glycan biosynthesis
Ferroptosis
Cholesterol metabolism
Complement and coagulation cascades
Fatty acid metabolism
Oxidative phosphorylation
ECM-receptor interaction
PPAR signaling pathway
Ar. right ventr. cardiomyopathy (ARVC)
Systemic lupus erythematosus

Enrichment Ratio

Pyruvate metabolism
Propanoate metabolism
Arginine biosynthesis
Glyoxylate and dicarboxylate metabolism
Pentose phosphate pathway
Biosynthesis of amino acids
Carbon metabolism
Arginine and proline metabolism
Glycolysis / Gluconeogenesis
Cysteine and methionine metabolism
Ar. right ventr. cardiomyopathy (ARVC)
PPAR signaling pathway

Enrichment Ratio

939

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 (+/+) 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.

A Glucose consumption per workload

B Last day of treatment

C OCTN2 (+/+)

OCTN2 (N32S)

OCTN2 (-/-)

D Acylcarnitine tissue content

E Ceramide tissue content
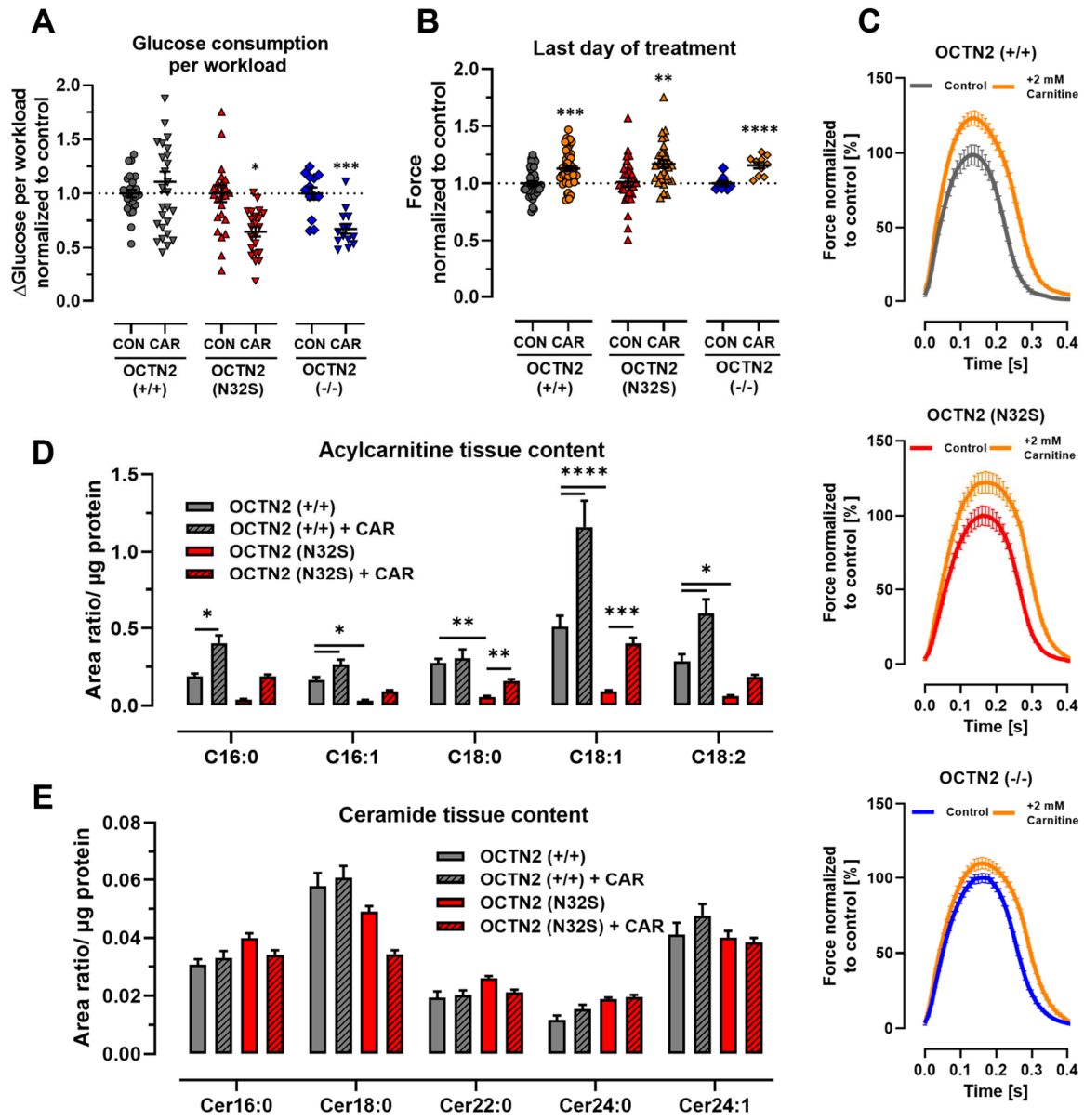
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972

