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**Mitochondrial fission process 1 (MTFP1) controls bioenergetic efficiency and prevents inflammatory cardiomyopathy and heart failure in mice**

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28 **Abstract**

29 Mitochondria are paramount to the metabolism and survival of cardiomyocytes. Here we show that  
30 Mitochondrial Fission Process 1 (MFTP1) is essential for cardiac structure and function.  
31 Constitutive knockout of cardiomyocyte MFTP1 in mice resulted in adult-onset dilated  
32 cardiomyopathy (DCM) characterized by sterile inflammation and cardiac fibrosis that progressed  
33 to heart failure and middle-aged death. Failing hearts from cardiomyocyte-restricted knockout  
34 mice displayed a general decline in mitochondrial gene expression and oxidative phosphorylation  
35 (OXPHOS) activity. Pre-DCM, we observed no defects in mitochondrial morphology, content, gene  
36 expression, OXPHOS assembly nor phosphorylation dependent respiration. However, knockout  
37 cardiac mitochondria displayed reduced membrane potential and increased non-phosphorylation  
38 dependent respiration, which could be rescued by pharmacological inhibition of the adenine  
39 nucleotide translocase ANT. Primary cardiomyocytes from pre-symptomatic knockout mice  
40 exhibited normal excitation-contraction coupling but increased sensitivity to programmed cell death  
41 (PCD), which was accompanied by an opening of the mitochondrial permeability transition pore  
42 (mPTP). Intriguingly, mouse embryonic fibroblasts deleted for *Mtfp1* recapitulated PCD sensitivity  
43 and mPTP opening, both of which could be rescued by pharmacological or genetic inhibition of the  
44 mPTP regulator Cyclophilin D. Collectively, our data demonstrate that contrary to previous in vitro  
45 studies, the loss of the MFTP1 promotes mitochondrial uncoupling and increases cell death  
46 sensitivity, causally mediating pathogenic cardiac remodeling.

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48

49 **Introduction**

50 Mitochondria are multifaceted organelles that are essential in every tissue of the body and are  
51 most abundant in the heart, where they control the metabolism and survival of cardiac cells  
52 (Kolwicz et al., 2013). Mitochondria are double membrane-bound organelles, composed of an  
53 inner (IMM) and an outer mitochondrial membrane (OMM), which separate the intermembrane  
54 space (IMS) from the matrix. The IMM extends internally to form cristae, which harbor essential  
55 macromolecular complexes such as the machinery of oxidative phosphorylation (OXPHOS). The  
56 OXPHOS system is comprised of two functional entities: the electron transport chain (ETC) and  
57 the phosphorylation system, which includes the integral membrane ATP synthase and carriers  
58 such as the adenine nucleotide translocase (ANT), which imports ADP into the matrix and exports  
59 ATP (Chance and Williams, 1956). The ETC is composed of four macromolecular complexes (I, II,  
60 III, and IV) and of mobile electrons carriers as coenzyme Q (CoQ) and cytochrome-c (Cyt c). The  
61 energy available for ATP synthesis is directly derived from the membrane potential ( $\Delta\Psi$ ) and  
62 proton motive force generated across the IMM by electron transfer from by the ETC, which is then  
63 harnessed by the ATP synthase (complex V) to generate ATP (Mitchell, 1961). Continuous  
64 generation of ATP is fundamental for the function of cardiomyocytes, enabling them to meet the  
65 enormous energy requirement for the contraction–relaxation cycles that drive their contractility  
66 (Kohlhaas et al., 2017). Defects that impair OXPHOS assembly and function can promote fatal  
67 cardiomyopathies (Antonicka et al., 2003a, 2003b; Graham et al., 1997; Hansson et al., 2004;  
68 Karamanlidis et al., 2013). The beating heart demands near-maximum OXPHOS capacity, with  
69 scant aerobic ATP reserves under normal conditions (Mootha et al., 1997). Consequently, even  
70 modest uncoupling of the ETC from ATP synthesis, which can occur when protons are diverted  
71 back across the IMM through uncoupling channels rather than being used by the ATP synthase,  
72 would be expected to yield fatal consequences for cardiac function and health, although this has  
73 never been tested.

74 While most famous for their role as the powerhouse of the cell, mitochondria have proven to be  
75 essential for cardiac homeostasis through the regulation of various biosynthetic and signaling  
76 functions beyond OXPHOS, such as calcium buffering, reactive oxygen species (ROS) generation  
77 and maintenance, programmed cell death (PCD) and innate immune responses (Zhou and Tian,  
78 2018). Under stress conditions, extrinsic or intrinsic signals can lead to a permeabilization of the

79 outer membrane (MOMP) resulting in activation of caspases and PCD (Bock and Tait, 2020). At  
80 the IMM, long-lasting opening of the mitochondrial permeability transition pore (mPTP) allows for  
81 unselective diffusion of low molecular weight solutes and water (<1.5 kDa), causing an osmotic  
82 pressure in the matrix that causes mitochondrial swelling (Carraro et al., 2020) and rupture,  
83 thereby releasing pro-apoptotic and pro-inflammatory mitochondrial factors into the cytosol (Bock  
84 and Tait, 2020). While the molecular identity of the mPTP is still the subject of feverous debate, to  
85 date several factors have been identified to be unequivocally crucial for its activation. Cyclophilin D  
86 (CypD, encoded by *Ppif*), a mitochondrial matrix isomerase which can be inhibited  
87 pharmacologically by cyclosporin A (CsA), calcium overload and ROS are known to promote  
88 mPTP opening and PCD induction (Baines et al., 2005; Carraro et al., 2020; Nakagawa et al.,  
89 2005). Notably, ablation of CypD or treatment with CsA can protect animals from cardiomyocyte  
90 death and cardiomyopathy induced by genetic (Song et al., 2015), infectious (Milduberger et al.,  
91 2021), and surgical lesions (Nakagawa et al., 2005).

92 The maintenance of mitochondrial morphology and structure is of critical importance for  
93 cardiac function (Sprenger and Langer, 2019). Mitochondrial morphology is regulated by opposing  
94 forces of mitochondrial fusion and division, which must be tightly regulated to ensure organellar  
95 function and quality control (Giacomello et al., 2020; Ng et al., 2021). Intrinsic (Ashrafian et al.,  
96 2010; Chen et al., 2015; Kageyama et al., 2014; Song et al., 2015; Wai et al., 2015) or extrinsic  
97 (Acin-Perez et al., 2018; Chen et al., 2009; Shirakabe et al., 2016) lesions that upset the balance  
98 between mitochondrial fission and fusion have devastating consequences for cardiomyocyte  
99 function and cardiac health. Mitochondrial dynamics is orchestrated by dynamin-like GTPases:  
100 OPA1 and mitofusins (MFN1/2) execute inner and outer membrane fusion, respectively, while  
101 DRP1 performs mitochondrial constriction and division once recruited to the OMM via interactions  
102 that require integral membrane proteins and receptors, such as MFF, MiD49/51, and FIS1. DRP1  
103 is the lynchpin of the mitochondrial fission apparatus, which is triggered to coalesce at contact  
104 sites with the ER, lysosomes, trans-golgi network, and actin by signals that can originate both  
105 outside (Cretin et al., 2021; Giacomello et al., 2020) and inside (Cho et al., 2017; Lewis et al.,  
106 2016; Tondera, 2005) mitochondria.

107 While it is unclear how IMM fission is executed, the aptly-named inner membrane protein  
108 Mitochondrial fission process 1 (MTPF1/MTP18), has emerged as a promising scaffold for the IMM

109 division apparatus, whose formal identification remains elusive (Ng et al., 2021). As per its  
110 namesake, *Mtfp1* was initially identified as a gene whose ablation was reported to reduce  
111 mitochondrial fission, as well as the proliferation and viability of cultured cells (Tondera, 2005;  
112 Tondera et al., 2004). The pro-apoptotic effects of *Mtfp1* depletion have been replicated in various  
113 cell lines (Duroux-Richard et al., 2016; Wang et al., 2017), however, more recent studies by the Li  
114 group (Aung et al., 2017a, 2017b; Wang et al., 2017) have reported the contrary in cultured cells,  
115 thus fueling the existing narrative that elongated mitochondria resulting from MTFP1 depletion  
116 protects cells from PCD. Whether this paradigm holds true in vivo has never been explored.

117 In this study, we created a cardiomyocyte-specific *Mtfp1* knockout mouse model to  
118 specifically investigate the role of this protein in vivo. Contrary to previous in vitro studies (Wang et  
119 al., 2017), we show that MTFP1 plays an essential role in maintaining cardiac energy metabolism  
120 as its deletion in post-natal cardiomyocytes drives a progressive dilated cardiomyopathy (DCM)  
121 culminating in HF and middle-aged death in mice. Surprisingly, MTFP1 ablation does not  
122 appreciably alter mitochondrial morphology in the heart and is entirely dispensable for  
123 mitochondrial fission. Unexpectedly, we discovered that MTFP1 depletion reduces OXPHOS  
124 efficiency in cardiac mitochondria by increasing proton leak through the adenine nucleotide  
125 translocase (ANT). Finally, we show MTFP1 ablation increases mPTP opening and renders  
126 cardiomyocytes and embryonic fibroblasts more sensitive to PCD. Altogether, our data reveal an  
127 unexpected role of MTFP1 in mitochondrial bioenergetics and provide mechanistic insights into  
128 how MTFP1 regulates the life and death of the cell.

129

130

## Results

131

### ***Mtfp1* deletion in cardiomyocytes causes dilated cardiomyopathy and middle-aged death**

132

MTFP1 is predicted to localize in mitochondrial inner membrane (Figure 1A), and *MTFP1* is highly

133

expressed in human cardiac tissue (GTEx plot, Figure S1A). To investigate the importance of

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MTFP1 for cardiac function we began by confirming its expression and submitochondrial

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localization in the mouse heart. Protease protection assays and alkaline carbonate extraction

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experiments performed on isolated cardiac mitochondria allowed us to demonstrate the inner

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membrane localization of MTFP1 in cardiac mitochondria (Figure S1B-C). To investigate its role in

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vivo, we generated a conditional mouse model for *Mtfp1* (*Mtfp1*<sup>LoxP/LoxP</sup>) on a C57Bl6/N

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background. Conditional mice were crossed with transgenic mice expressing Cre recombinase

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under the control of alpha myosin heavy chain promoter (Agah et al., 1997) (*Myh6*) to specifically

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ablate MTFP1 in post-natal cardiomyocytes (Figure 1B, S1D). We previously showed that genetic

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deletion mediated by *Myh6*-Cre occurs during the perinatal period (Wai et al., 2015) and by 8

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weeks of age cardiomyocyte-specific *Mtfp1* KO mice (*Myh6*-Cre<sup>Tg/+</sup> *Mtfp1*<sup>LoxP/LoxP</sup>, cMKO mice)

144

exhibited a 7-fold reduction in *Mtfp1* mRNA (Figure 1C) and undetectable levels of MTFP1 protein

145

in cardiac lysates assessed by immunoblot analysis (Figure 1D) and shotgun proteomics (Figure

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1E, Dataset EV1). Both male and female cMKO mice were generated according to Mendelian

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proportions and were outwardly normal and viable. However, cMKO mice had significantly

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shortened lifespans [median life span: 26.4 weeks (male), 37.5 weeks (female)] relative to wild

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type (WT) littermates (Figure 1F, Figure S1E), demonstrating that MTFP1 is required to protect

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against middle-aged death.

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To directly assess the importance of MTFP1 for cardiac structure and function, we

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performed longitudinal echocardiographic (echo) studies in male and female mice (Figure 1G-L,

153

S1F-K). Echo analyses beginning at 10 weeks of age revealed normal cardiac structure and

154

function in cMKO mice. By 18 weeks, however, despite normal cardiac structure we observed a

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progressive decrease in systolic function, culminating in dilated cardiomyopathy (DCM) and left

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ventricle (LV) remodeling. By 34 weeks, cMKO mice exhibited all the hallmarks of DCM and heart

157

failure (HF): significantly reduced LV ejection fraction [% EF, WT 60.31 ± 5.4 % versus cMKO

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25.87 ± 11.8 %; Figure 1H], thinning of the interventricular septum in systole [IVS (mm), WT 1.23

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± 0.097 versus cMKO 0.9 ± 0.092; Figure 1I), posterior wall during systole [PWs (mm), WT 1.39 ±

160 0.10 *versus* cMKO  $0.952 \pm 0.17$ ; Figure 1J), dilated LV chamber during the cardiac cycle of  
161 diastole [LVDD (mm), WT  $4.00 \pm 0.15$  *versus* cMKO  $5.02 \pm 0.65$ , Figure 1K] and systole [LVSD  
162 (mm), WT  $2.72 \pm 0.16$  *versus* cMKO  $4.43 \pm 0.84$ ; Figure 1L) with pulmonary congestion (Figure  
163 S1L) and increased heart mass at severe HF (Figure S1M).

164 To determine whether the progressive cardiac contractile dysfunction observed in cMKO  
165 mice was caused by primary defects in cardiomyocyte function, we assessed sarcomere length  
166 and shortening (Figure S1N-P) coupled to intracellular  $Ca^{2+}$  levels  $[Ca^{2+}]_c$  (Figure S1Q-S) in field-  
167 stimulated cMKO cardiomyocytes isolated from pre-symptomatic mice (8-10 weeks of age).  
168 Diastolic and systolic sarcomere length as well as  $[Ca^{2+}]_c$  transients were similar between WT and  
169 cMKO myocytes both at baseline (0.5 Hz stimulation frequency) and during  $\beta$ -adrenergic  
170 stimulation (5 Hz stimulation frequency) despite a modest increase of sarcomere shortening of  
171 cMKO myocytes under stress conditions and a normal sarcomere re-lengthening at baseline  
172 conditions. Together, these data indicate that *Mtfp1* deletion does not impinge upon cardiomyocyte  
173 excitation-contraction coupling before the onset of cardiomyopathy in vivo.

174 Histological analyses of cMKO hearts of mice at HF (34 weeks) confirmed defects in  
175 cardiac structure: hematoxylin-eosin (H&E) staining of cardiac cross sections showed LV chamber  
176 expansion and myocardial wall thinning, while Masson's trichrome and Picrosirius Red staining  
177 showed disruption of the myofibril architecture by dramatic fibrosis and collagen deposition at DCM  
178 (Figure 1M). We also found increased serum levels of cardiac troponin I (cTNI, Figure 1N) and  
179 myosin light chain 1 (MLC-1, Figure 1O) in cMKO mice sampled at 18 and 34 weeks, further  
180 substantiating the ongoing cardiomyocyte damage and death. We did not observe gender  
181 differences in the development of the cardiac dysfunction (Figure S1F-K), highlighting the essential  
182 nature of MTFP1 for cardiac structure and function.