Figure 5. A: Effect of carnitine supplementation Δglucose per workload (Δglucose= glucose concentration at baseline minus glucose concentration after 24 hours of incubation; workload = force × 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. 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. **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<0.001. 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. **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.
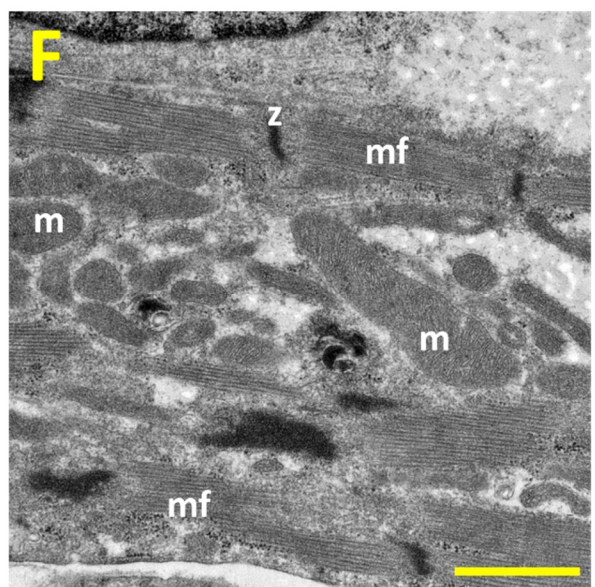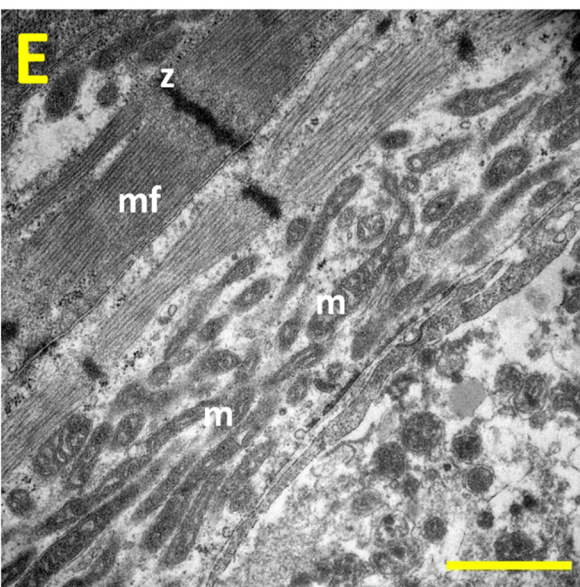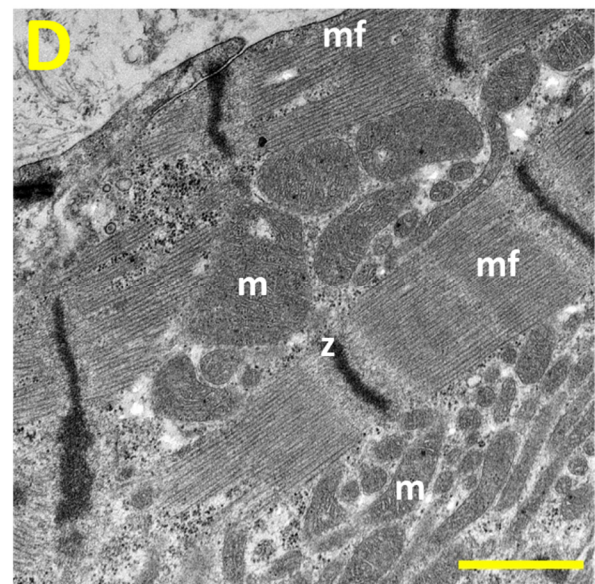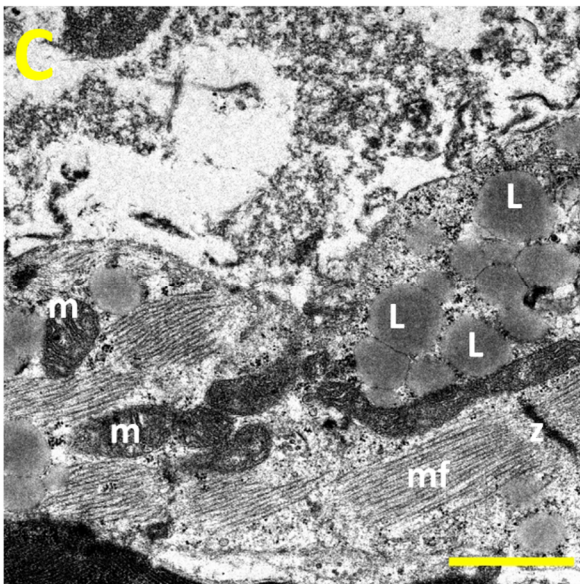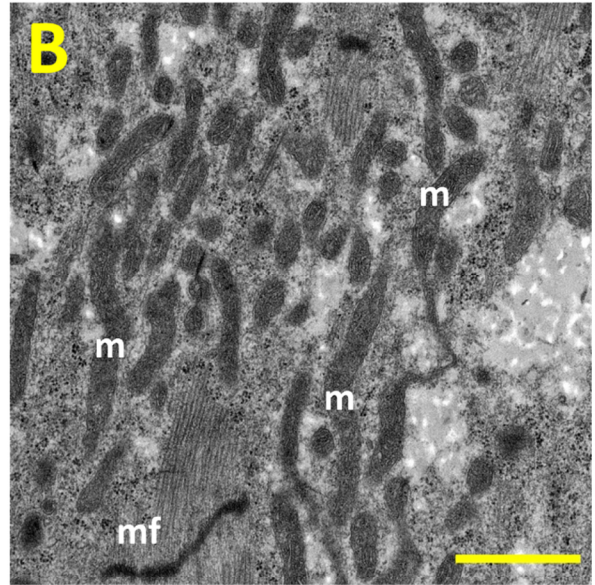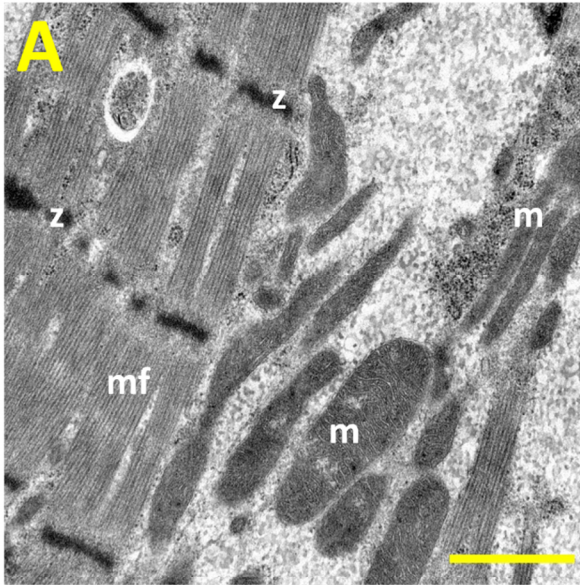
Figure 6. Transmission electron microscopy of OCTN2 EHTs. A + B: OCTN2 (+/+), C + D: OCTN2 (N32S), E + F: OCTN2 (-/-). A, C and E untreated, B, D and F supplemented with carnitine (2 mM). mf: myofilaments, z: z-line, m: mitochondria, L: lipid droplet. Scale bar = 1 μm.

A

All samples

Cardiomyocytes · Endothelial cells · Myeloid
Cardiomyocytes (proliferating) · Fibroblasts

OCTN2 (+/+)   OCTN2 (N32S)   OCTN2 (-/-)

B

C

D

All samples

FB1 · FB2 · FB3 · FB4

OCTN2 (+/+)   OCTN2 (N32S)   OCTN2 (-/-)

E

F
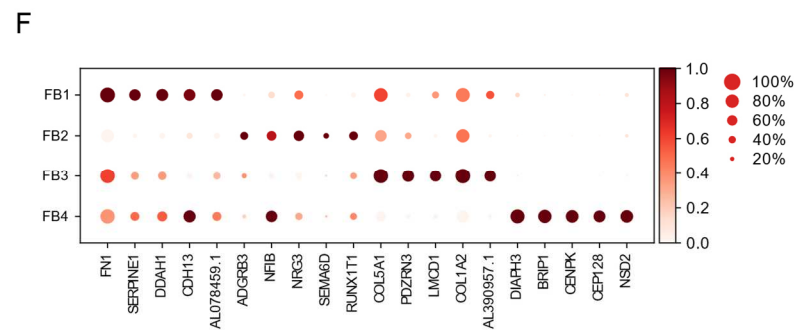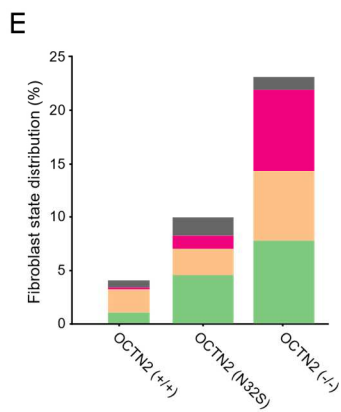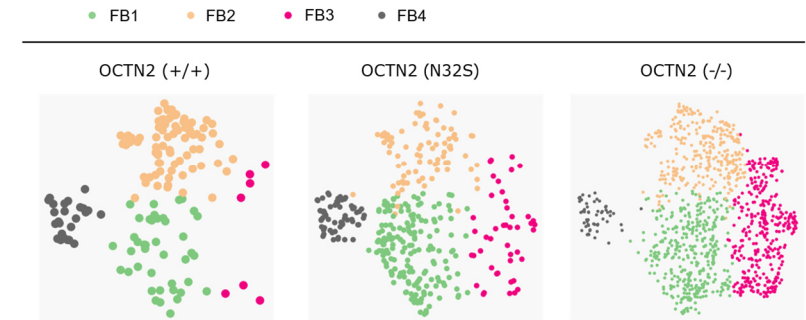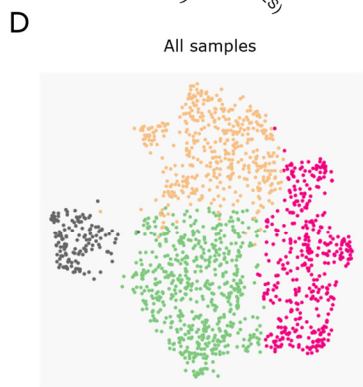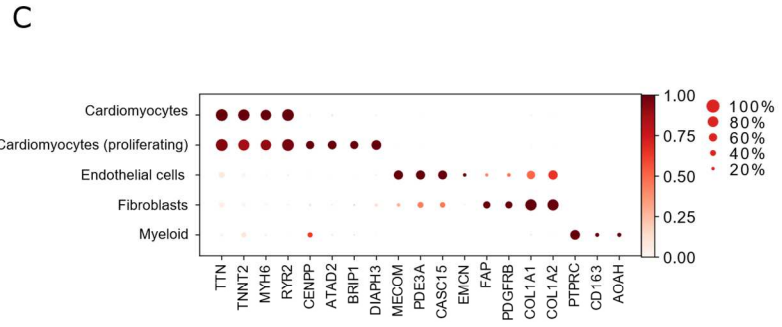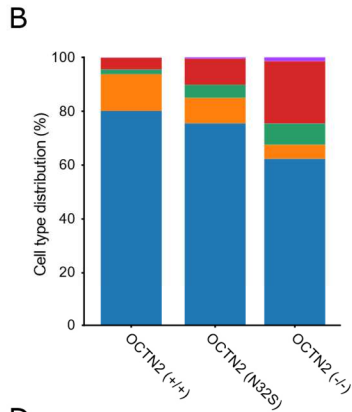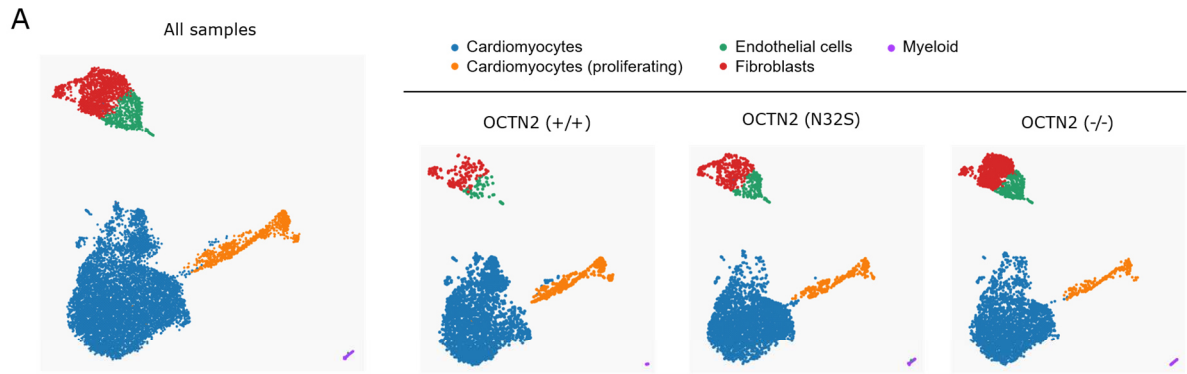
1002
1003
1004
1005

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.
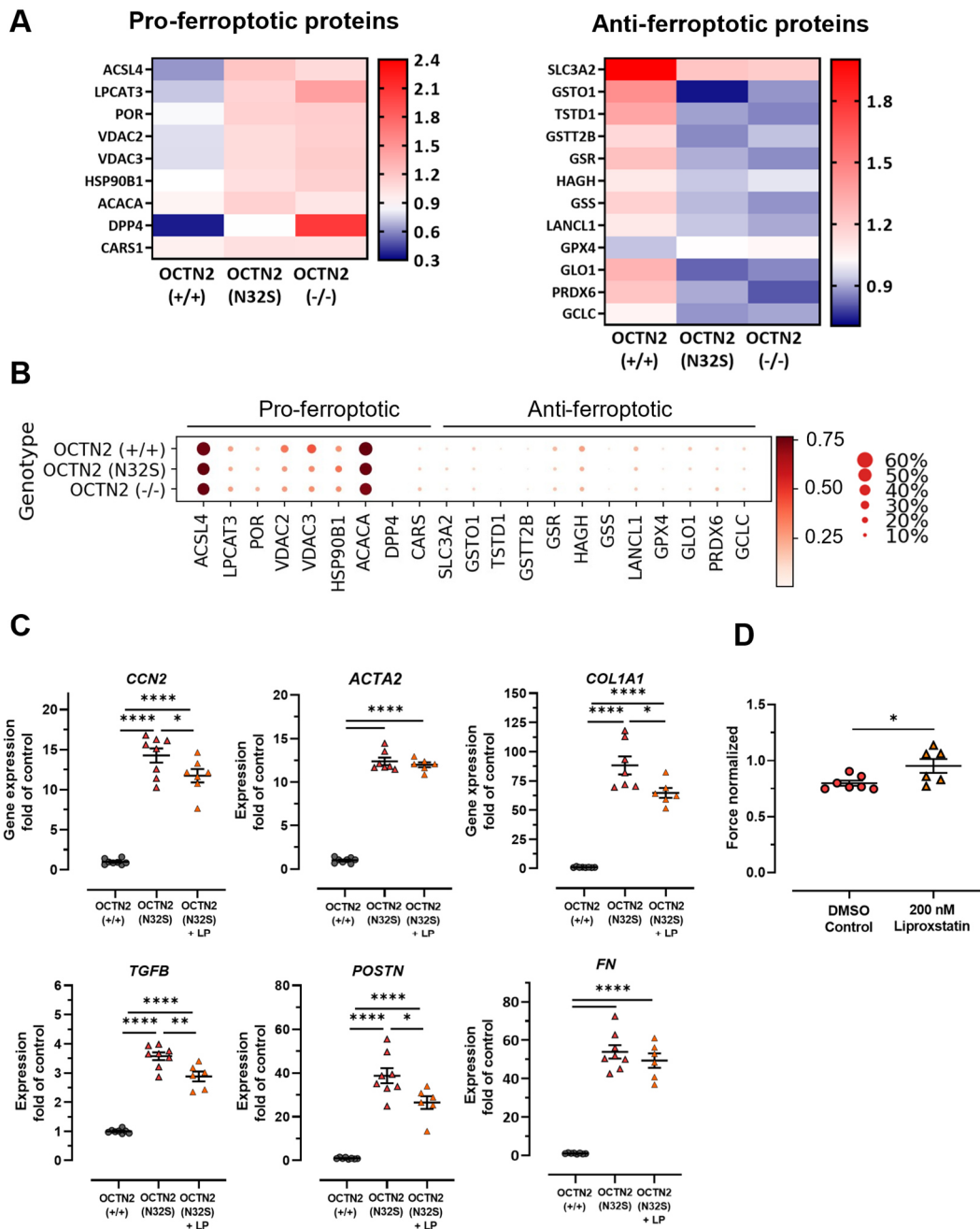
Figure 8. Evidence for ferroptosis pathway activation in TMT-based quantitative proteomic analysis and pharmacological inhibitor experiments. A: Proteomic analysis heatmaps display the relative abundance of pro- and anti-ferroptotic proteins of all genotypes. OCNT2 (+/+): mean of 10 EHTs from 1 batch; 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). **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. OCNT2 (+/+): 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 information