183

#### 184 **Metabolic and inflammatory gene expression dysregulation in cMKO mice**

185 To gain further insights into the molecular and cellular mechanisms underlying cardiac pathology in  
186 cMKO mice, we performed transcriptomic analyses by bulk RNA sequencing (RNAseq) of LVs  
187 isolated from WT and cMKO mice at a pre-symptomatic stage (8 weeks of age) and at the onset of  
188 DCM (18 weeks). We observed up-regulation of *Nppa* in pre-symptomatic cMKO mice, which was  
189 associated to up-regulation of *Nppb* at the onset of DCM, prototypical cardiomyocyte stress-



190 response genes that are activated in response to hemodynamic load (Hohl et al., 2013) and  
191 metabolic or contractile abnormalities (Houweling et al., 2005) (Figure 1P). At the pre-symptomatic  
192 stage, we observed limited transcriptional remodeling in hearts from cMKO mice with differential  
193 expression of only ~1% of 25815 genes: 137 genes were downregulated, and 122 genes were  
194 upregulated in cMKO mice (Dataset EV2, Figure 1P), whereas at 18 weeks, we observed a much  
195 broader transcriptional response with 3642 differentially expressed genes in cMKO mice  
196 manifesting early signs of DCM (Dataset EV2, Figure 1P).

197 Functional enrichment analyses performed with g:Profiler (Reimand et al., 2019) and  
198 Enrichr (Chen et al., 2013) revealed a number of dysregulated genes involved in various metabolic  
199 processes (Figure 1Q, left). Among the downregulated genes with the gene ontology (GO) term  
200 metabolic process (Dataset EV2) were genes required for OXPHOS, TCA cycle, fatty acid  
201 oxidation and pyruvate metabolism, suggestive of cardiometabolic changes previously observed in  
202 mitochondrial models of HF (Hansson et al., 2004; Liao et al., 2015; Wai et al., 2015; Zhou and  
203 Tian, 2018). In fact, further examination revealed that half of all mitochondrial genes referenced on  
204 MitoCarta (Rath et al., 2021) were downregulated (Figure 1Q, middle). On the other hand, we  
205 observed a strong sterile inflammatory gene expression signature and innate immune engagement  
206 (Figure 1Q, right), which together with dysregulated extracellular matrix-remodeling genes  
207 [profibrotic cytokines such as TGF $\beta$ , collagen precursor genes (*Col11a1*, *Col14a1*, *Col8a1*) and  
208 matrix metalloproteinases (*Mmp-2*, *Mmp-14*, *Mmp-3*)] (Figure 1R) corroborates the cardiac fibrosis  
209 revealed by histological analysis of cMKO hearts (Figure 1M). Notably, the suppression of  
210 mitochondrial gene expression and the activation of sterile inflammation measured in cMKO mice  
211 at 18 weeks was absent in pre-symptomatic cMKO mice, implying that these transcriptional  
212 responses are downstream consequences *Mtfp1* deletion in adult cardiomyocytes.

213

#### 214 ***Mtfp1* is required for bioenergetic efficiency in cardiac mitochondria**

215 To directly assess the effects of energy metabolism in cMKO mice, we measured mitochondrial  
216 respiration in cardiac mitochondria from WT and cMKO mice (Figure 2A). High resolution  
217 respirometry studies showed a general impairment of mitochondrial O<sub>2</sub> consumption in cMKO  
218 hearts at both early and late stage of DCM: complex I-, complex II- and complex IV-driven  
219 mitochondrial respiration were all significantly lower at either age (Figure 2B, C). Interestingly,

220 reduced mitochondrial respiration was not accompanied by reduced levels of mitochondrial  
221 proteins, including those involved in OXPHOS (Figure S2A-B, Dataset EV1), demonstrating that  
222 bioenergetic decline was not the result of increased wholesale mitophagy.

223 Since reduced mitochondrial content and/or OXPHOS activity has been observed in other  
224 genetic models of cardiomyopathy (Burke et al., 2016), we wondered whether impaired  
225 mitochondrial respiration observed in failing cMKO hearts reflected the consequences of cardiac  
226 dysfunction and cardiac remodeling, rather than the ablation of an essential component or  
227 regulator of OXPHOS. We observed no deficits of the mitochondrial Krebs cycle in intact field-  
228 stimulated cMKO cardiomyocytes assessed by determining the autofluorescence of  
229 NAD(P)H/NAD(P)<sup>+</sup> and FADH<sub>2</sub>/FAD, intrinsic biomarkers of mitochondrial metabolic activity.  
230 NAD(P)H/FAD redox state was monitored during a protocol in which cells were exposed to an  
231 increase in stimulation frequency and β-adrenergic stimulation (Bertero et al.), creating a typical  
232 ADP-induced oxidation (“undershoot”) followed by Ca<sup>2+</sup>-dependent regeneration (“recovery”)  
233 behavior (Cortassa et al., 2003) (Figure S2C). This behavior was similar between cMKO and WT  
234 myocytes (Figure S2C), ruling out gross alterations in Krebs cycle activity and Ca<sup>2+</sup>-induced redox  
235 adaptation.

236 To uncover the functional alterations of OXPHOS that potentiate the development of DCM,  
237 we performed bioenergetic measurements in cardiac mitochondria isolated from pre-symptomatic  
238 mice. Using a dual respirometer-fluorimeter (O2k, Oroboros), simultaneous kinetic measurements  
239 of oxygen consumption rates (JO<sub>2</sub>) and mitochondrial membrane potential (ΔΨ) were performed in  
240 cardiac mitochondria isolated from pre-symptomatic WT and cMKO mice (8-10 weeks). Active  
241 mitochondria were pre-labeled with the potentiometric dye Rhodamine-123 (RH-123) and  
242 energized with substrates whose metabolism promotes complex I- [state 2; pyruvate, glutamate,  
243 and malate (PGM)] or complex II-linked respiration (state 2; succinate and rotenone), followed by  
244 ADP to promote phosphorylating (state 3: ADP) respiration. Finally, the ATP synthase inhibitor  
245 oligomycin (Omy) was applied to assess non-phosphorylating (state 4) respiration (Figure 2A).

246 We observed no differences in state 3 JO<sub>2</sub> rates between WT and cMKO cardiac  
247 mitochondria fueled with either PGM, succinate with rotenone or palmitoyl-carnitine with malate,  
248 respectively (Figure 2D (left), Figure S2D (left), F). However, respiration was ~30% higher in cMKO  
249 mitochondria in state 2 (+27.4 %) and state 4 (+30.3 %) when complex I was fueled with PGM

250 (Figure 2D), significantly decreasing respiratory control ratios [State 3:2, State 3:4, Figure 2D  
251 (middle, right)]. In addition, altered  $JO_2$  rates in cMKO mitochondria were accompanied by  
252 impaired RH-123 quenching in state 2 and state 4 (Figure 2E, S2E), indicating defective IMM  
253 substrate-dependent hyperpolarization. Supporting the notion that MTFP1 ablation increases  
254 proton leak, we consistently observed reduced respiratory control ratios when complex II was  
255 energized (Figure S2D, middle-right) and lower mitochondrial membrane potential under both state  
256 2 and state 4 conditions regardless of the respiratory substrates that were provided (Figure S2D-  
257 E).

258 The elevated  $JO_2$  rates and reduced RH-123 quenching in state 4 could be explained either  
259 by a reduced sensitivity of the ATP synthase to Omy treatment or by uncoupling caused by proton  
260 leak across the IMM. In the mouse heart, reduced Omy sensitivity can result from defects in the  
261 assembly of the ATP synthase that alter the affinity of Omy binding to Complex V via OSCP  
262 (Mourier et al., 2014). However, BN-PAGE analyses of cardiac mitochondria isolated from pre-  
263 symptomatic cMKO mice revealed no defects in ATP synthase assembly/maintenance (Figure 2F),  
264 leaving us with increased proton leak as the most parsimonious explanation for the observed state  
265 4 respiration and membrane potential differences (Figure 2D, E; Figure S2D-E).

266 Next, we sought to corroborate our findings in cultured cells. To this end, we generated MTFP1-  
267 deficient mouse embryonic fibroblasts (MEFs) (*Mtfp1*<sup>-/-</sup>) and MTFP1-deficient human U2OS cells  
268 by Crispr/Cas9 genome editing (*MTFP1*<sup>Crispr</sup>) and corresponding WT (*MTFP1*<sup>+/+</sup>) controls (Figure  
269 S2H, I) and then assessed oxygen consumption by Seahorse FluxAnalyzer. Intriguingly, we  
270 observed no changes in basal or maximal respiration rates nor any evidence of mitochondrial  
271 uncoupling (Figure S2J-Q) suggesting that MTFP1-dependent proton leak may be cell type  
272 specific.

273 Taken together, our data reveal an unappreciated and critical role of MTFP1 in bioenergetic  
274 efficiency and mitochondrial uncoupling, particularly evident in metabolically active  
275 cardiomyocytes, which precedes the manifestation of cardiac dysfunction and heart failure in  
276 cMKO mice.

277  
278 ***Mitochondrial uncoupling is mediated by the Adenine Nucleotide Translocase***

279 To uncover the mechanism responsible for the mild mitochondrial uncoupling caused by *Mtfp1*  
280 deletion in cardiomyocytes, we turned our attention to known uncoupling proteins. Uncoupling  
281 proteins (UCPs, UCP 1/2/3) and adenine nucleotide translocase (ANT) IMM proteins have been  
282 reported to be the two main catalysts of futile proton leak in mammalian mitochondria (Brand et al.,  
283 2004; Echtay et al., 2002). UCP1 is a bona fide uncoupler that is primarily expressed in brown  
284 adipose tissue (Adams, 2000) and shares significant sequence similarity with UCP2 and 3, which  
285 are expressed in other tissues (Woyda-Ploszczyca and Jarmuszkiewicz, 2014). ANT is an integral  
286 IMM transporter that imports ADP and concomitantly exports ATP between the mitochondrial  
287 matrix and IMS (Ruprecht and Kunji, 2021). ANT exists in four different tissue specific isoforms  
288 (ANT1, 2, 3, and 4), with ANT1 being the most abundant protein in mitochondria (Lu YW et al,  
289 2017, Brand MD et al, 2005; Karch et al., 2019; JE Kokoszka et al., 2016). ANT1 has long been  
290 known for its namesake role as a nucleotide translocator, and recent studies have proven it to be  
291 an essential transporter of protons across the IMM in mammals (Bertholet et al., 2019) and a rate-  
292 limiting factor for proton leak in *Drosophila* (Brand et al., 2005). While the steady state levels of  
293 ANT in cardiac tissue of WT and cMKO mice were identical (Figure 2G), we nevertheless sought to  
294 functionally assess the contribution of ANT to MTFP1-dependent proton leak in freshly isolated  
295 cardiac mitochondria by using the ANT antagonist carboxyatractyloside (CATR), which binds  
296 irreversibly to ANT on the IMS side of the IMM, blocking its activity (Todisco et al., 2016). The  
297 addition of CATR alone or after Omy treatment rescued the reduced  $\Delta\Psi$  and elevated  $JO_2$  rates  
298 back to WT levels (Figure 2H-I), normalizing the respiratory control ratio for state 3:4 (Figure 2J),  
299 suggesting that *Mtfp1* deletion increases ANT-dependent proton leak.

300 To exclude the unlikely possibility that other uncoupling proteins such as UCPs might  
301 contribute to increased proton leak caused by *Mtfp1* ablation, we measured  $\Delta\Psi$  of cardiac  
302 mitochondria in the presence of GTP, a pyrimidine nucleotide previously demonstrated to potently  
303 inhibit uncoupling in vitro (Macher et al., 2018; Woyda-Ploszczyca and Jarmuszkiewicz, 2014).  
304 UCP inhibition with GTP was not able to rescue the defective membrane potential observed in  
305 Omy-treated cMKO mitochondria (Figure 2K) suggesting that UCPs do not contribute to the futile  
306 proton leak in cMKO cardiac mitochondria. Taken together, these data demonstrate that depletion  
307 of MTFP1 in the IMM leads to an increased uncoupling activity of ANT, resulting in proton leak and  
308 bioenergetic inefficiency preceding the development of cardiac dysfunction (Figure S2R).

309

310 ***MTFP1 is dispensable for mitochondrial fission.***

311 Transient MTFP1 knock-down has previously been reported to promote mitochondrial elongation in  
312 a variety of cultured cell types including neonatal cardiomyocytes (Duroux-Richard et al., 2016;  
313 Morita and Terada, 2015; Tondera et al., 2009; Wang et al., 2017), yet the consequence in the  
314 adult heart has never been explored. Therefore, to determine the impact of *Mtfp1* deletion on  
315 mitochondrial morphology, we co-labeled primary WT and cMKO primary adult cardiomyocytes  
316 (CMs) with tetramethylrhodamine ethyl ester (TMRE) and Mitotracker Deep Red (MTDR) to  
317 visualize mitochondria. Contrary to previous reports purporting that *Mtfp1* depletion causes  
318 mitochondrial elongation (Aung et al., 2017a, 2019), we failed to observe any obvious effects on  
319 the morphology, distribution, or content of mitochondria in primary adult CMs deleted of *Mtfp1*  
320 (Figure 3A-B). Similarly, transmission electron microscopy (TEM) analyses of pre-symptomatic  
321 cMKO and WT hearts (LV) showed no indications of mitochondrial elongation (median  
322 mitochondrial surface area: WT 3198  $\mu\text{m}^2$  versus cMKO 2954  $\mu\text{m}^2$ ) nor altered cristae organization  
323 (Figure 3C-D). We also did not observe changes in mtDNA content (Figure 3E) or mitochondrial  
324 shaping proteins in cardiac biopsies: the steady-state levels of fusion (MFN1, MFN2, OPA1) and  
325 fission (DRP1, MID51, FIS1) proteins were no different between WT and cMKO mice (Figure 3F).  
326 Thus, MTFP1 is dispensable for mitochondrial dynamics in the heart.