Supplementary Figures
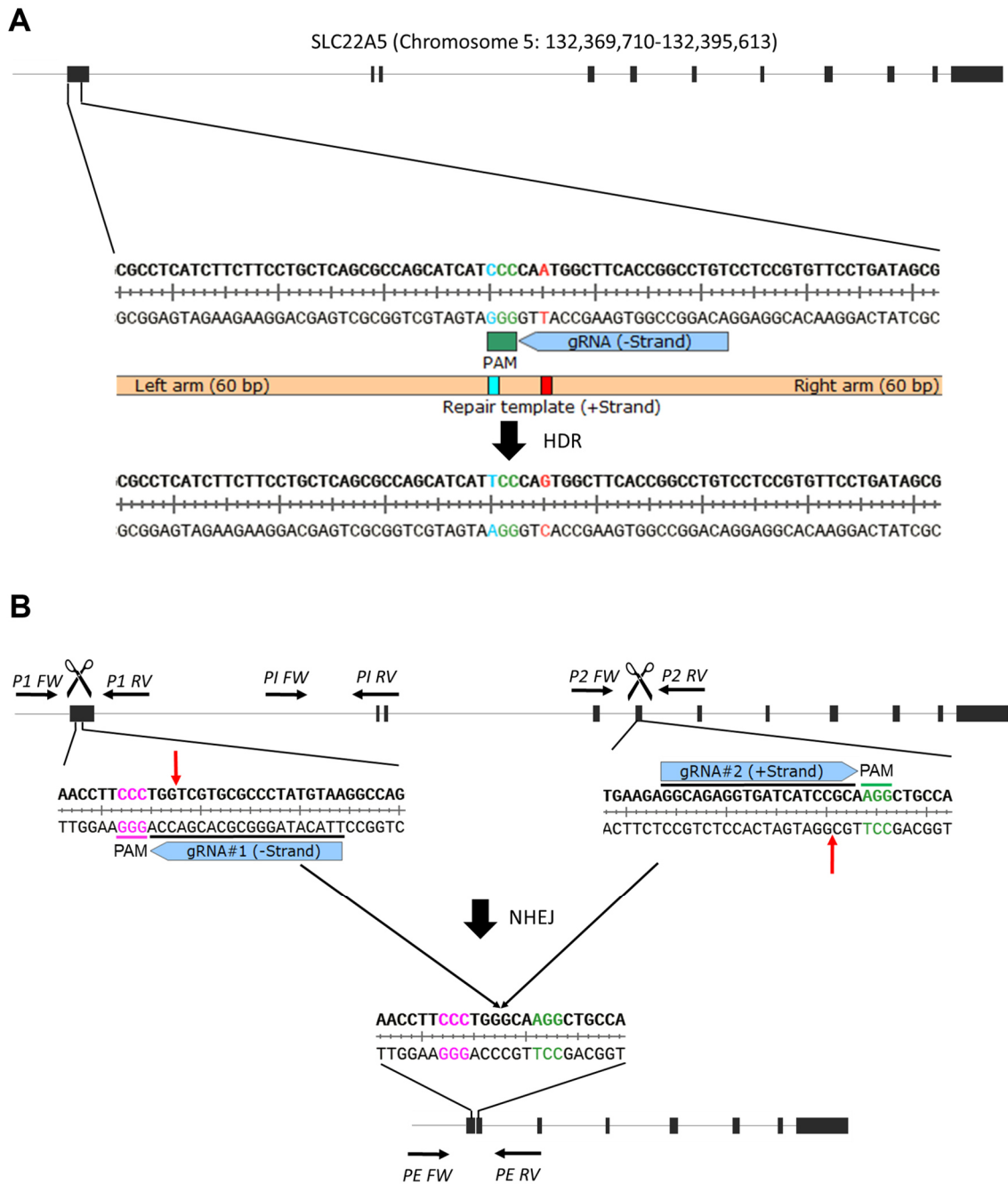
1036 **Supplementary Figure 1. A:** Schematic overview of CRISPR/Cas9 strategy for OCTN2 (N32S)
1037 generation. A ssODN containing the desired point mutation was co-transfected with CRISPR
1038 components to introduce the mutation c.95A>G, p.N32S in exon1 of the *SLC22A5* gene.
1039 Depicted is the gRNA target site and the predictive DNA sequence after successful integration
1040 of the repair template. **B**: Schematic overview over CRISPR/Cas9 strategy for OCTN2 (-/-)
1041 generation. Two gRNA were co-transfected to induce simultaneous cutting and a large deletion
1042 in the *SLC22A5* gene. Depicted are the gRNA target sites and the predictive DNA sequence
1043 after successful editing. Red arrows indicate the predicted Cas9 cutting sites. Black arrows
1044 indicate the primer binding sites for PCR validation. P1: Primer target region gRNA1; P2: Primer
1045 target region gRNA2; PI: Primer internal; PE: Primer external; Pink: PAM1; Green: PAM2.
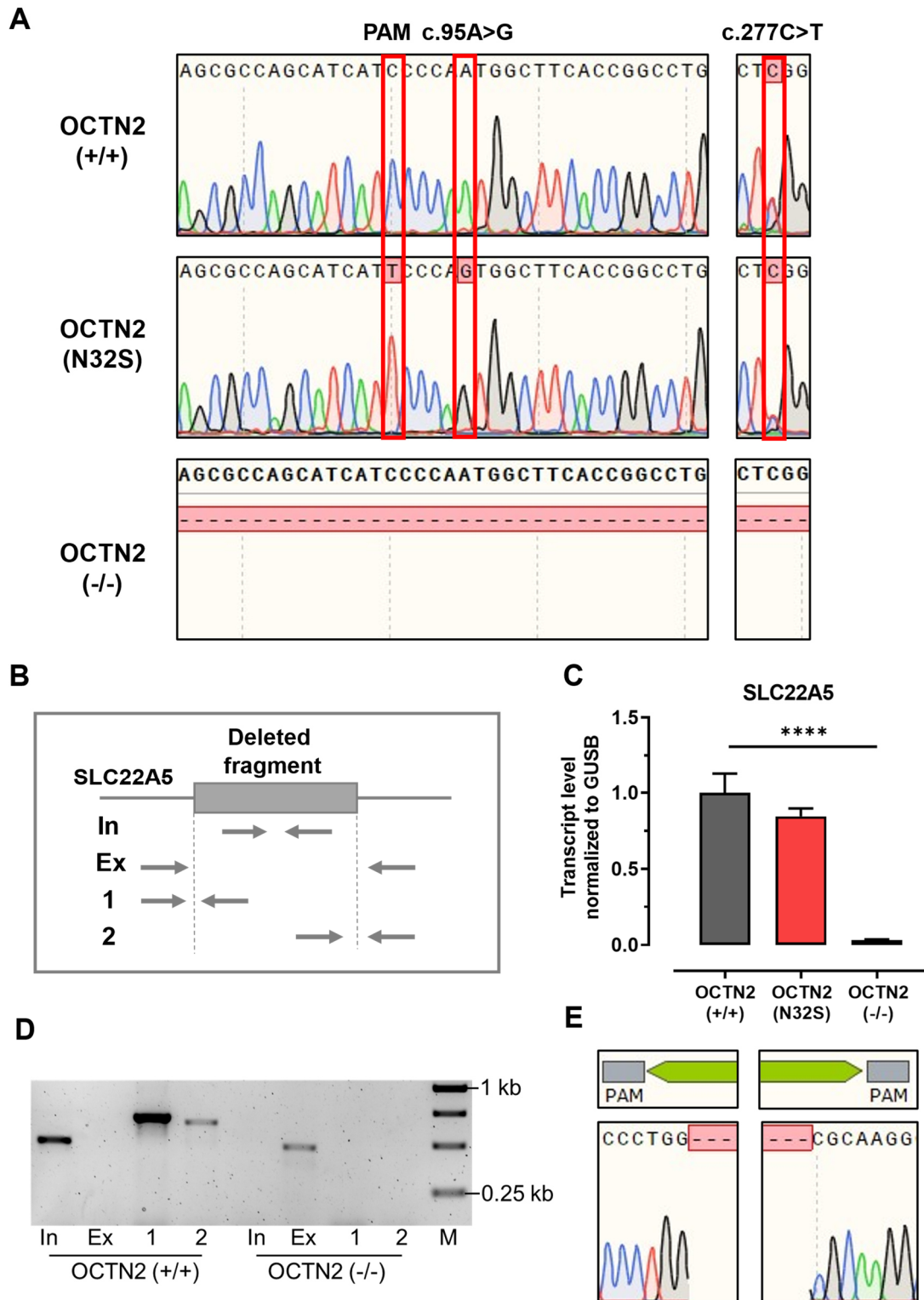
Supplementary Figure 2. Genotype characterization of CRISPR/Cas9-edited hiPSCs. A: Sanger sequencing traces of OCTN2 (+/+) and derived single cell clones for OCTN2 (N32S) and OCTN2 (-/-) genotypes. The red boxes indicate the silent PAM- and c.95A>G mutation and the heterozygous silent c277C>T mutation 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.
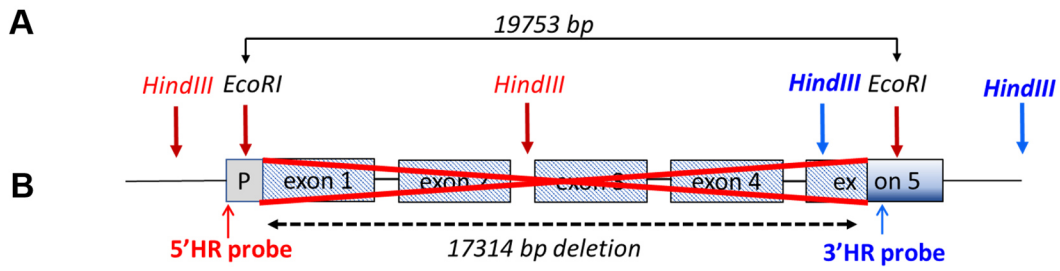
1055