327 In light of these surprising findings, we decided to measure mitochondrial morphology in MEFs  
328 depleted of MTFP1 under basal and stress conditions using a recent supervised machine learning  
329 (ML) approach we developed for high-throughput image acquisition and analyses (Cretin et al.,  
330 2021). In contrast to previous reports, MEFs depleted (siRNA *Mtfp1*) or deleted (*Mtfp1*<sup>-/-</sup>) of *Mtfp1*  
331 showed only modest elongation of the mitochondrial network: both transient (siRNA) or chronic  
332 (knockout) ablation of MTFP1 resulted in ~15% increase in hypertubular mitochondria (Figure 3I-  
333 J), and *Mtfp1*<sup>-/-</sup> MEFs showed unaltered steady-state levels of mitochondrial fusion and fission  
334 proteins (Figure S3A). Contrary to DRP1-deficient cells (Cretin et al., 2021), *Mtfp1*<sup>-/-</sup> MEFs were  
335 not protected from mitochondrial fragmentation induced by established pharmacological triggers of  
336 mitochondrial fragmentation, such as oligomycin (Omy), Rotenone, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), or  
337 carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Figure 3G-H). Similarly, *Mtfp1*<sup>-/-</sup> MEFs were  
338 not protected from genetic induction of mitochondrial fragmentation by depletion of *Yme1l* or *Opa1*

339 (Figure 3I-J). While we could confirm that MTFP1 overexpression is able to promote mitochondrial  
340 fragmentation in MEFs, without affecting steady state level of fusion and fission proteins (Figure  
341 S3B-C), our data collectively indicate that MTFP1, unlike DRP1, is not an essential fission protein.  
342 Taken together, these results strongly argue that contrary to its namesake, MTFP1 is not an  
343 essential fission factor either in vitro or in vivo.

344  
345 ***MTFP1 deletion promotes mitochondrial permeability transition pore opening and***  
346 ***programmed cell death.***

347 Cardiomyocyte death is catastrophic for adult cardiac function because of the limited regenerative  
348 capacity of these post-mitotic cells. Given the appearance of cardiac cell damage and death in  
349 cMKO mice during DCM (Figure 1N-O), we sought investigate whether MTFP1 loss specifically  
350 increased cell death sensitivity. To this end, we isolated adult primary cardiac myocytes (CMs)  
351 from WT and pre-symptomatic cMKO mice between 8-10 weeks of age and kinetically measured  
352 cell survival in response to a variety of cell death triggers using supervised ML-assisted high-  
353 throughput live-cell imaging (Cretin et al., 2021). We were able to isolate equally viable CMs from  
354 both WT and cMKO mice, yet upon dissipation of the membrane potential with CCCP (Figure 4A-  
355 B) or treatment with H<sub>2</sub>O<sub>2</sub> (Figure 4C-D), *Mtfp1*<sup>-/-</sup> CMs succumbed to cell death more rapidly than  
356 WT CMs. Moreover, the induction of cell death with doxorubicin (DOXO, Figure 4E-F), a  
357 cardiotoxic chemotherapeutic agent that triggers programmed cell death (PCD) (Christidi and  
358 Brunham, 2021), induced a significant increase of death in *Mtfp1*<sup>-/-</sup> compared to WT CMs,  
359 indicating that MTFP1 is essential for cell survival.

360 Prolonged opening of mitochondrial permeability transition pore (mPTP) causes  
361 mitochondria swelling, membrane potential dissipation and bioenergetic collapse, becoming a  
362 determinant of cell death (Carraro et al., 2020). To test whether MTFP1 loss causes increased  
363 susceptibility to mPTP opening, we assessed mitochondria swelling by exposing cardiac  
364 mitochondria of pre-symptomatic WT and cMKO mice to a high concentration of Ca<sup>2+</sup> and  
365 kinetically measured the light scattering (Karch et al., 2013). Ca<sup>2+</sup> overload induced an increased  
366 mPTP dependent swelling of MTFP1-deficient cardiac mitochondria, which could be inhibited by  
367 the mPTP inhibitor cyclosporin A (CsA), indicating that *Mtfp1* deletion sensitizes cardiac  
368 mitochondria to mPTP opening (Figure 4G-H). These findings were corroborated in *Mtfp1*<sup>-/-</sup> MEFs,

369 in which we observed increased mPTP sensitivity that could be suppressed by CsA treatment  
370 (Figure S4A) or by knocking out Cyclophilin D (Cyp D, encoded by *Ppif*), the pharmacological  
371 target of CsA in mitochondria (Figure 4I) (Halestrap and Davidson, 1990). Thus, these data clearly  
372 indicate that loss of MTFP1 at the level of the IMM sensitizes mitochondria to mPTP opening.  
373 To define the molecular mechanisms underlying the increased sensitivity PCD and mPTP opening  
374 caused by MTFP1 ablation, we used WT and *Mtfp1*<sup>-/-</sup> MEFs. We began by confirming that like  
375 *Mtfp1*<sup>-/-</sup> CMs, MEFs deleted of *Mtfp1* were more sensitive to PCD. As expected, *Mtfp1*<sup>-/-</sup> MEFs  
376 showed increased sensitivity to multiple cell death stimuli, as evidenced by more rapid kinetics of  
377 caspase 3/7 activation and cell death monitored by ML-assisted live-cell imaging of CellEvent (CE)  
378 and Propidium Iodide (PI) uptake, respectively (Cretin et al., 2021). Treatment with cell death  
379 triggers actinomycin D (ActD) and ABT-737 (Figure 4J-O), staurosporine (STS, figure S4B-D) or  
380 DOXO (Figure 4P-R) all promoted a more rapid and robust cell death response in *Mtfp1*<sup>-/-</sup> MEFs  
381 relative to WT MEFs, which could be blocked with the pan-caspase inhibitor Q-VD-OPh (qVD).  
382 These effects were independent of cell proliferation or mitochondrial respiration, neither of which  
383 were altered in *Mtfp1*<sup>-/-</sup> MEFs (Figure S4E, S2J-M). Taken together, our data clearly demonstrate a  
384 protective role of MTFP1 in maintaining cell integrity and survival.

385

### 386 **Doxorubicin induced-cardiotoxicity accelerates the onset of cardiomyopathy in cMKO mice**

387 To test whether MTFP1 protected against PCD induction *in vivo*, we injected pre-symptomatic  
388 (aged 8 weeks) cMKO and WT mice with DOXO and assessed cardiac function at 14 days post  
389 treatment by echo (Figure S4F-J). Consistent with the data obtained *in vitro*, we observed that  
390 DOXO accelerated the onset of cardiac dysfunction in cMKO mice by lowering LVEF (WT 60.85 ±  
391 6.2% vs cMKO 47.02 ± 9.1%), PW thickness during systole (WT 1.082 ± 0.099 mm vs cMKO 0.887  
392 ± 0.062 mm), while increasing systolic LV diameter (WT 2.393 ± 0.27 mm vs cMKO 3.009 ± 0.36  
393 mm) and diastolic LV diameter (WT 3.505 ± 0.20 mm vs cMKO 3.918 ± 0.25 mm). These results  
394 clearly indicate that cMKO mice are more susceptible to DOXO induced cardiotoxicity, accelerating  
395 the onset of cardiomyopathy.

396

### 397 ***Inhibition of mPTP rescues cell death sensitivity of MTFP1 deficient cells***

398 It has been previously reported that DOXO mediates mPTP opening and cell death in lung cancer  
399 cells (Lu et al., 2014) and cardiac myocytes, and that H<sub>2</sub>O<sub>2</sub> activates necrosis through the induction  
400 of mPTP opening (Vaseva et al., 2012). We observed that H<sub>2</sub>O<sub>2</sub>-induced cell death was  
401 accelerated in *Mtftp1*<sup>-/-</sup> cells and was reduced, yet not totally abolished by caspase inhibition with  
402 qVD, indicating that MTFP1 loss also renders cells more susceptible to caspase-independent cell  
403 death (Figure 5A-C). Since MTFP1-deficient cardiac and MEF mitochondria were more susceptible  
404 to mPTP opening (Figure 4G-I, S4A), we investigated whether prolonged mPTP opening  
405 contributes to increased cell death sensitivity of MTFP1-deficient cells. To test the dependence of  
406 PCD on Cyp D, we disrupted Cyp D in WT and *Mtftp1*<sup>-/-</sup> MEFs by introducing a truncating,  
407 homozygous frame shift mutation (p.Val65\*) by Crispr/Cas9 genome editing (Figure 5D) and  
408 subjected cells to H<sub>2</sub>O<sub>2</sub> (Figure 5E-G) or DOXO treatment (Figure 5H-J). By tracking the kinetics of  
409 PI or CE uptake respectively, we observed that Cyp D ablation in *Mtftp1*<sup>-/-</sup> cells (*Mtftp1*<sup>-/-</sup>*Ppif1*<sup>-/-</sup>  
410 MEFs) rescued the cell death sensitivity back to WT levels (Figure 5E-J). Consistent with a  
411 cytoprotective effect of Cyp D ablation, we observed that the association of CsA to qVD treatment  
412 had a synergic effect in suppressing cell death sensitivity of *Mtftp1*<sup>-/-</sup> MEFs to WT levels (Figure 5A-  
413 C). Taken together, these results clearly indicate that loss of MTFP1 promotes mPTP opening via  
414 Cyp D to lower the resistance to programmed cell death. To gain insights into the molecular  
415 regulation of the mPTP by MTFP1 we sought to define the cardiac interactome of MTFP1. We  
416 expressed FLAG-MTFP1 at the *Rosa26* locus in C57Bl6/N mouse hearts via targeted transgenesis  
417 (Figure S5A) and confirmed that the modest level of over-expression did negatively impact cardiac  
418 function in vivo (Figure S5B-C). Next, we performed a co-immunoprecipitation study coupled to  
419 mass spectrometry (MS) for the analysis of cardiac MTFP1 interactome. We identified 60  
420 mitochondrial proteins besides the bait protein (MTFP1) that were exclusively present in FLAG-  
421 MTFP1 eluates or significantly enriched greater than two-fold (Dataset EV3, Figure S5D). Among  
422 these interactors we found factors involved in OXPHOS function (Figure S5E), notably proteins  
423 required for the assembly and functions of Complex I (Ndufa10, NDUFA7, NDUFS6, NDUFB4,  
424 NDUFS6, Mtnd5), Complex IV (CMC2, COX4I1, COX6B1, COX7A1, SCO2), and Complex V  
425 (ATP5L, USMG5). In addition, we identified a number of proteins that have previously been  
426 implicated in mPTP regulation including the ADP/ATP translocase SLC25A4 (Karch et al., 2019),  
427 the inorganic phosphate carrier SLC25A3 (Kwong et al., 2014), and the heat shock protein TRAP1



428 (Kang et al., 2007) (Figure S5D-E). Altogether, our data have uncovered a functional and physical  
429 link between MTFP1 and the mPTP complex in the inner mitochondrial membrane.

## 431 **Discussion**

432  
433 We initially chose to focus studies on Mitochondrial Fission Process 1 (MTFP1) because we  
434 viewed this protein as a promising entry point to study the hitherto molecularly undefined process  
435 of inner membrane division (Wai and Langer, 2016), based largely on previous studies that had  
436 purported a pro-fission role upon over-expression and an anti-fission role upon depletion (Aung et  
437 al., 2017a, 2017b; Duroux-Richard et al., 2016; Morita et al., 2017; Wang et al., 2017). While we  
438 demonstrate MTFP1 to be a bona fide inner membrane protein in vivo (Figure S1B-C), confirming  
439 previous in vitro studies (Tondera, 2005; Tondera et al., 2004), careful, unbiased mitochondrial  
440 morphology analyses unequivocally excluded this protein as an essential fission factor (Figure 3G-  
441 J) for the following reasons: 1) acute or chronic depletion of MTFP1 in vitro (Figure 3G-J) or  
442 deletion in vivo (Figure 3C-D) had little impact on the elongation of the mitochondrial network and  
443 2) MTFP1-deficient cells were not protected against mitochondrial fragmentation caused either by  
444 accelerated fission or impaired fusion induced either by genetic or pharmacological triggers (Figure  
445 3G-J). Consistent with these findings, we did not identify MTFP1 as a regulator of mitochondrial  
446 morphology in a recent, comprehensive siRNA-based phenotypic imaging screens of all  
447 mitochondrial genes performed in human fibroblasts (Cretin et al., 2021), which did identify  
448 essential fission proteins like DRP1 and receptors (Fonseca et al., 2019; Losón et al., 2013;  
449 Osellame et al., 2016), forcing us to reconsider the existing models of mitochondrial fission we (Ng  
450 et al., 2021; Wai and Langer, 2016) and others had proposed (Giacomello et al., 2020). In  
451 contrast, other studies have reported that chemical inhibitors of mTOR (Morita et al., 2017), PI3K  
452 (Tondera et al., 2004), or miRNAs (Duroux-Richard et al., 2016; Wang et al., 2017) can deplete  
453 MTFP1 protein levels and thus inhibit mitochondrial fragmentation, although the pleiotropic effects  
454 of these molecules and the signaling cascades they regulate make it the interpretation of these  
455 data very challenging. However, our in vitro studies confirmed that over-expression of MTFP1  
456 does indeed promote mitochondrial fragmentation, which appears to be independent of stress-  
457 induced OPA1 processing and alterations in the levels of the fission and fusion executors (Figure

458 S3B-C). In vivo, stable over-expression in cardiomyocytes does not appear to impact basal  
459 cardiac function assessed by echocardiography (Figure S5C), although future studies will be  
460 required to determine whether MTFP1 overexpression can impact cardiac physiology under stress  
461 conditions.

462 If MTFP1 is dispensable for fission, what role, if any, does this metazoan-specific factor play in  
463 mitochondria? Our in vivo studies revealed that cardiomyocyte-specific deletion of MTFP1 (cMKO)  
464 drives the progressive development of dilated cardiomyopathy (DCM) beginning at 18 weeks of  
465 age culminating in chronic heart failure and middle-aged death in both male and female mice.  
466 Cardiac dysfunction was accompanied by a reduction in mitochondrial gene expression and  
467 respiratory chain function, fibrotic remodeling (Figure 1P-R) and a general dysregulation of  
468 metabolic genes (Figure 1Q), which are features that have been observed in other cardiomyocyte-  
469 specific knockout mouse models of mitochondrial genes (Fernandez-Caggiano et al., 2020; Ghazal  
470 et al., 2021; Hansson et al., 2004; Liao et al., 2015; Wai et al., 2015; Zhang et al., 2020b).  
471 Similarly, accumulation of pathogenic mutations in mitochondrial DNA have recently been shown  
472 to drive mitochondrial dysfunction and sterile inflammation in a number of different tissues  
473 including the heart and genetic inhibition of the latter phenotype appears to resolve tissue  
474 dysfunction, supporting a pathological role of cardiac inflammation triggered by mitochondrial  
475 dysfunction (Lei et al., 2021). In cMKO mice, the aforementioned phenotypes were absent before  
476 the onset of DCM, indicating that MTFP1 deletion in perinatal cardiomyocytes does not  
477 compromise post-natal cardiac development nor function in mice. Thus, we hypothesize that the  
478 metabolic and inflammatory remodeling that accompanies DCM manifests as downstream  
479 response to cardiac dysfunction, which we are currently testing. Surprisingly, functional  
480 characterization of field-stimulated primary adult cardiomyocytes from pre-symptomatic mice did  
481 not reveal defects in contractile capacity, excitation contraction (EC) coupling, or sarcomere  
482 integrity (Figure S1N-S), prompting us to search for other homeostatic dysfunctions of  
483 cardiomyocytes that could account for the contractile defects of the beating heart caused by  
484 MTFP1 deletion. Indeed, characterization of primary cardiomyocytes and cardiac mitochondria  
485 from cMKO mice revealed an increased sensitivity to programmed cell death and increased  
486 sensitivity to opening of the mitochondrial permeability transition pore (mPTP), respectively  
487 (Baines et al., 2005; Carraro et al., 2020; Nakagawa et al., 2005). Accelerated opening of the

488 mPTP has been shown to control cardiomyocyte viability and cardiac function in genetic (Song et  
489 al., 2015), infectious (Milduberger et al., 2021), and surgically-induced mouse models of  
490 cardiomyopathy (Nakagawa et al., 2005). The identity of the mPTP has been hotly debated  
491 (Carraro et al., 2020; Kwong and Molkenin, 2015; Morciano et al.) and a consensus has yet to be  
492 achieved regarding its structure and molecular constitution, although compelling evidence from  
493 knockout mice and in vitro reconstitution experiments have identified a number of mitochondrial  
494 proteins associated with or integrated into the IMM subunits that are required for the efficient  
495 opening of the mPTP including subunits of the ATP synthase, cyclophilin D (Cyp D, encoded by  
496 the nuclear gene *Ppif*), and IMM carrier proteins of the SLC25 family (Baines et al., 2005; Basso et  
497 al., 2008; Bernardi et al., 2015; Carraro et al., 2018, 2020; Karch et al., 2013, 2019; Kwong et al.,  
498 2014; Nakagawa et al., 2005), several of which we identified by coimmunoprecipitation studies  
499 performed on cardiac mitochondria to physically interact with FLAG-MTFP1, including SLC25A3  
500 (Phosphate carrier), SLC25A4 (ANT1), TRAP1, ATP5L and USMG5 (Figure S5D-E).  
501 Complexosome profiling of rat cardiac mitochondria revealed co-migration of MTFP1 with several  
502 proteins we identified by coimmunoprecipitation (Heide et al., 2012), including SLC25A3,  
503 SLC25A4, and Cyp D, implying that MTFP1 may act as a scaffold in the IMM to participate in the  
504 maintenance, assembly or regulation of multi-protein complexes such as the mPTP complex. In  
505 MTFP1-deficient cells, inhibiting mPTP opening by pharmacological inhibition with Cyclosporin A  
506 (CsA) or genetic deletion of Cyp D rescues the sensitivity to mPTP opening and accelerated cell  
507 death. Our discovery that the genetic deletion of *Mtfp1* sensitizes both post-mitotic cardiac cells  
508 and mitotic epithelial cells to programmed cell death (PCD) without affecting cardiomyocyte  
509 differentiation nor cell proliferation argues for a general role for MTFP1 in cell survival, which is  
510 supported by studies in other cell lines (Duroux-Richard et al., 2016; Morita et al., 2017). Why  
511 other groups have reported that MTFP1 depletion can protect gastric cancer and cardiomyocyte  
512 cell lines from PCD induced by various cell death triggers including doxorubicin is unclear (Aung et  
513 al., 2017a, 2017b; Wang et al., 2017). Although our in vivo data demonstrate that MTFP1 deletion  
514 in cardiomyocytes accelerates, rather than retards, the cardiotoxic effects of doxorubicin (Figure  
515 S4F-J), which is consistent with the PCD sensitivity we measured in MEFs and primary  
516 cardiomyocytes, we cannot exclude that different cell lines and tissue may respond differently to  
517 the loss of MTFP1.

518 Indeed, MTFP1 ablation significantly impacts mitochondrial respiration in cardiac cells but not in  
519 highly glycolytic epithelial cells such as MEFs and human osteosarcoma cells (U2OS) (Figure S2J-  
520 Q), in which the bioenergetic efficiency was unaffected.

521 Efficiently coupled oxidative phosphorylation requires proton gradient formed across the IMM by  
522 the mitochondrial ETC, which is then harnessed by ATP synthase to generate ATP from ADP and  
523 inorganic phosphate, imported into the matrix by the ADP/ATP translocase (ANT) and phosphate  
524 carrier (SLC25A3), respectively. Mitochondrial uncoupling occurs when proton motive force is  
525 dissipated by proton leak into the matrix and oxygen consumption is not coupled to ATP  
526 generation. Maintenance of constant cellular ATP concentration is critical for cell survival and the  
527 function, therefore, uncoupling of the ETC from ATP generation can have deleterious effects in  
528 cardiac cells, whose constant energy supply is essential for the beating heart.

529 While the oxygen consumption rate (OCR) (Figure S2J-Q) and the maximal phosphorylating  
530 respiration (Figure 2D, 2H, S2D, S2F), equivalent to state 3, were normal in cells and cardiac  
531 mitochondria isolated from cMKO mice, MTFP1 loss significantly reduced the respiratory control  
532 ratios (RCR) (Figure 2D, S2D) in cardiac mitochondria energized with complex I or complex II  
533 substrates, suggesting a general mechanism of uncoupling of the respiration from the ATP  
534 production as a result of increased proton leak through the IMM.

535 Proton leak has marked influence on energy metabolism. Enhancement of this process in various  
536 tissues can counteract the deleterious effects of nutrient overload via UCP1-dependent and  
537 independent pathways (Roesler and Kazak, 2020) and thus may be beneficial in some settings. In  
538 the heart, whose bioenergetic efficiency has evolved to maximize ATP output, excessive proton  
539 leak has been shown to drive age-related cardiomyocyte and cardiac dysfunction in mice (Chiao et  
540 al., 2020; Zhang et al., 2020a). Studies performed by the Rabinovitch lab clearly demonstrated that  
541 ANT-dependent proton leak is increased in cardiomyocytes from old, but not young mice and can  
542 be rejuvenated by blocking ANT and reducing sensitivity to mPTP opening.

543 In line with the notion that increased proton leak is maladaptive for the heart, our study shows for  
544 the first time that MTFP1 loss in cardiomyocytes reduces the mitochondrial membrane potential as  
545 a result of increased proton leak through the IMM (Figure 2D-E, S2D-G) preceding the onset of  
546 cardiomyopathy. We provide direct evidence that ANT is the most likely site of proton leak in  
547 cardiac mitochondria, as its inhibition with carboxyatractyloside (CATR) suppresses proton leak

548 and re-establishes normal membrane potential and respiratory control ratios in MTFP1-deficient  
549 mitochondria (Figure 2H-J). While we have clear evidence of uncoupling via ANT on one hand  
550 and increased sensitivity to mPTP opening and mitochondria swelling on the other, future studies  
551 are still required to decipher whether these mechanisms are interdependent and whether they  
552 must synergize to drive cardiac decline and heart failure. While a number of genetic mouse  
553 models of cardiomyopathy targeting mitochondrial genes have been generated over the last 20  
554 years, to the best of our knowledge cMKO mice represent the first in which bioenergetic efficiency  
555 is compromised without affecting maximal respiratory capacity (state 3), thus providing a novel  
556 model to study the relevance of cardiac mitochondrial uncoupling and its progressive impact on  
557 cardiac homeostasis.

558 In summary our study reveals new and essential roles of MTFP1 in cardiac homeostasis that are  
559 distinct from its previously reported impact on mitochondrial fission, the latter of which our data  
560 conclusively show is unaffected in vitro and in vivo. Thus, our work now positions MTFP1 as a  
561 critical regulator of mitochondrial coupling through ANT in cardiomyocytes and its loss leads to  
562 membrane potential dissipation associated to mPTP opening, cell death and progressive DCM that  
563 leads to heart failure and middle-aged death. These findings advance our understanding of the  
564 mitochondrial defects that can trigger the development of dilated cardiomyopathy and heart failure.  
565 We propose that MTFP1 to be a valuable tool for the molecular dissection of mitochondrial  
566 uncoupling and mPTP function and thus a promising target to mitigate the pathological events of  
567 cardiac and metabolic remodeling in heart disease.

568

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570

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572

573 **Figure Legends**

574 **Figure 1. *Mtfp1* deletion in cardiomyocytes causes dilated cardiomyopathy and middle-**  
575 **aged death in mice**

576 **A)** AlphaFold prediction of MTFP1 as integral protein of the inner mitochondrial membrane (IMM)  
577 associated with mitochondrial fission. DRP1 binds to its receptors MFF or MiD49/51 to initiate  
578 mitochondrial constriction. IMM fission occurs coincidentally with mtDNA replication mediated by  
579 TFAM and POLG2. S-OPA1 accumulation generated by OMA1 accelerates fission.

580 **B)** Generation of a cardiomyocyte-specific *Mtfp1* KO (cMKO) mouse model.

581 **C)** *Mtfp1* mRNA expression measured by bulk RNAseq (arbitrary units; AU) in heart tissue of WT  
582 (n=5) and cMKO (n=5) mice at 8 weeks (Dataset EV2). Data represent mean  $\pm$  SD; 2-tailed  
583 unpaired Student's t-test, \*\*\*\*p<0.0001.

584 **D)** Densitometric quantification of immunoblot analysis of cardiac lysates from WT (n=5) and  
585 cMKO (n=5) mice at 8 weeks using the indicated antibodies. Data represent mean  $\pm$  SD; Unpaired  
586 t-test, \*\*p<0.01.

587 **E)** MTFP1 protein expression in cardiac tissue of WT (n=4) and cMKO (n=4) at 18 weeks  
588 measured by label free quantification (LFQ) mass spectrometry (MS) (Dataset EV1). Data  
589 represent mean  $\pm$  SD; 2-tailed unpaired Student's t-test, \*\*\*p<0.001.

590 **F)** Kaplan-Meier survival curve of WT (n=9) and cMKO (n=15) male mice. Median lifespan of  
591 cMKO mice is 26.4 weeks. Log-rank (Mantel-Cox) test, \*\*\*\*p < 0.0001.

592 **G-L)** Longitudinal echocardiography of WT and cMKO mice from 10 to 34 weeks of age.

593 **G)** Representative M-Mode echocardiographic images of left ventricles from WT (left) and cMKO  
594 (right) of male mice at 34 weeks.

595 **H)** Quantification of left ventricular ejection fraction (% LVEF) of WT and cMKO mice at indicated  
596 ages. Data represent mean  $\pm$  SD; 2way Anova - Sidak's multiple comparison test: 10 week WT  
597 (n=13) vs cMKO (n=18); 14 week WT (n=4) vs cMKO (n=6); 18 week WT (n=10) vs cMKO (n=13)  
598 \*\*\*p<0.001; 22 week WT (n=4) vs cMKO (n=6) \*\*p<0.01; 30 week WT (n=3) vs cMKO (n=7)  
599 \*\*p<0.01; 34 week WT (n=4) vs cMKO (n=10)\*\*\*\*p<0.0001.

600 **I)** Quantification of systolic interventricular septum thickness (IVSs, mm) of WT and cMKO mice at  
601 indicated ages. Data represent mean  $\pm$  SD; 2way Anova - Sidak's multiple comparison test, 18  
602 weeks \*\*\*p<0.001; 22 weeks \*p<0.05; 34 weeks \*\*\*p<0.001.

603 **J)** Quantification of left ventricle posterior wall thickness at systole (LVPWs, mm) of WT and  
604 cMKO mice at indicated ages. Data represent mean  $\pm$  SD; 2way Anova - Sidak's multiple  
605 comparison test, 18 weeks \*\* $p < 0.01$ ; 22 weeks \* $p < 0.05$ ; 30 weeks \*\*\* $p < 0.001$ , 34 weeks  
606 \*\*\*\* $p < 0.0001$ .

607 **K)** Quantification of left ventricle end diastolic diameter (LVDD, mm) of WT and cMKO mice at  
608 indicated ages. Data represent mean  $\pm$  SD; 2way Anova - Sidak's multiple comparison test, 34  
609 week \*\*\*\* $p < 0.0001$ .

610 **L)** Quantification of left ventricle end systolic diameter (LVSD, mm) of WT and cMKO mice at  
611 indicated ages. Data represent mean  $\pm$  SD; 2way Anova - Sidak's multiple comparison test, 30  
612 weeks \* $p < 0.05$ ; 34 weeks \*\*\* $p < 0.001$ .

613 **M)** Representative histological images of cardiac short axis view of WT (n=4) and cMKO (n=4) at  
614 34 weeks. H&E (left), Massons's Trichrome (middle) and Picrosirius red (right) staining show  
615 cardiac remodeling and collagen deposition within the myocardium of cMKO mice. Scale bar 500  
616  $\mu$ M.

617 **N)** Circulating levels of cardiac troponin-I (cTNI) measured in serum of WT and cMKO mice at 18  
618 [WT (n=6) vs cMKO (n=6)] and 34 weeks [WT (n=11) vs cMKO (n=20)]. Data represent mean  $\pm$   
619 SD; 2-tailed unpaired Student's t-test at 18 weeks and 34 weeks (w); \*\*\* $p < 0.001$ .

620 **O)** Myosin light chain 1 (MLC-1) levels measured in serum of WT and cMKO mice at 18 [WT (n=5)  
621 vs cMKO (n=6)] and 34 weeks (w) [WT (n=11) vs cMKO (n=17)]. Data represent mean  $\pm$  SD; 2-  
622 tailed unpaired Student's t-test at 18w and 34w; \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

623 **P)** Volcano plots generated from the RNAseq analysis (Dataset EV2) of the differentially expressed  
624 genes in cardiac tissue of WT and cMKO mice at 8 (left) and 18 weeks (right).

625 **Q)** Number of genes up-regulated and down-regulated in cMKO mice at 8 (blue) and 18 (pink)  
626 weeks (w) within the gene ontology (GO) term: metabolic process (left), mitochondrial genes  
627 (MitoCarta, middle) and inflammation (right) obtained from RNAseq analysis (Dataset EV2).

628 **R)** mRNA expression measured by bulk RNAseq (arbitrary units; AU) of the indicated profibrotic  
629 genes in heart tissue of WT (n=6) and cMKO (n=6) mice at 18 weeks. Data represent mean  $\pm$  SD;  
630 2-tailed unpaired Student's t-test.