1056
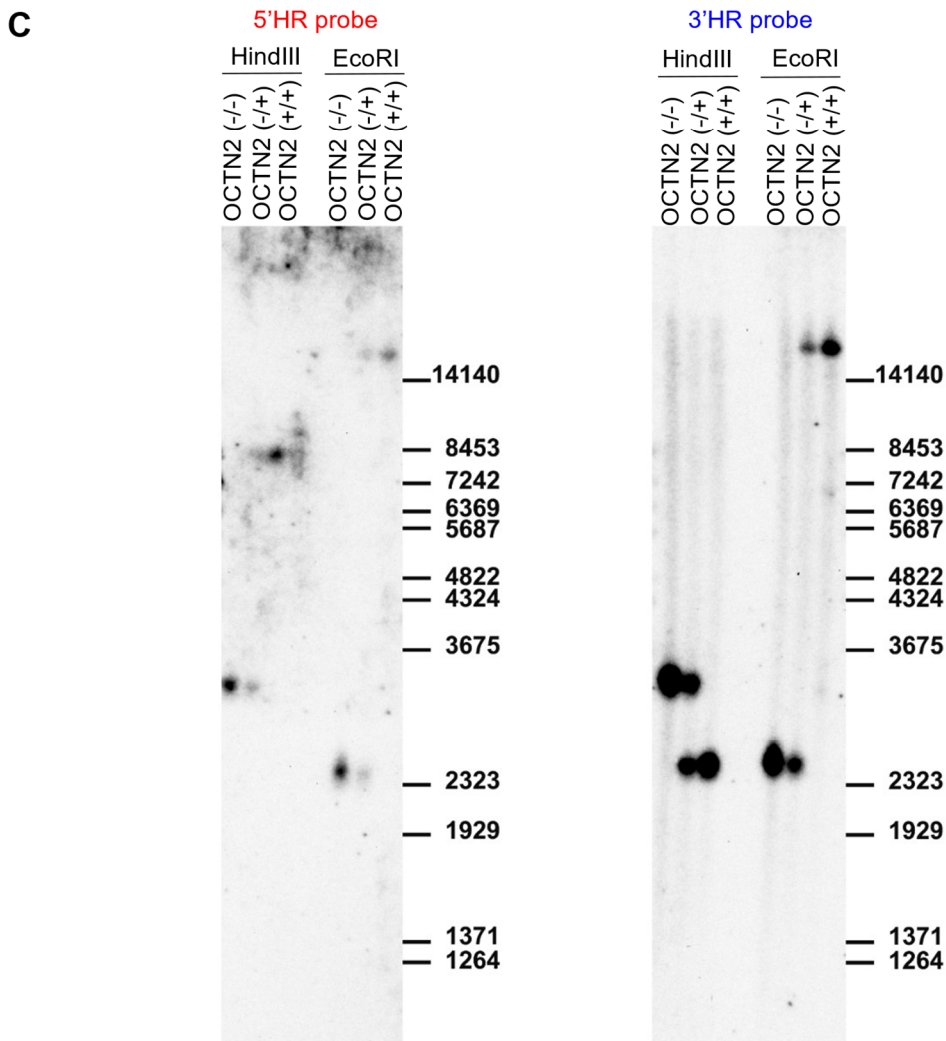
1057 **Supplementary Figure 3**. **Southern blot analysis of genomic DNA from OCTN2 (+/+), OCTN2**
1058 **(-/+) and OCTN2 (-/-) hiPSC. A**: Schematic presentation of Southern blot probes hybridization
1059 position. HindIII and EcoRI enzymes were used for enzymatic digestion of genomic DNA
1060 samples. **B**: Fragment size prediction of 5' HR and 3'HR hybridization after HindIII or EcoRI
1061 restriction enzyme digest. **C**: Southern blot analysis of OCTN2 (+/+), OCTN2 (-/+) and OCTN2 (-
1062 /-) hiPSC.

A OCTN2 (+/+)
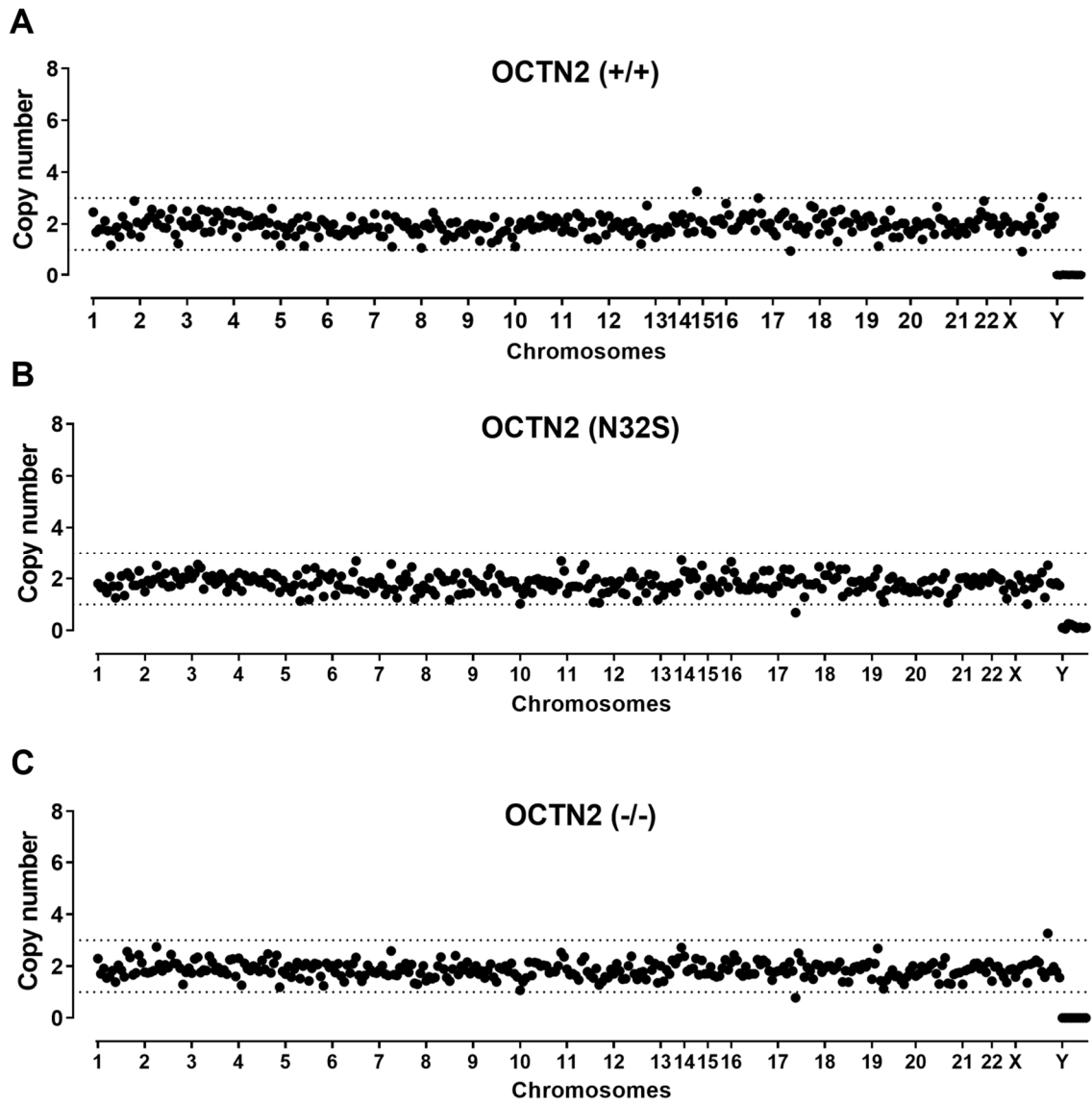
B OCTN2 (N32S)

C OCTN2 (-/-)