631

632 **Figure 2. *Mtfp1* is required for bioenergetic efficiency in cardiac mitochondria**

633 **A)** Substrates from fatty acid oxidation (mustard) and glycolysis (purple, blue) are metabolized in  
634 the TCA cycle which delivers fuels the electron transport chain (ETC) complexes located in  
635 the inner mitochondrial membrane by providing NADH and FADH to complexes I (purple) and II  
636 (blue), respectively. Complexes I, III and IV extrude protons from matrix into the intermembranous  
637 space creating an electrochemical gradient driving the phosphorylation of ADP at the ATP  
638 synthase (complex V). The electron flow is limited by the availability of oxygen, a terminal acceptor  
639 of the energy depleted electron at the complex IV (cytochrome oxidase). Uncoupling proteins such  
640 as ANT promote a proton leak, playing an important role in regulation of membrane potential and  
641 oxidative phosphorylation efficiency. Specific inhibitors of complex I (rotenone), complex V  
642 (oligomycin), and ANT (carboxyatractyloside, CATR).

643 **B)** Oxygen consumption rates ( $JO_2$ ) measured by high-resolution respirometry of cardiac  
644 mitochondria from WT (n=5) and cMKO (n=5) male mice at 18 weeks. Complex I driven respiration  
645 (left) was measured in presence of pyruvate, malate, glutamate (PGM) and ADP followed by the  
646 addition of rotenone and succinate to assess complex II driven respiration (middle) and antimycin  
647 A, carbonyl cyanide m-chlorophenyl hydrazine (CCCP), and N,N,N',N'-Tetramethyl-p-  
648 phenylenediamine (TMPD) and ascorbate to measure complex IV driven respiration (right). Data  
649 represent mean  $\pm$  SD; unpaired Student's t-test, \*p<0.05.

650 **C)** Oxygen consumption rates ( $JO_2$ ) measured by high-resolution respirometry of cardiac  
651 mitochondria from WT (n=5) and cMKO (n=5) male mice at 34 weeks (w). Complex I driven  
652 respiration (left) was measured in presence of pyruvate, malate, glutamate (PGM) and ADP  
653 followed by the addition of rotenone and succinate to assess complex II driven respiration (middle)  
654 and antimycin A, carbonyl cyanide m-chlorophenyl hydrazine (CCCP), and N,N,N',N'-Tetramethyl-  
655 p-phenylenediamine (TMPD) and ascorbate to measure complex IV driven respiration (right). Data  
656 represent mean  $\pm$  SD; unpaired Student's t-test, \*p<0.05.

657 **D)** Oxygen consumption rates ( $JO_2$ ) measured by high-resolution respirometry of cardiac  
658 mitochondria isolated from WT (n=11) and cMKO (n=11) mice between 8-10 weeks (left).  
659 Respiration was measured in presence of pyruvate, malate, and glutamate (PGM) (state 2)  
660 followed by the addition of ADP (state 3) and Oligomycin (Omy- state 4). Data represent mean  $\pm$   
661 SD. Multiple t-test, state 2 \*\*\*p<0.001, state 4 \*p<0.05. Respiratory control ratios (RCR) of state



662 3:2 (middle:  $JO_2$  ADP/PGM) and respiratory control ratios (RCR) of state 3:4 (right:  $JO_2$  ADP/Omy).

663 Data represent mean  $\pm$  SD; 2-tailed unpaired Student's t-test, \*\*\* $p < 0.001$ .

664 **E)** Mitochondrial membrane potential ( $\Delta\Psi$ ) measured by quenching of Rhodamine 123 (RH123)

665 fluorescence in cardiac mitochondria isolated from WT (n=11) and cMKO (n=11) mice between 8-

666 10 weeks.  $\Delta\Psi$  was measured in presence of pyruvate, malate, and glutamate (PGM) (state 2)

667 followed by the addition of ADP (state 3) and Oligomycin (state 4). Data represent mean  $\pm$  SD;

668 Multiple t-test, \*\*\*\* $p < 0.0001$ .

669 **F)** Representative BN-PAGE immunoblot analysis of cardiac OXPHOS complexes isolated from

670 WT and cMKO mice at 8-10 weeks using the indicated antibodies.

671 **G)** Equal amounts of protein extracted from WT (n=5) and cMKO (n=5) hearts between 8-10

672 weeks were separated by SDS-PAGE and immunoblotted with the indicated antibodies and

673 quantified by densitometry using VINCULIN as a loading control. Data represent mean  $\pm$  SD.

674 **H)** Oxygen consumption rates measured by high-resolution respirometry (left;  $JO_2$ ) and

675 mitochondrial membrane potential (right;  $\Delta\Psi$ ) measured by quenching of Rhodamine 123 (RH123)

676 fluorescence in cardiac mitochondria of WT (n=4) and cMKO (n=4) mice between 8-10 week of

677 age.  $JO_2$  and  $\Delta\Psi$  were measured in presence of pyruvate, malate, and glutamate (state 2) followed

678 by the addition of ADP (state 3) and carboxyatractyloside (CATR) (state 4). Data represent mean

679  $\pm$  SD; Multiple t-test, \*\*\* $p < 0.001$ .

680 **I)** Oxygen consumption rates measured by high-resolution respirometry (left;  $JO_2$ ) and

681 mitochondrial membrane potential (right;  $\Delta\Psi$ ) measured by quenching of Rhodamine 123 (RH123)

682 fluorescence in cardiac mitochondria of WT and cMKO mice between 8-10 week of age.

683 Respiration was measured in presence of pyruvate, malate, and glutamate (PGM) (state 2)

684 followed by the addition of ADP (state 3), Oligomycin (state 4) and carboxyatractyloside (state 4-

685 CATR) [WT (n=3) cMKO (n=3)]. Data represent mean  $\pm$  SD. Multiple t-test, state 2 \*\*\* $p < 0.001$ ,

686 state 4 \* $p < 0.05$ .

687 **J)** Respiratory control ratio (RCR) of state 3:4 (ADP/CATR) between WT (n=4) and cMKO (n=4)

688 calculated from H). Data represent mean  $\pm$  SD.

689 **K)** Mitochondrial membrane potential ( $\Delta\Psi$ ) measured by quenching of Rhodamine 123 (RH123)

690 fluorescence in cardiac mitochondria of WT and cMKO mice between 8-10 week of age.  $\Delta\Psi$  was

691 measured in presence of pyruvate, malate, and glutamate (state 2) followed by the addition of ADP

692 (state 3), oligomycin (state 4) and GTP (state 4 - GTP) [ WT (n=3), cMKO (n=3)]. Data represent  
693 mean  $\pm$  SD; Multiple t-test, \* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

694  
695 **Figure 3. MTFP1 is dispensable for mitochondrial fission.**

696 **A)** Representative confocal images of primary adult cardiomyocytes isolated from WT and cMKO  
697 mice at 8 weeks. Mitochondria were stained with MitoTracker Deep Red (MTDR) and  
698 tetramethylrhodamine, ethyl ester (TMRE), nuclei were stained with NucBlue (NB). Scale bar=50  
699  $\mu\text{m}$ .

700 **B)** Violin plot of mitochondrial content (MTDR Intensity/Area) of WT (n=6085) and cMKO CMs  
701 (n=3647) measured in A).

702 **C)** Representative transmission electron micrographs of cardiac posterior walls of WT (top, n=3)  
703 and cMKO (bottom, n=3) mice at 8-10 weeks. Scale bar: 500 nm.

704 **D)** Violin plot of mitochondrial surface area ( $\mu\text{m}^2$ ) within cardiac posterior wall measured in C (WT  
705 mitochondria n=659; cMKO mitochondria n=966). Dotted line represents quartiles and dashed line  
706 represents median; \*\* $p < 0.01$  Mann-Whitney test.

707 **E)** Mitochondrial DNA (mtDNA) content in WT (n=5) and cMKO (n=5) heart tissue quantified by  
708 amplification of the mitochondrial *Mtntl1* gene relative to nuclear gene *b-Actin*. Data represent mean  
709  $\pm$  SD.

710 **F)** Immunoblot of mitochondrial fission and fusion proteins. Equal amounts of protein from cardiac  
711 WT and cMKO (8-10 week) extracts were separated by SDS-PAGE and immunoblotted with the  
712 indicated antibodies (horizontal line denotes different membranes). VINCULIN or ACTIN are used  
713 as loading controls.

714 **G)** Representative confocal images of WT and *Mtfp1*<sup>-/-</sup> MEFs treated with the fission inducing  
715 drugs: oligomycin (Omy), Rotenone, H<sub>2</sub>O<sub>2</sub> and carbonyl cyanide m-chlorophenyl hydrazine  
716 (CCCP). Mitochondria were stained with MitoTracker Deep Red (MTDR, green) and nuclei with  
717 NucBlue (NB, blue). Scale bar=100  $\mu\text{m}$ .

718 **H)** Quantification of mitochondrial morphology in G) by supervised ML using WT cells with normal  
719 (UT), fragmented (CCCP-treated) or hypertubular (cycloheximide-treated) mitochondria as ground  
720 truths. Data are means  $\pm$  SEM of 4 independent replicates. 2way-ANOVA, Dunnet's multiple  
721 comparison test: % fragmentation \*\*\*\* $p < 0.0001$  treatment vs WT UT or *Mtfp1*<sup>-/-</sup> UT.

722 **I)** Representative confocal images of WT and *Mtfp1*<sup>-/-</sup> MEFs treated with indicated siRNAs (20 nM)  
723 for 72 h. Mitochondria were stained with MitoTracker Deep Red (MTDR, green) and nuclei with  
724 NucBlue (NB, blue). Scale bar=100  $\mu$ m.

725 **J)** Quantification of mitochondrial morphology in I) by supervised machine learning (ML) using WT  
726 cells with normal (non-targeting NT siRNA), fragmented (*Opa1* siRNA) or hypertubular (*Dnm1l*  
727 siRNA) mitochondria as ground truths. Data are means  $\pm$  SEM of 4-8 technical replicates. 2way-  
728 ANOVA, Dunnet's multiple comparison test: % hypertubular \*  $p < 0.05$ ; \*\*\* $p < 0.001$  versus WT NT  
729 siRNA; % fragmented \*\*\*\* $p < 0.0001$  versus *Mtfp1*<sup>-/-</sup> NT siRNA.

730

731 **Figure 4. MTFP1 protects against mitochondrial PTP opening and cell death**

732 **A)** Representative confocal images of adult cardiomyocytes (CMs) isolated from WT and cMKO at  
733 8 weeks stained with tetramethylrhodamine, ethyl ester (TMRE) treated with or without cyanide m-  
734 chlorophenyl hydrazine treatment (CCCP) for 15 minutes. Rod-shaped CMs: live cells, round-  
735 shaped CMs: dead cells. Scale bar: 500  $\mu$ m.

736 **B)** Quantification of number of live cells (% survival) in A) by supervised machine learning. Data  
737 are means  $\pm$  SD of n=3 independent experiments. Unpaired Student's t-test; \* $p < 0.05$ .

738 **C)** Representative confocal images of adult cardiomyocytes (CMs) isolated from WT and cMKO at  
739 8 weeks stained with tetramethylrhodamine, ethyl ester (TMRE) and subjected to H<sub>2</sub>O<sub>2</sub> treatment  
740 for 1 hour. Rod-shaped CMs: live cells, round-shaped CMs: dead cells. Scale bar: 500  $\mu$ m.

741 **D)** Quantification of number of live cells (% survival) over time measured in C) by supervised  
742 machine learning. Data are means  $\pm$  SD of 2-3 culture replicates and representative of n=3  
743 experiments. 2wayANOVA, Tukey's multiple comparison test, \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  vs WT  
744 H<sub>2</sub>O<sub>2</sub>.

745 **E)** Representative confocal images of adult cardiomyocytes (CMs) isolated from WT and cMKO at  
746 8 weeks stained with tetramethylrhodamine, ethyl ester (TMRE) and subjected to Doxorubicin  
747 (DOXO). Scale bar: 500  $\mu$ m.

748 **F)** Quantification of number of live cells (% survival) in E) by supervised machine learning. Data  
749 are means  $\pm$  SD of 2-3 culture replicates and representative of n=3 experiments. 2wayANOVA,  
750 Tukey's multiple comparison test, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  vs WT DOXO.  
751 Scale bar: 1 mm.

752 **G)** Mitochondrial permeability transition pore (mPTP) opening. Swelling assay performed on  
753 cardiac mitochondria extracted from hearts of WT (n=3) and cMKO (n=3) mice at 8-10 weeks.  
754 Light scattering (absorbance 540 nm, % relative to T<sub>0</sub>) was measured every 20 s before and after  
755 addition of a single pulse of CaCl<sub>2</sub> (arrowhead). Cyclosporin A (CsA) was added prior CaCl<sub>2</sub> pulse  
756 to inhibit mPTP-induced mitochondrial swelling. Data are means ± SD.

757 **H)** one-way ANOVA of maximal absorbance<sub>540 nm</sub> (% relative to T<sub>0</sub>) change in G) \*p<0.05,  
758 \*\*\*\*p<0.0001.

759 **I)** Mitochondrial permeability transition pore (mPTP) opening. Swelling assay performed on  
760 mitochondria purified from WT and *Mtfp1*<sup>-/-</sup>, *Ppif*<sup>-/-</sup> and *Mtfp1*<sup>-/-</sup>*Ppif*<sup>-/-</sup> MEFs. Mitochondrial  
761 absorbance changes (absorbance 540 nm, % relative to T<sub>0</sub>) are measured every 20 s prior and  
762 after addition of a single pulse of CaCl<sub>2</sub> (arrowhead). Cyclosporin A (CsA) was added prior Ca<sup>2+</sup> to  
763 inhibit mPTP-dependent swelling. Data are means ± SD.

764 **J)** Representative confocal images of WT and *Mtfp1*<sup>-/-</sup> MEFs subjected to actinomycin D (ActD)  
765 plus ABT-737 treatment in the presence or absence of the pan-caspase inhibitor q-VD-OPh  
766 hydrate (qVD). Cell death was monitored by using nuclear Propidium Iodide uptake (PI, orange)  
767 and imaging cells every hour (h) for 20h. Scale bar = 100 μm.

768 **K)** Kinetics of PI uptake was determined by counting the number of PI<sup>+</sup> positive cells (orange) over  
769 total number cells nuclear stained with NucBlue (NB, blue) and expressed as % PI<sup>+</sup>/NucBlue. Data  
770 are means ± SD of n=4 independent experiments.

771 **L)** one-way ANOVA of K) at 18h, \*\*\*\*p<0.0001.

772 **M)** Representative confocal images of WT and *Mtfp1*<sup>-/-</sup> MEFs subjected to actinomycin D (ActD)  
773 plus ABT-737 treatment in the presence or absence of the pan-caspase inhibitor q-VD-OPh  
774 hydrate (qVD). Live induction of the caspase 3/7 activation was monitored by using the CellEvent  
775 (CE, green) reagent and imaging cells every hour (h) for 20h. Scale bar = 100 μm.

776 **N)** Kinetics of caspase 3/7 activation was determined by counting the number of CE<sup>+</sup> positive cells  
777 (green) over total number cells nuclear stained with NucBlue (NB, blue) and expressed as %  
778 CE<sup>+</sup>/NucBlue. Data are means ± SD of n=4 independent experiments.

779 **O)** one-way ANOVA of N) at 12h, \*\*\*\*p<0.0001.

780 **P)** Representative confocal images of WT and *Mtfp1*<sup>-/-</sup> MEFs subjected to doxorubicin treatment in  
781 the presence or absence of the pan-caspase inhibitor q-VD-OPh hydrate (qVD). Live induction of

782 the caspase 3/7 activation was monitored by using the CellEvent (CE, green) reagent and imaging  
783 cells every hour (h) for 18h. Scale bar = 100  $\mu$ m.  
784 **Q)** Kinetics of caspase 3/7 activation was determined by counting the number of CE<sup>+</sup> positive cells  
785 (green) over total number cells nuclear stained with NucBlue (NB, blue) and expressed as %  
786 CE<sup>+</sup>/NucBlue. Data are means  $\pm$  SD and representative of at least n=3 independent experiments.  
787 **R)** one-way ANOVA of Q) at 12h, p<0.0001.

788

789 **Figure 5. mPTP accounts for cell death sensitivity in MTFP1 deficient cells**

790 **A)** Representative confocal images of WT and *Mtfp1*<sup>-/-</sup> MEFs subjected to H<sub>2</sub>O<sub>2</sub> treatment. The  
791 pan-caspase and cyclophilin D inhibitors, q-VD-OPh hydrate (qVD) and cyclosporin A (CsA)  
792 respectively, were used to block both caspase and mPTP dependent cell death. Cell death was  
793 monitored by Propidium Iodide uptake (PI, orange) and imaging cells every hour (h) for 18h. Scale  
794 bar = 100  $\mu$ m.

795 **B)** Kinetics of PI uptake was determined by counting the number of PI<sup>+</sup> positive cells (orange) over  
796 total number cells nuclear stained with NucBlue (NB, blue) and expressed as % PI<sup>+</sup>/NucBlue. Data  
797 are means  $\pm$  SD of n=6 independent experiments.

798 **C)** one-way ANOVA of B) at 18h, \*p<0.05

799 **D)** Validation of Cyclophilin D (CYP D) deletion by Crispr/Cas9 genome editing in WT and *Mtfp1*<sup>-/-</sup>  
800 MEFs. Equal amounts of protein extracted from WT, *Mtfp1*<sup>-/-</sup>, *Ppif*<sup>-/-</sup>, *Mtfp1*<sup>-/-</sup> *Ppif*<sup>-/-</sup> MEFs were  
801 separated by SDS-PAGE and immunoblotted with the indicated antibodies. SDHA was used as  
802 mitochondrial marker and loading control.

803 **E)** Representative confocal images of WT, *Mtfp1*<sup>-/-</sup>, *Ppif*<sup>-/-</sup>, *Mtfp1*<sup>-/-</sup> *Ppif*<sup>-/-</sup> MEFs subjected to H<sub>2</sub>O<sub>2</sub>  
804 treatment. Cell death was monitored by Propidium Iodide uptake (PI, orange) and imaging cells  
805 every hour (h) for 18h. Scale bar = 100  $\mu$ m.

806 **F)** Kinetics of PI uptake was determined by counting the number of PI<sup>+</sup> positive cells (orange) over  
807 total number cells nuclear stained with NucBlue (NB, blue) and expressed as % PI<sup>+</sup>/NucBlue. Data  
808 are means  $\pm$  SD of n=3 independent experiments.

809 **G)** one-way ANOVA of F) at 18h; \*\*p<0.01, \*\*\*\*p<0.0001.

810 **H)** Representative confocal images of WT, *Mtfp1*<sup>-/-</sup>, *Ppif*<sup>-/-</sup>, *Mtfp1*<sup>-/-</sup> *Ppif*<sup>-/-</sup> MEFs subjected to  
811 doxorubicin (DOXO) treatment. Live induction of the caspase 3/7 activation was monitored by

812 using the CellEvent (CE, green). CellEvent positive cells (CE<sup>+</sup>, green) over total number over cells  
813 NucBlue labeled (blue) were imaged every hour (h) for 18h. Scale bar = 100  $\mu$ m.  
814 **I)** Kinetics of caspase 3/7 activation was determined by counting the number of CE<sup>+</sup> positive cells  
815 (green) over total number cells nuclear stained with NucBlue (NB, blue) and expressed as %  
816 CE<sup>+</sup>/NucBlue. Data are means  $\pm$  SD and representative of n=3 independent experiments.  
817 **J)** one-way ANOVA of I) at 16h, \*\*\*\*p<0.0001.

818

### 819 **Figure 6. Model for the regulation of mitochondrial and cardiac function by MTFP1**

820 *Mtfp1* deletion in cardiomyocytes occurs at birth (cMKO) and sensitizes cardiac myocytes to  
821 mitochondrial permeability transition pore (mPTP) opening, cell death and increases mitochondria  
822 uncoupling of the inner membrane. At the adult age of 8-10 weeks heart of cMKO mice have  
823 normal structure and function but undergoes to the development of a progressive dilated  
824 cardiomyopathy (DCM) at 18 weeks which progresses to severe heart failure and middle-aged  
825 death by 34 weeks. At onset of DCM, cMKO mice exhibit increased cardiac cell death, reduced  
826 mitochondrial respiration, and induction of a sterile inflammatory response.

827

828

### 829 **Figure S1. *Mtfp1* deletion in cardiomyocytes causes dilated cardiomyopathy and middle-** 830 **aged death in mice**

831 **A)** Genotype tissue expression plot (GTEx) of MTFP1 in human tissues.  
832 **B)** Alkaline carbonate extraction assay demonstrating solubility of MTFP1 similar to other multi-  
833 pass integral membrane proteins. Immunoblot analysis of soluble (S) and insoluble (pellet, P)  
834 fractions of wild type (WT) cardiac mitochondria extracted with Na<sub>2</sub>CO<sub>3</sub> at the indicated pH. SDHA,  
835 SDHB and MT-CO2, ANT1, NDUFA9 were used as markers for soluble and integral membrane  
836 proteins, respectively.  
837 **C)** Determination of sub-mitochondrial localization of MTFP1 in cardiac mitochondria by protease K  
838 protection assay. TOMM40 and MFN2, were used as OMM markers, CYT C as an IMS marker,  
839 and ANT1, ATP5B and SDHA as IMM markers.  
840 **D)** Targeting strategy for conditional inactivation of mouse *Mtfp1*. To allow deletion of *Mtfp1* exons  
841 2 and 3 were flanked in both cases by LoxP sites (blue arrowheads). Flox denotes NeoR cassette

842 containing LoxP targeted locus. LoxP denotes NeoR cassette-deleted targeted locus.  $\Delta$  denotes  
843 deletion induced by Cre-recombinase. FRT sites (green) initially flank NeoR cassette (yellow).

844 **E)** Kaplan-Meier survival curve of WT (n=11) and cMKO (n=14) female mice. Median lifespan of  
845 cMKO mice is 37.5 weeks. Log-rank (Mantel-Cox) test, \*\*\*p<0.001.

846 **F)** M-Mode echocardiography images of WT (top) and cMKO (bottom) female mice at 34 weeks.

847 **G)** Quantification of left ventricular ejection fraction (% LVEF) of WT (n=7) and cMKO (n=6) female  
848 mice at 34 weeks. Data represent mean  $\pm$  SD. 2-tailed unpaired Student's t-test, \*\*\*\*p<0.0001.

849 **H)** Quantification of systolic interventricular septum thickness (IVSs, mm) of WT (n=7) and cMKO  
850 (n=6) female mice at 34 weeks. Data represent mean  $\pm$  SD. 2-tailed unpaired Student's t-test,  
851 \*p<0.05.

852 **I)** Quantification of systolic left ventricular posterior wall thickness (LVPWs, mm) of WT (n=7) and  
853 cMKO (n=6) female mice at 34 weeks. Data represent mean  $\pm$  SD. 2-tailed unpaired Student's t-  
854 test, \*\*\*p<0.001.

855 **J)** Quantification of left ventricle end systolic diameter (LVSD, mm) of WT (n=7) and cMKO (n=6)  
856 female mice at 34 weeks. Data represent mean  $\pm$  SD. 2-tailed unpaired Student's t-test,  
857 \*\*\*p<0.001.

858 **K)** Quantification of left ventricle end diastolic diameter (LVDD, mm) of WT (n=7) and cMKO (n=6)  
859 female mice at 34 weeks. Data represent mean  $\pm$  SD. 2-tailed unpaired Student's t-test,  
860 \*\*\*p<0.001.

861 **L)** Quantification of pulmonary edema. Wet Lung/Body Weight (BW) of WT (n=8) and cMKO (n= 8)  
862 at 34 weeks. Data represent mean  $\pm$  SD; 2-tailed unpaired Student's t-test, \*p<0.05.

863 **M)** Representative images (left) of WT (top) and cMKO (bottom) hearts isolated from female mice  
864 at 34 weeks; (right) quantification of ratio of heart mass to tibia length (mg/mm). Data represent  
865 mean  $\pm$  SD, 2-tailed unpaired Student's t-test, \*\*\*p<0.001.

866 **N-S)** Isolated primary adult cardiomyocytes from WT (n=51) and cMKO (n=52 ) mice at 8 weeks  
867 were i) field-stimulated at 0.5 Hz, ii) exposed to the  $\beta$ -adrenergic agonist isoproterenol and iii)  
868 stimulated at frequency of 5 Hz, iv) and then stepped back to 0.5 Hz washing out (wo)  
869 isoproterenol. **N)** Diastolic and systolic sarcomere length ( $\mu$ m) of WT and cMKO; **O)** fractional  
870 sarcomere shortening; **P)** Time to 50% or 90% decay of sarcomere shortening in WT or cMKO  
871 myocytes, \*\*\*p<0.001.

872 **Q-S)** Isolated primary adult cardiomyocytes from WT (n=29) and cMKO (n=52) mice at 8 weeks  
873 were i) field-stimulated at 0.5 Hz, ii) exposed to the  $\beta$ -adrenergic agonist isoproterenol and iii)  
874 stimulated at frequency of 5 Hz, iv) and then stepped back to 0.5 Hz washing out (wo)  
875 isoproterenol. Intracellular  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_c$ ) were assessed by loading cells with Indo-1  
876 AM and recording calcium transients at 405 nm/485 nm. **Q)**  $[\text{Ca}^{2+}]_c$  at diastole or systole; **R)**  
877 amplitude of  $[\text{Ca}^{2+}]_c$  transients; **S)** Time to 50% or 90% decay (RT) of  $[\text{Ca}^{2+}]_c$  in WT and cMKO  
878 myocytes, \*\*p<0.01, \*\*\*\*p<0.0001.

879

## 880 **Figure S2. *Mtfp1* is required for bioenergetic efficiency in cardiac mitochondria**

881 **A)** Equal amounts of protein extracted from WT (n=4) and cMKO (n=6) hearts at 18 weeks were  
882 separated by SDS-PAGE and immunoblotted with the indicated antibodies.

883 **B)** Volcano plot of the Label Free Quantification of the cardiac proteome in WT and cMKO mice  
884 (18 weeks) listed in Dataset EV1.

885 **C)** Ratio of NAD(P)H/FAD of the redox states of NAD(P)H/NAD(P)<sup>+</sup> and FADH<sub>2</sub>/FAD assessed in  
886 field-stimulated WT (n=51) and cMKO (n=51) cardiomyocytes isolated from 8-10 weeks old mice  
887 (n=3).

888 **D)** Oxygen consumption rates (left;  $\text{JO}_2$ ) measured by high-resolution respirometry of cardiac  
889 mitochondria isolated from WT (n=5) and cMKO (n=5) mice between 8-10 weeks. Respiration was  
890 measured in presence of succinate and rotenone (state 2) followed by the addition of ADP (state 3)  
891 and Oligomycin (Omy- state 4). Data represent mean  $\pm$  SD. Multiple t-test, state 2 \*\*\*p<0.001,  
892 state 4 \*p<0.05. Respiratory control ratios (RCR) of state 3:2 (middle; ADP/Succinate (SUCC)) and  
893 state 3:4 (right; ADP/Omy) under complex-II driven respiration. Data represent mean  $\pm$  SD;  
894 multiple t-test, \*p<0.05.

895 **E)** Mitochondrial membrane potential ( $\Delta\Psi$ ) measured by quenching of Rhodamine 123 (RH123)  
896 fluorescence in cardiac mitochondria from WT (n=8) and cMKO (n=8) mice between 8-10 weeks.  
897  $\Delta\Psi$  was measured in presence of rotenone and succinate (state 2) followed by the addition of ADP  
898 (state 3) and Oligomycin (state 4). Data represent mean  $\pm$  SD; Multiple t-test, \*p<0.05, \*\*\*p<0.001.

899 **F)** Oxygen consumption rates ( $\text{JO}_2$ ) measured by high-resolution respirometry of cardiac  
900 mitochondria isolated from WT (n=6) and cMKO (n=6) mice between 8-10 weeks. Respiration was



901 measured in presence of malate (state 2) followed by the addition of ADP (state 3) and palmitoyl-  
902 carnitine (PC). Data represent mean  $\pm$  SD.

903 **G)** Mitochondrial membrane potential ( $\Delta\Psi$ ) measured by quenching of Rhodamine 123 (RH123)  
904 fluorescence in cardiac mitochondria isolated from WT (n=6) and cMKO (n=6) mice between 8-10  
905 weeks.  $\Delta\Psi$  sustained by fatty acid oxidation was measured in presence of malate (state 2) followed  
906 by the addition of ADP (state 3) and palmitoyl-carnitine (PC). Data represent mean  $\pm$  SD; Multiple  
907 t-test, \*\*\*p<0.001.

908 **H)** PCR genotyping of *Mtfp1* alleles in mice. Deletion of exons 2 and 3 was obtained by crossing  
909 *Mtfp1*<sup>LoxP/LoxP</sup> mice with *CMV-Cre* recombinase mice to generate heterozygous knockout *Mtfp1*<sup>+/-</sup>  
910 offspring. Agarose gel of PCR amplicons of genomic DNA. The wild type (387 bp) and the deleted  
911 (737 bp) alleles are shown for *Mtfp1*<sup>+/+</sup> and *Mtfp1*<sup>-/-</sup> cells.

912 **I)** Equal amounts of protein extracted from WT and *MTFP1*<sup>Crispr</sup> U2OS cells separated by SDS-  
913 PAGE and immunoblotted with antibodies against MTFP1 and normalized against Stain Free as a  
914 loading control.