1063

Supplementary Figure 4. Nanostring karyotype analysis of hiPSC master cell bank samples.
**A**: OCTN2 (+/+), **B**: OCTN2 (N32S) and **C**: OCTN2 (-/-).
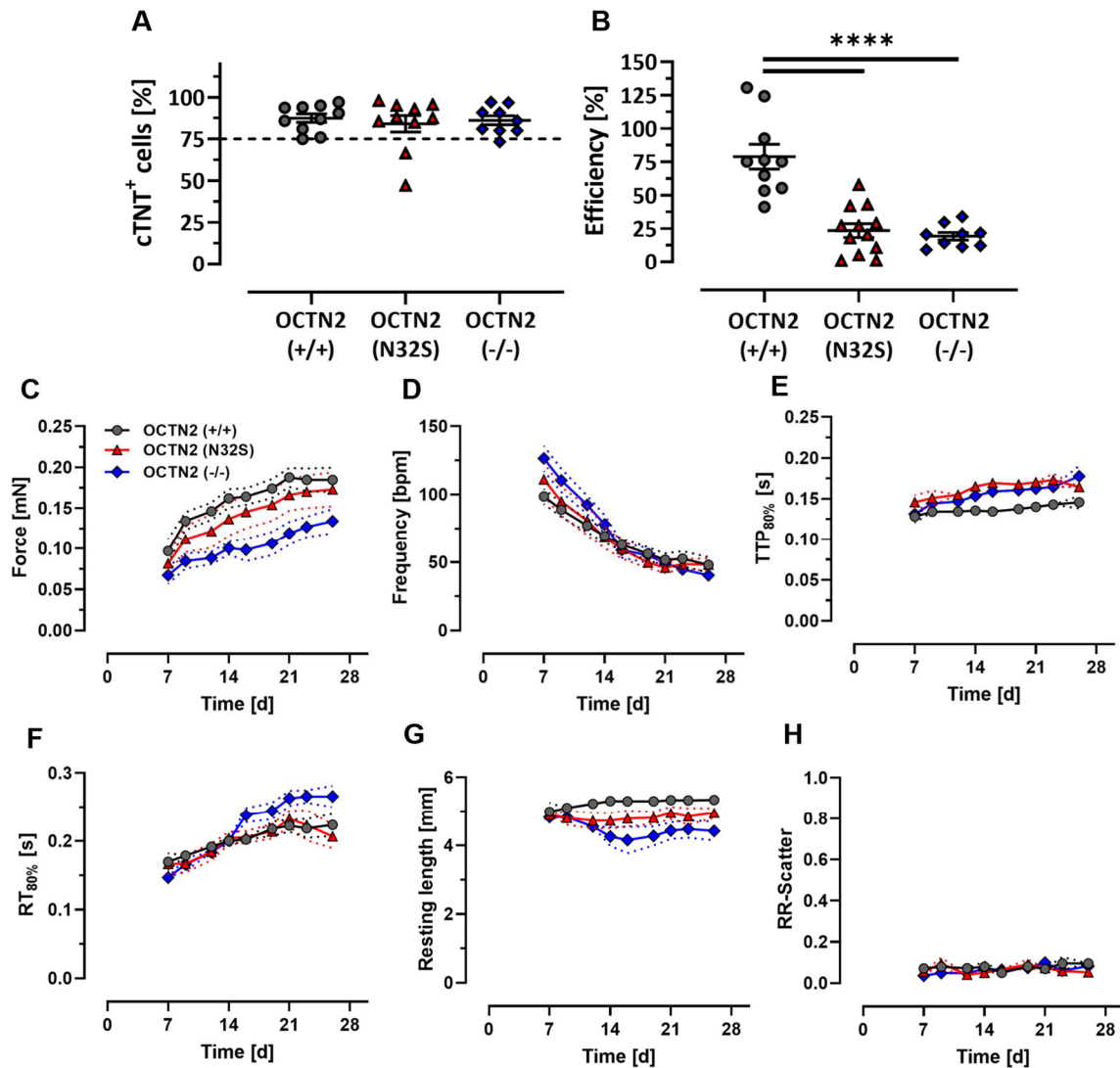
1066

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, OCTN2 (N32S): n=10, OCTN2 (-/-): 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 RR-Scatter (parameter of irregularity) of spontaneous beating EHTs between day 7 and day 26. OCNT2 (+/+): n=152 EHTs from 9 batches, OCTN2 (N32S): n=108 EHTs from 7 batches, OCTN2 (-/-): n=91 EHTs from 5 batches, data are expressed as EHT batch mean±SEM.
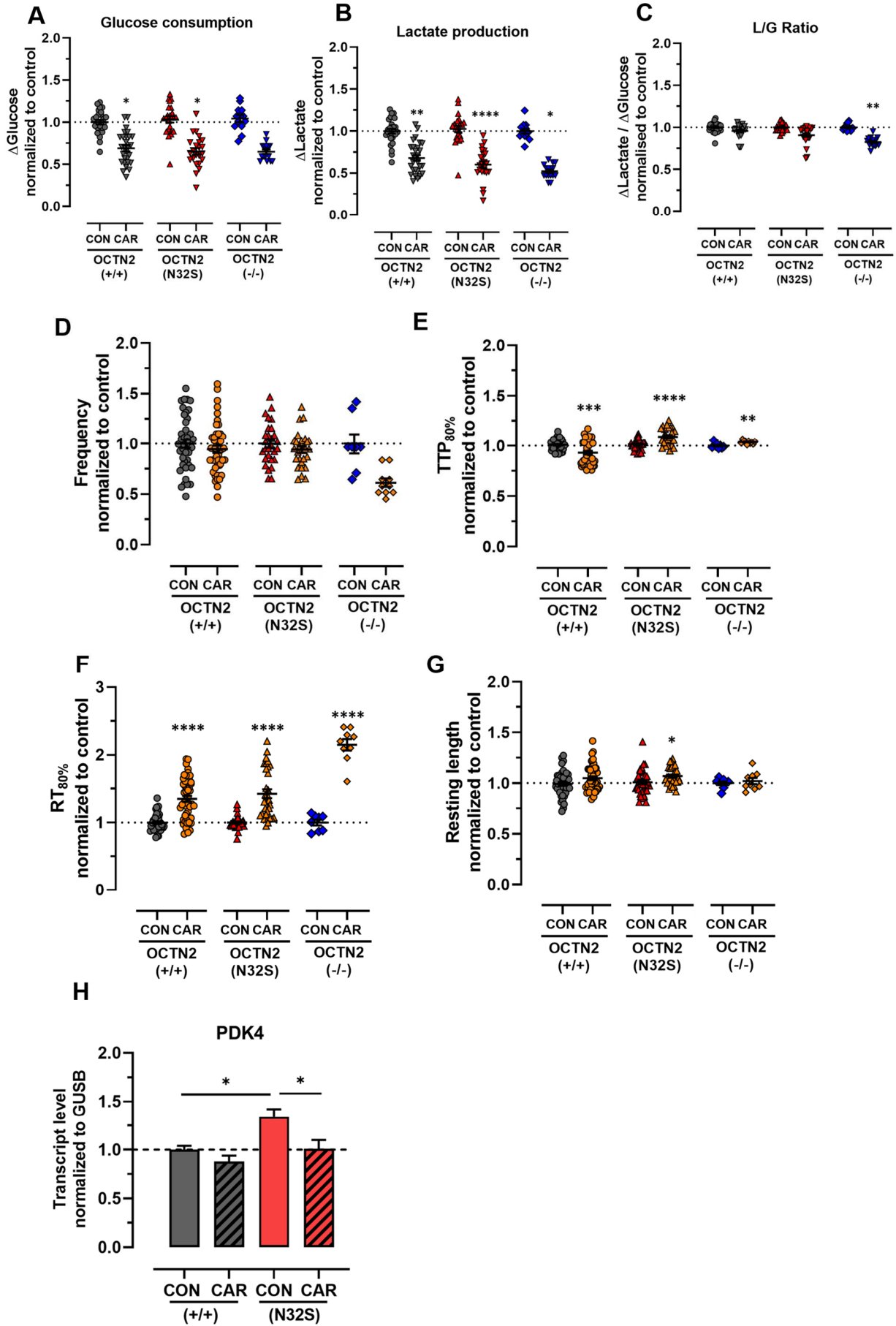
A

B

C
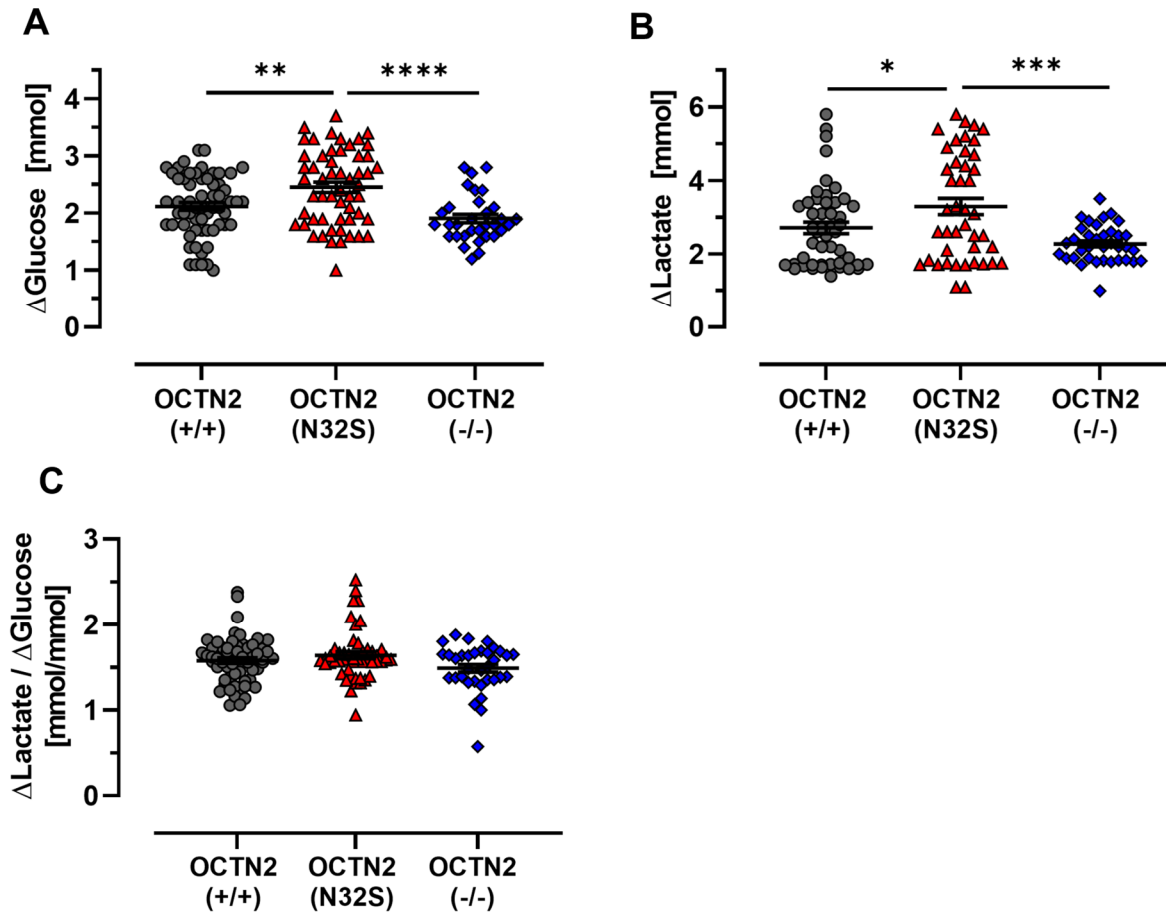
1078

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. 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 independent EHT. Data are expressed as mean±SEM.

1087
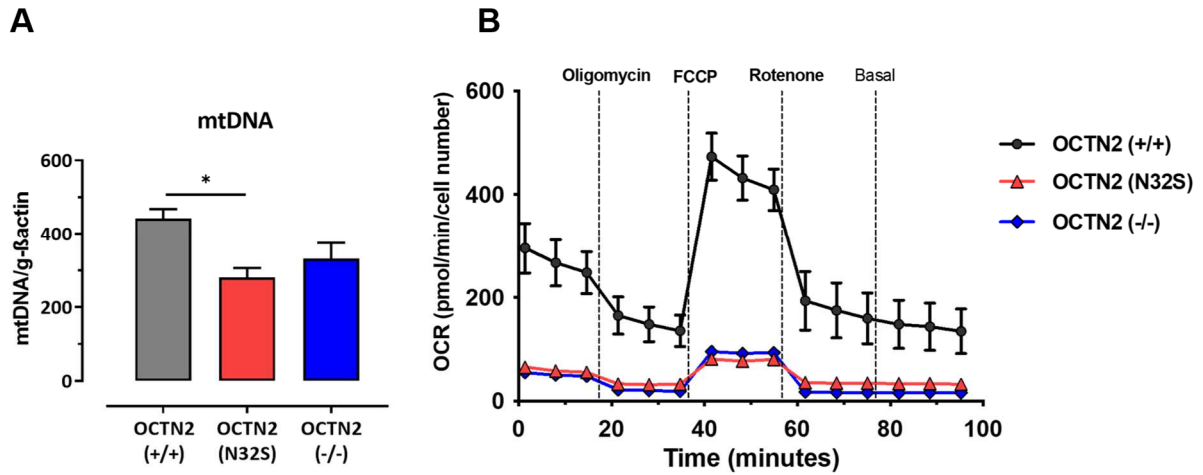
1088

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). OCNT2 (+/+): 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 OCNT2 (+/+), (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.