915 **J-M)** Mitochondrial respiration measured in adherent wild type (WT) and *Mtfp1*<sup>-/-</sup> immortalized  
916 mouse embryonic fibroblasts (MEFs) cells using the Seahorse Flux Analyzer. **J)** Oxygen  
917 consumption rate (OCR) normalized to protein concentration under **K)** Basal **L)** Maximal (FCCP)  
918 and **M)** non-phosphorylating (state 4) respiration (Oligomycin treated - Omy). Data are means  $\pm$   
919 SD of n=4 independent experiments measured on different days. Each point represents the mean  
920 of 7-12 technical OCR measurements replicates of each independent experiment.

921 **N-Q)** Mitochondrial respiration measured in adherent wild type (WT) and *MTFP1*<sup>Crispr</sup> human U2OS  
922 osteosarcoma cells using the Seahorse Flux Analyzer. **N)** Oxygen consumption rate (OCR)  
923 normalized to protein concentration under **O)** Basal **P)** Maximal (FCCP) and **Q)** non-  
924 phosphorylating (state 4) respiration (Oligomycin treated - Omy). Data are means  $\pm$  SD of 7-12  
925 technical replicates.

926 **R)** Inhibition of proton leak by MTFP1. Proposed model of the regulation of ANT-dependent proton  
927 leak by MTFP1 (red). Proton gradient generated by the electron transport chain (ETC) is  
928 harnessed by ATP synthase in WT cells to generate ATP from ADP and Pi. Upon *Mtfp1* ablation  
929 (*Mtfp1*<sup>-/-</sup>), ANT-mediated proton leak increases thus compromising bioenergetic efficiency.

930

931

**Figure S3. MTFP1 is dispensable for mitochondrial fission.**

932

**A)** Immunoblot of mitochondrial fission and fusion proteins in WT and *Mtfp1*<sup>-/-</sup> MEFs. Equal amounts of protein extracted from WT and *Mtfp1*<sup>-/-</sup> MEFs were separated by SDS-PAGE and immunoblotted with the indicated antibodies (horizontal line denotes different membranes). VINCULIN was used as loading control.

936

**B)** Representative confocal images of fragmented mitochondria morphology in MEFs stably expressing FLAG-MTFP1 (mitoYFP, green). EV (empty vector, top), FLAG-MTFP1 (bottom). Scale bar=100  $\mu$ m.

939

**C)** Representative immunoblot of mitochondrial fission (red) and fusion (green) proteins in WT and FLAG-MTFP1 stably overexpressing MEFs. Equal amounts of protein were separated by SDS-PAGE and immunoblotted with the indicated antibodies (horizontal line denotes different membranes).

943

944

**Figure S4. MTFP1 protects against mitochondrial PTP opening and cell death**

945

**A)** Mitochondrial permeability transition pore (mPTP) opening. Swelling assay performed on mitochondria purified from WT and *Mtfp1*<sup>-/-</sup> MEFs. Mitochondrial absorbance changes (absorbance 540 nm, % relative to T<sub>0</sub>) are measured every 20 s prior and after addition of a single pulse of CaCl<sub>2</sub> (arrowhead). Cyclosporin A (CsA) was added prior Ca<sup>2+</sup> to inhibit mPTP-dependent swelling. Data are means  $\pm$  SD.

950

**B)** Representative confocal images of WT and *Mtfp1*<sup>-/-</sup> MEFs subjected to staurosporine (STS) treatment in the presence or absence of the pan-caspase inhibitor q-VD-Oph hydrate (qVD). Cell death was monitored by measuring Propidium Iodide uptake (PI) and imaging cells every hour (h) for 18 h. Scale bar = 100  $\mu$ m.

954

**C)** Kinetics of PI uptake was determined by counting the number of PI<sup>+</sup> positive cells (orange) over total number cells nuclear stained with NucBlue (NB, blue) and expressed as % PI<sup>+</sup>/NucBlue. Data are means  $\pm$  SD of n=4 independent experiments.

957

**D)** one-way ANOVA of C) at 12h, \*\*\*\*p<0.0001.

958

**E)** Cell proliferation curves of WT and *Mtfp1*<sup>-/-</sup> MEFs in glucose (4.5 g/L) containing medium. Number of cells was determined by counting the number of NucBlue (NB) labeled nuclei every 24 h.

960

- 961 **F)** Representative M-Mode echocardiographic images of left ventricles of WT (left) and cMKO  
962 (right) mice treated with doxorubicin (Doxo, cumulative 20 mg/kg) at 10 weeks.
- 963 **G)** Quantification of left ventricular ejection fraction (% LVEF) of WT (n=5) and cMKO (n=5) after 2  
964 weeks of Doxo administration. Data represent mean  $\pm$  SD. 2-tailed unpaired Student's t-test,  
965 \*p<0.05.
- 966 **H)** Quantification of systolic left ventricular diameter (LVSD, mm) of WT (n=5) and cMKO (n=5)  
967 mice after Doxo treatment. Data represent mean  $\pm$  SD. 2-tailed unpaired Student's t test, \*p<0.05.
- 968 **I)** Quantification of diastolic left ventricular diameter (LVDD, mm) of WT (n=5) and cMKO (n=5)  
969 mice after Doxo treatment. Data represent mean  $\pm$  SD. 2-tailed unpaired Student's t test, \*p<0.05.
- 970 **J)** Quantification of systolic left ventricular posterior wall thickness (LVPWs, mm) of WT (n=5) and  
971 cMKO (n=5) mice after Doxo treatment. Data represent mean  $\pm$  SD. 2-tailed unpaired Student's t  
972 test, \*p<0.05.
- 973
- 974 **Figure S5. Generation of FLAG-MTFP1 mouse model to define the cardiac interactome**
- 975 **A)** For inducible overexpression of mCherry-P2A-Flag-Mtp18, two mouse models were generated  
976 from one construct. The first (upper pane) allowing inducible expression from the CAG-promoter,  
977 the second (lower panel) from the mouse Rosa26 promoter. To do so, the construct in the upper  
978 panel is generated and recombined into the mouse Rosa26 locus. The genuine Rosa26 promoter  
979 is further upstream, and its transcript is spliced into the splice acceptor of exon 2 ("s.acc.", purple).  
980 In the native state, transcription is aborted at the insulator site (H19, black), and instead started at  
981 the CAG promoter (pCAG, yellow arrow). The loxP-flanked (lox, blue arrow heads) neo-STOP  
982 cassette for conditional activation has a stop site that is a large region of SV40 intron plus late  
983 polyA (neo, green arrow; STOP, blue box). The cDNA is inserted downstream of the neo/stop  
984 cassette. Both, Rosa26-driven and CAG-driven expression, is polyadenylated at a bovine growth  
985 hormone polyA site (bGH pA, small black box). For exchange of the promoters, the CAG-cassette  
986 is flanked by FRT-sites. This cassette will be excised by Flp-mediated deletion in vivo, leaving  
987 mCherry-P2A-Flag-Mtp18 expression under control of the Rosa26 promoter. Upon Cre mediated  
988 deletion, the mCherry-P2A-Flag-Mtp18 cDNA is expressed from the CAG promoter (upper panel)  
989 or the intrinsic Rosa26 promoter (lower panel).

990 **B)** Representative immunoblot of the expression of Flag-MTFP1 compared to endogenous MTFP1  
991 levels in KI mice.

992 **C)** Quantification of left ventricular ejection fraction (% LVEF) of WT (n=4) and KI (n=3) mice at 20  
993 weeks. Data represent mean  $\pm$  SD.

994 **D)** Volcano plot of the FLAG-MTFP1 interactome analyzed by mass spectrometry. Mitochondrial  
995 proteins exclusively present in FLAG-MTFP1 eluates or significantly enriched greater than two-  
996 fold, listed in Dataset EV3.

997 **E)** Functional classification of 60 mitochondrial proteins identified in Co-IP eluates in C) (Dataset  
998 EV3).

999

1000

1001

1002

## 1003 **Material and Methods**

### 1004 **Animals**

1005 Animals were handled according to the European legislation for animal welfare ([Directive](#)  
1006 [2010/63/EU](#)). All animal protocols were reviewed and approved by the local and national  
1007 authorities. Mice were housed within a specific pathogen free facility and maintained under  
1008 standard housing conditions of a 14-10h light-dark cycle, 50-70% humidity, 19-21°C with free  
1009 access to food and water in cages enriched with bedding material and gnawing sticks. *Mtfp1*  
1010 conditional mice (*Mtfp1<sup>LoxP/LoxP</sup>*) were generated by PolyGene AG (Switzerland) on a C57Bl6/N  
1011 background. Cardiomyocyte specific *Mtfp1* KO mice (cMKO; *Myh6-Cre<sup>tg/+</sup>Mtfp1<sup>LoxP/LoxP</sup>*) were  
1012 generated by crossing *Mtfp1* conditional mice (*Mtfp1<sup>LoxP/LoxP</sup>*) with transgenic (Tg) mice expressing  
1013 the Cre recombinase under the control of the cardiac alpha myosin heavy chain 6 promoter (*Myh6-*  
1014 *Cre*)(Agah et al., 1997). Littermates that were homozygous for the conditional allele and negative  
1015 for *Myh6-Cre* were used as controls (WT; *Myh6-Cre<sup>+/+</sup>Mtfp1<sup>LoxP/LoxP</sup>*). Heterozygous whole-body  
1016 *Mtfp1* KO mice (*Mtfp1<sup>+/-</sup>*) were generated by crossing *Mtfp1* conditional mice (*Mtfp1<sup>LoxP/LoxP</sup>*) with  
1017 transgenic (Tg) mice expressing the Cre recombinase under the control of the CMV promoter  
1018 (*CMV-Cre*)(Schwenk et al., 1995) and backcrossing to C57Bl6/N wild type (WT) mice.  
1019 Cardiomyocyte specific FLAG-MTFP1 Knock-In (KI) mice (*Myh6-Cre<sup>tg/+</sup>Mtfp1<sup>+/+</sup>*, *CAG<sup>tg/+</sup>*) were  
1020 generated by crossing an inducible mouse model for mCherry-P2A-FLAG-MTFP1 generated by  
1021 PolyGene AG (Switzerland) on a C57Bl6/N background expression under the CAG promoter with  
1022 mice expressing the Cre recombinase under the control of the cardiac alpha-( $\alpha$ ) myosin heavy  
1023 chain 6 promoter (*Myh6-Cre*).

### 1024 **Cell Lines**

1025 WT (*Mtfp1<sup>+/+</sup>*) and knockout (KO) *Mtfp1<sup>-/-</sup>* embryos were isolated at E13.5 following F1  
1026 heterozygous intercrosses of *Mtfp1<sup>+/-</sup>* whole-body KO mice. immortalization of WT (*Mtfp1<sup>+/+</sup>*) and  
1027 *Mtfp1<sup>-/-</sup>* primary mouse embryonic fibroblasts (MEFs) was performed as previously  
1028 described(Cretin et al., 2021, 2021) using a plasmid encoding SV40 large T antigen. MEFs cells  
1029 were maintained in Dulbecco's modified Eagle's medium (DMEM + GlutaMAX, 4.5 g/L D-Glucose,  
1030 pyruvate) supplemented with 5% FBS and 1% penicillin/streptomycin (P/S, 50  $\mu$ g/ml) in a 5% CO<sub>2</sub>  
1031 atmosphere at 37°C.

1033 Genetic disruption of *Ppif* in WT and *Mtfp1*<sup>-/-</sup> MEFs was performed via CRISPR-Cas9 gene editing  
1034 targeting Exon 1 of *Ppif* (sgRNA: forward: aaacCCGGGAACCCGCTCGTGTAC and reverse:  
1035 CACCGTACACGAGCGGGTTCCTGG. sgDNA oligonucleotides were annealed and cloned into  
1036 pSpCas9(BB)-2A-GFP PX458 to generate pTW363 (pSpCas9(BB)-2A-GFP PPIFsgDNA). WT  
1037 (*Mtfp1*<sup>+/+</sup>) and *Mtfp1*<sup>-/-</sup> immortalized MEFs were transfected with 2.5 µg of plasmid pTW363 using  
1038 Lipofectamine 2000 (Life Technologies). After 48h incubation, cells were individually isolated in 96  
1039 well plates by FACS. Clones were then expanded and validated by Sanger sequencing and  
1040 western blotting. Both single *Ppif* KO MEFs (*Ppif*<sup>-/-</sup>) and double KO *Mtfp1*<sup>-/-</sup>*Ppif*<sup>-/-</sup> cells carry the  
1041 same homozygous c.126delG insertion that is predicted to yield a truncated polypeptide product at  
1042 amino acid position 65 (1-64 protein) p.Val65\*. Immunoblot analysis was used to confirm the  
1043 absence of PPIF protein.

1044 MEFs expressing mitochondrially targeted YFP (mitoYFP) were generated from  
1045 Gt(ROSA26)Sor<sup>mitoYFP/+</sup> embryos on a C57Bl6/N genetic background at E13.5 and immortalized  
1046 using a plasmid encoding SV40 large T antigen as previously described (Cretin et al., 2021). MEFs  
1047 stably expressing FLAG-MTFP1 were generated by lentiviral transduction with pTW142 (pLVX-  
1048 EF1α-MTFP1) containing a puromycin resistant marker. The empty vector (EV) pLVX-EF1α  
1049 (pTW122) was used to generate control cells.

1050 Human U2OS osteosarcoma cells were depleted for MTFP1 by CRISPR-Cas9 gene editing. The  
1051 single-guide RNAs (sgRNAs) were designed using the CRISPR-Cas9 design tool (benchling.com)  
1052 to target Exon 1 of *MTFP1*. sgDNA oligonucleotides (forward: 5'-  
1053 caccgGCGCAGAGCGCGATCTCTAC -3' and reverse: 5'-aaacGTAGAGATCGCGCTCTGCGCc-  
1054 3') were annealed and cloned into the BbsI digested pSpCas9(BB)-2A-GFP vector (SpCas9(BB)-  
1055 2A-GFP (PX458) which was a gift from Feng Zhang (Addgene plasmid # 48138). U2OS cells were  
1056 transfected in 6-well dishes with 2µg of pSpCas9(BB)-2A-GFP plasmid containing the respective  
1057 sgRNA using Lipofectamine 2000 (Life Technologies, 11668027). After 24h incubation, GFP  
1058 positive cells were individually isolated by fluorescence-activated cell sorting. Clones were  
1059 expanded and were validated by western blotting and DNA sequencing.