A Glucose consumption

B Lactate production

C L/G Ratio

D Frequency

E $TTP_{80\%}$

F $RT_{80\%}$

G Resting length

H PDK4

1096

1097 **Supplementary Figure 8. A-C: Effect of carnitine supplementation on glucose consumption**
1098 **and lactate production. A:** ΔGlucose (Δglucose= glucose concentration at baseline minus
1099 glucose concentration after 24 hours of incubation); **B**: ΔLactate (Δlactate= lactate
1100 concentration after 24 hours of incubation minus lactate concentration at baseline). Nested t-
1101 test vs CON, **p<0.01, ***p<0.001, ****p<0.0001. OCNT2 (+/+) control: n=27 EHTs from 3
1102 batches. OCNT2 (+/+) + carnitine (2 mM): n=28 EHTs from 3 batches. OCTN2 (N32S) control:
1103 n=23 EHTs from 3 batches. OCTN2 (N32S) + carnitine (2 mM): n=23 EHTs from 3 batches,
1104 OCTN2 (-/-) control: n=13 EHTs from 3 batches, OCTN2 (-/-) + carnitine (2 mM): n=16 EHTs from
1105 3 batches. Data are expressed as mean±SEM. **D-G:** Effect of carnitine supplementation on
1106 spontaneous beating EHTs at the last day of treatment (Day 33-42). **D**: Frequency, **E**: Time to
1107 peak, **F**: Relaxation time and **G**: Resting length. Values were normalized to last day of treatment
1108 of untreated control. Student's t-test vs CON, **p<0.01, ***p<0.001, ****p<0.001. OCNT2
1109 (+/+) control: n=54 EHTs from 4 batches. OCNT2 (+/+) + carnitine (2 mM): n=49 EHTs from 4
1110 batches. OCTN2 (N32S) control: n=36 EHTs from 3 batches, OCTN2 (N32S) + carnitine (2 mM):
1111 n=33 EHTs from 3 batches, OCTN2 (-/-) control: n=9 EHTs from 1 batch, OCTN2 (-/-) + carnitine
1112 (2 mM): n=9 EHTs from 1 batch. Data are expressed as mean±SEM. **H:** Effect of carnitine
1113 supplementation on PDK4 mRNA expression. OCNT2 (+/+) and OCTN2 (N32S) EHTs were
1114 treated over the entire culture time harvested on day 42. Gene expression was normalized to
1115 GUSB over OCTN2 (+/+) control. n=7 EHTs per genotype and carnitine treatment from 1 batch.
1116 2-way ANOVA followed by Bonferroni's post-test for multiple comparisons, *p<0.05. Data are
1117 expressed as mean±SEM.
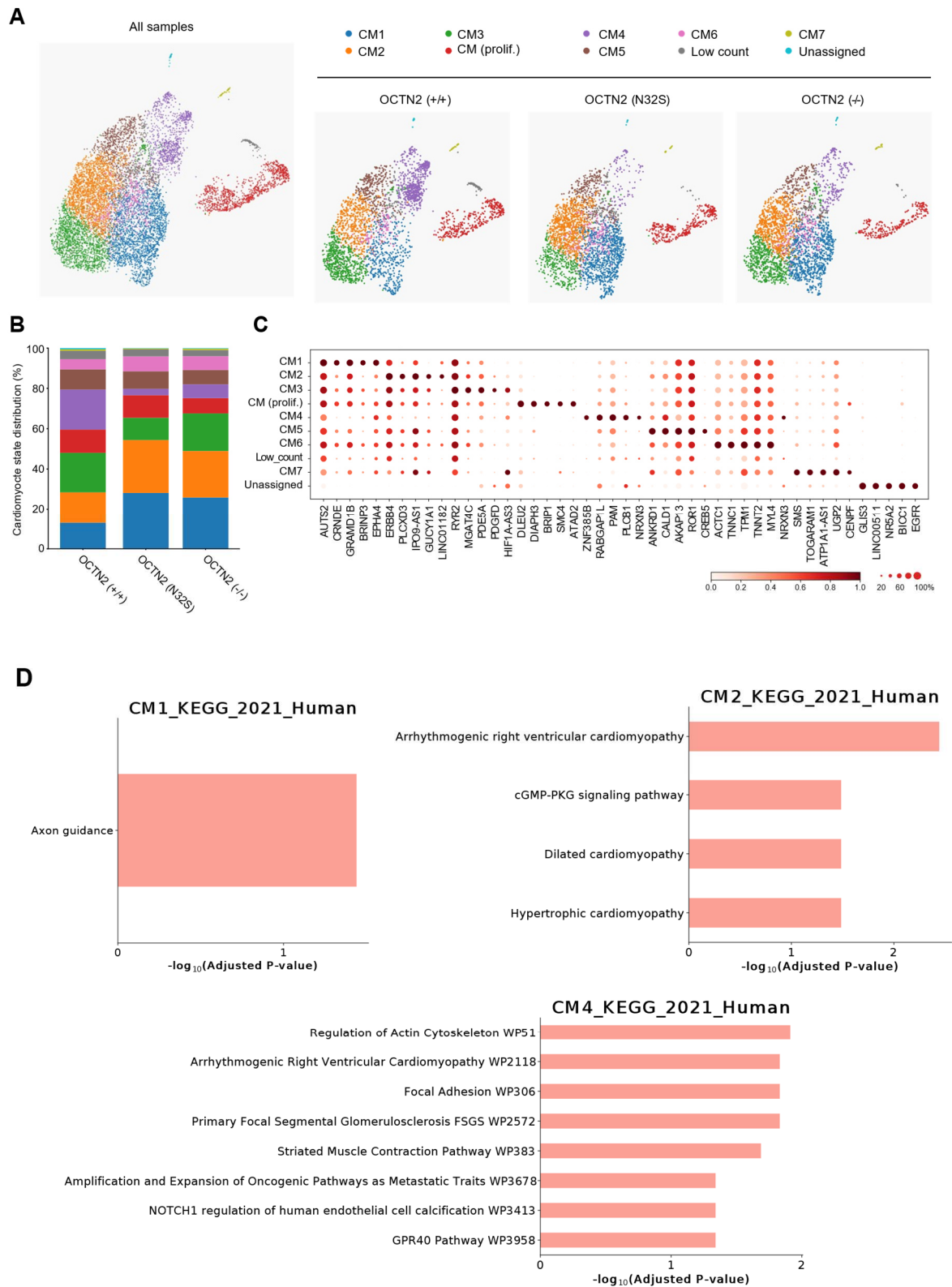
Supplementary Figure 9. Cardiomyocyte and fibroblast subcluster analysis in OCTN2 genotypes. A: Representative UMAP plot after single-nucleus RNA sequencing of all samples and individual genotypes. **B:** Percentage of cardiomyocyte subcluster per genotype. **C:** Dot plot graph showing the relative 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.

A

B
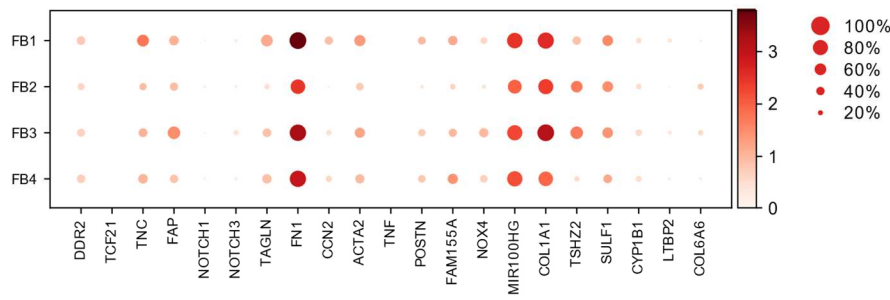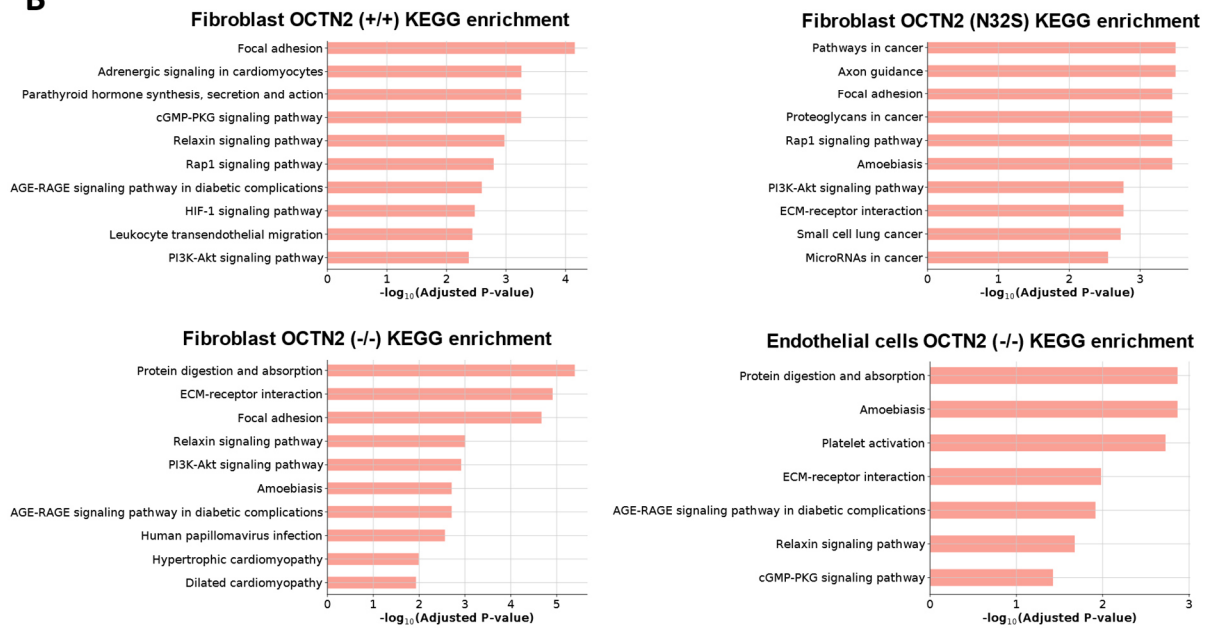
1125

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 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.

1133   Supplementary Tables

1134   Supplementary Table 1. Detected proteins proteomic analysis

1135   Supplementary Table 2. KEGG Enrichment analysis of proteins with significant higher
1136   abundance in OCTN2 (N32) vs OCTN2 (++)

1137   Supplementary Table 3. KEGG Enrichment analysis of proteins with significant lower
1138   abundance in OCTN2 (N32) vs OCTN2 (++)

1139   Supplementary Table 4. Composition of stem cell related culture media

1140   Supplementary Table 5. SsODN- and gRNA sequences

1141   Supplementary Table 6. Primer pairs for PCR and Sanger sequencing

1142   Supplementary Table 7. Primary antibodies used for flow cytometry/FACS

1143   Supplementary Table 8. Primer pairs for qPCR

1144   Supplementary Table 9. Primer pairs for 5'HR probe and 3' HR probe design

1145

1146   Supplementary Videos

1147   Supplementary Video 1. Spontaneously beating OCTN2 (+/+) EHT

1148   Supplementary Video 2. Spontaneously beating OCTN2 (N32S) EHT

1149   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
- SupplementaryTables49.pdf
- SupplementaryVideo1.SpontaneouslybeatingOCTN2EHT.avi
- SupplementaryVideo2.SpontaneouslybeatingOCTN2N32SEHT.avi
- SupplementaryVideo3SpontaneouslybeatingOCTN2EHT.avi