1060

1061 **SDS-PAGE immunoblot analysis**

1062 Immunoblot analysis was used to assess steady-state protein levels in cardiac tissue and cell  
1063 lysates. For tissue lysates, mice were sacrificed by cervical dislocation, the chests were opened,  
1064 and the hearts were excised, weighed, flash frozen in liquid nitrogen, and stored at -80°C until use.  
1065 The LV tissue or MEFs cellular pellet was homogenized in cold RIPA buffer [1 mg/ 20 µL, 1%  
1066 Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 7.8), 1 mM  
1067 EDTA, and 1 mM EGTA] in presence of protease and phosphatase inhibitors and kept on ice for  
1068 30 minutes. The homogenate was then centrifuged for 15 min at 16,000g, 4°C. The protein  
1069 concentration was determined by Bradford assay (Bio-Rad) using a BSA standard curve. The  
1070 protein absorbance was measured at 595 nm by using a microplate reader Infinite M2000 (Tecan).  
1071 Equal amounts of protein were reconstituted in 4x Laemmli Sample Buffer [355 mM, 2-  
1072 mercaptoethanol, 62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 1%(w/v) SDS, 0.005% (v/v)  
1073 Bromophenol Blue] and heated at 95°C for 5 min. Samples (10 µg) were resolved on 4-20%  
1074 polyacrylamide gels (Mini Protean TGX Stain-Free gels, BioRad) and transferred to nitrocellulose  
1075 membrane with Trans-Blot® Turbo™ Transfer system (Bio-Rad). Equal protein amount across  
1076 membrane lanes were checked by Ponceau S staining or Stain-free detection. Membranes were  
1077 blocked for at least 1h with 5% (w/v) semi-skimmed dry milk dissolved in Tris-buffered saline  
1078 Tween 0.1% (TBST), incubated overnight at 4°C with primary antibodies dissolved 1:1,000 in 2%  
1079 (w/v) Bovine Serum Albumin (BSA), 0.1% TBST. The next day membranes were incubated in  
1080 secondary antibodies conjugated to horseradish peroxidase (HRP) at room temperature for 2h  
1081 (diluted 1:10,000 in BSA 2% TBST 0.1%). Finally, membranes were incubated in Clarity™ Western  
1082 ECL Substrate (Bio-Rad) for 2 min and luminescence was detected using the ChemiDoc® Gel  
1083 Imaging System. Densitometric analysis of the immunoblots was performed using Image Lab  
1084 Software (Bio-Rad).

1085

### 1086 **Mitochondrial isolation**

1087 Isolated cardiac mitochondria were freshly isolated as previously described (Mourier et al., 2014)  
1088 with some modifications. Briefly, the heart was washed in ice-cold PBS solution. Ventricles were  
1089 separated from atria and non-myocardial tissue, cut in small pieces, and then transferred to an ice-  
1090 cold 2-ml homogenizer (Teflon pestle) and manually homogenized in IB buffer (sucrose 275 mM,  
1091 Tris 20 mM, EGTA-KOH 1 mM, pH 7.2) containing Trypsin-EDTA (0.05%). Trypsin activity was

1092 then inhibited by adding to the homogenate bovine serum albumin (BSA) fatty acid free (0.25  
1093 mg/mL) and protease inhibitor cocktail (PIC, Roche).  
1094 Mitochondria isolation from MEFs was performed starting from 10 x 150 mm dishes at 100%  
1095 confluence. Cells were collected into 10 mL of IB Buffer containing BSA fatty acid free (0.25  
1096 mg/mL) and PIC and homogenized with 30 strokes of the plunger at 1500 rpm on ice.  
1097 Cardiac and/or cellular homogenates were then centrifuged at low speed (1000 g, 10 min, 4 °C) to  
1098 discard nuclei and debris, and further centrifuged (3200 g, 15 min, 4°C) to obtain the crude  
1099 mitochondrial pellet and the cytosolic fraction. The crude mitochondrial pellet was finally  
1100 resuspended in IB buffer containing BSA and PIC and protein concentration was determined by  
1101 using the Bradford assay.

### 1103 **Protease Protection Assay**

1104 Crude mitochondria isolated from WT mouse hearts was subjected to protease protection assay as  
1105 previously described (Anand et al., 2014). 50 µg of crude mitochondria were resuspended into the  
1106 following buffers without protease inhibitors: 1) Mitochondrial isolation IB buffer 2) Mitochondrial  
1107 isolation buffer with Proteinase K (100 µg/mL) 3) Swelling buffer (EDTA 1 mM, HEPES 40 mM) 4)  
1108 Swelling buffer + Proteinase K (100 µg/mL) 5) Swelling buffer + Proteinase K (100 µg/ml) + Triton  
1109 X-100 0.5% and incubated at 37°C for 30 minutes at 750 rpm. Mitochondrial proteins were then  
1110 precipitated in trichloric acid buffer (14% TCA , 40 mM HEPES, 0.02% Triton X-100) for 3 hours at  
1111 -20°C and centrifugated for 20 min. Pellets were washed twice with ice-cold acetone and air-dried  
1112 prior to re-suspension in 1x Laemilli sample buffer (Bio-Rad) for SDS-PAGE and immunoblot  
1113 analysis.

### 1115 **Alkaline Carbonate Extraction**

1116 Alkaline carbonate extraction of membrane proteins was performed as previously described  
1117 (Anand et al., 2014). Crude mitochondria isolated from WT mouse hearts were resuspended and  
1118 incubated for 30 min on ice with 0.1 M Na<sub>2</sub>CO<sub>3</sub> at the following pH: 12.5, 11.5, 10.5, or 9.5. The  
1119 suspensions were ultra-centrifuged at 4°C for 30 min at 90,000 g in Beckman polycarbonate tubes  
1120 in a TLA 110 rotor. Supernatants and pellets were then incubated in trichloric acid buffer (14%  
1121 TCA, 40 mM HEPES, 0.02% Triton X-100) for 15 min on ice, followed by centrifugation for 20 min



1122 at 28,000 g at 4°C. The samples were washed 3x with 100% acetone, subsequently dried for 30  
1123 min at RT. The dried pellet was then resuspended with 1x Laemilli sample buffer (Bio-Rad) for  
1124 SDS-PAGE and western blot analysis.

### 1125 1126 **siRNA transfection**

1127 Silencing of the indicated genes was performed using forward transfection: 20 nM of the indicated  
1128 SmartPool siRNAs listed in Dataset EV4 were mixed with Lipofectamine RNAiMax (Invitrogen),  
1129 added on top of cells seeded in 6-well dishes (300,000 cells/well) and incubated at 37°C in a CO<sub>2</sub>  
1130 incubator. For live imaging experiments, cells were seeded in 96 well plates (Cell Carrier Ultra,  
1131 Perkin Elmer) 48h after transfection to perform live imaging after 72h post-transfection.

### 1132 1133 **Cardiac RNA sequencing and RT-qPCR**

1134 WT and cMKO mice aged 8 and 18 weeks were sacrificed by cervical dislocation and hearts were  
1135 quickly excised and rinsed with cold sterile PBS. Left ventricle posterior walls were collected and  
1136 snap frozen in liquid nitrogen and stored at -80°C until use. Total RNA was extracted by using  
1137 TRIzol (Invitrogen, NY, USA) according to standard procedures. The Trizol/chloroform mixture was  
1138 centrifuged at 12000g for 15 min at 4°C, the supernatant corresponding to the aqueous phase was  
1139 transferred to a new tube, mixed with ethanol 70%, and then applied to an RNA mini-column  
1140 extraction kit (NucleoSpin RNA kit, MACHEREY-NAGEL) according to the manufacturer's  
1141 recommendations. After DNA digestion, total RNA was eluted with RNase-free water and stored in  
1142 liquid nitrogen. RNA was quantified by using the Bioanalyzer 2100 (Agilent) and only RNA samples  
1143 ( $\geq 50$  ng/ $\mu$ L) with an RNA integrity value (RIN)  $\geq 6.8$ ,  $OD^{260/280} \geq 2$ ,  $OD^{260/230} \geq 2$  were considered  
1144 for the transcriptome profiling. For RNA sequencing, libraries were built using a TruSeq Stranded  
1145 mRNA library Preparation Kit (Illumina, USA) following the manufacturer's protocol. Two runs of  
1146 RNA sequencing were performed for each library on an Illumina NextSeq 500 platform using  
1147 single-end 75bp. The RNA-seq analysis was performed with Sequana 0.8.5(Cokelaer et al., 2017)  
1148 using an RNA-seq pipeline 0.9.13 ([https://github.com/sequana/sequana\\_rnaseq](https://github.com/sequana/sequana_rnaseq)) built on top of  
1149 Snakemake 5.8.1(Köster and Rahmann, 2012). Reads were trimmed from adapters using  
1150 Cutadapt 2.10(Martin, 2011) then mapped to the mouse reference genome GRCm38 using STAR  
1151 2.7.3a(Dobin et al., 2013). FeatureCounts 2.0.0 was used to produce the count matrix, assigning

1152 reads to features using annotation from Ensembl GRCm38\_92 with strand-specificity  
1153 information(Liao et al., 2014). Quality control statistics were summarized using MultiQC 1.8(Ewels  
1154 et al., 2016). Statistical analysis on the count matrix was performed to identify differentially  
1155 expressed genes (DEGs), comparing WT and cMKO. Clustering of transcriptomic profiles were  
1156 assessed using a Principal Component Analysis (PCA). Differential expression testing was  
1157 conducted using DESeq2 library 1.24.0 scripts based on SARTools 1.7.0(Varet et al., 2016)  
1158 indicating the significance (Benjamini-Hochberg adjusted p-values, false discovery rate FDR <  
1159 0.05) and the effect size (fold-change) for each comparison.

1160 For RT-qPCR, 1 µg of total RNA was converted into cDNA using the iScript Reverse Transcription  
1161 Supermix (Bio-Rad). RT-qPCR was performed using the CFX384 Touch Real-Time PCR Detection  
1162 System (Bio-Rad) and SYBR® Green Master Mix (Bio-Rad) using the primers listed in Dataset  
1163 EV1. Gapdh was amplified as internal standard. Data were analyzed according to the  $2^{-\Delta\Delta CT}$   
1164 method(Livak and Schmittgen, 2001).

## 1166 **Proteomics**

1167 Heart extract proteins were extracted and denatured in RIPA buffer. Samples were sonicated using  
1168 a Vibracell 75186 and a miniprobe 2 mm (Amp 80% // Pulse 10 off 0.8, 3 cycles) and further  
1169 centrifuged. Protein assay was performed on supernatant (Pierce 660 nm, according to  
1170 manufacturer instructions) and 100 µg of each extract was delipidated and cleaned using a  
1171 Chloroform / Methanol / Water precipitation method. Briefly, 4 volume of ice-cold methanol were  
1172 added to the sample and vortex, 2 volume of ice-cold chloroform were added and vortex and 3  
1173 volume of ice-cold water was added and vortex. Samples were centrifuged 5 min at 5000 g. The  
1174 upper layer was removed and proteins at the interface were kept. Tubes were filled with ice-cold  
1175 methanol and centrifuged at max speed for 5 min. Resulting protein pellet was air-dried and then  
1176 dissolved in 130 µl of 100mM NaOH before adding 170 µl of Tris 50 mM pH 8.0, tris (2-  
1177 carboxyethyl)phosphine (TCEP) 5 mM and chloroacetamide (CAA) 20 mM. The mixture was  
1178 heated 5 min at 95 °C and then cooled on ice. Endoprotease LysC (1µg) was use for a 8h  
1179 digestion step at (37° C) followed with a trypsin digestion (1 µg) at 37°C for 4 h. Digestion was  
1180 stopped adding 0.1% final of trifluoroacetic acid (TFA). Resulting peptides were desalted using a  
1181 C18 stage tips strategy (Elution at 80% Acetonitrile (ACN) on Empore C18 discs stacked in a P200

1182 tips) and 30 µg of peptides were further fractionated in 4 fractions using  
1183 poly(styrenedivinylbenzene) reverse phase sulfonate (SDB-RPS) stage-tips method as previously  
1184 described(Kulak et al., 2014; Rappsilber et al., 2007). Four serial elutions were applied as  
1185 following: elution 1 (80 mM Ammonium formate (AmF), 20% (v/v) ACN, 0.5% (v/v) formic acid  
1186 (FA)), elution 2 (110 mM AmF, 35% (v/v) ACN, 0.5% (v/v) FA), elution 3 (150 mM AmmF, 50%  
1187 (v/v) ACN, 0.5% (v/v) FA) and elution 4 (80% (v/v) ACN, 5 % (v/v) ammonium hydroxide). All  
1188 fractions were dried and resuspended in 0.1% FA before injection.

### 1189 NanoLC-MS/MS

1190 LC-MS/MS analysis of digested peptides was performed on an Orbitrap Q Exactive Plus mass  
1191 spectrometer (Thermo Fisher Scientific, Bremen) coupled to an EASY-nLC 1200 (Thermo Fisher  
1192 Scientific). A home-made column was used for peptide separation (C<sub>18</sub> 50 cm capillary column  
1193 picotip silica emitter tip (75 µm diameter filled with 1.9 µm Reprosil-Pur Basic C<sub>18</sub>-HD resin, (Dr.  
1194 Maisch GmbH, Ammerbuch-Entringen, Germany)). It was equilibrated and peptide were loaded in  
1195 solvent A (0.1 % FA) at 900 bars. Peptides were separated at 250 nl.min<sup>-1</sup>. Peptides were eluted  
1196 using a gradient of solvent B (ACN, 0.1 % FA) from 3% to 7% in 8 min, 7% to 23% in 95 min, 23%  
1197 to 45% in 45 min (total length of the chromatographic run was 170 min including high ACN level  
1198 step and column regeneration). Mass spectra were acquired in data-dependent acquisition mode  
1199 with the XCalibur 2.2 software (Thermo Fisher Scientific, Bremen) with automatic switching  
1200 between MS and MS/MS scans using a top 12 method. MS spectra were acquired at a resolution  
1201 of 35000 (at *m/z* 400) with a target value of 3 × 10<sup>6</sup> ions. The scan range was limited from 300 to  
1202 1700 *m/z*. Peptide fragmentation was performed using higher-energy collision dissociation (HCD)  
1203 with the energy set at 27 NCE. Intensity threshold for ions selection was set at 1 × 10<sup>6</sup> ions with  
1204 charge exclusion of *z* = 1 and *z* > 7. The MS/MS spectra were acquired at a resolution of 17500 (at  
1205 *m/z* 400). Isolation window was set at 1.6 Th. Dynamic exclusion was employed within 45 s.

### 1206 Data Processing

1207 Data were searched using MaxQuant (version 1.5.3.8) using the Andromeda search  
1208 engine(Tyanova et al., 2016) against a reference proteome of *Mus musculus* (53449 entries,  
1209 downloaded from Uniprot the 24<sup>th</sup> of July 2018). The following search parameters were applied:  
1210 carbamidomethylation of cysteines was set as a fixed modification, oxidation of methionine and  
1211 protein N-terminal acetylation were set as variable modifications. The mass tolerances in MS and

1212 MS/MS were set to 5 ppm and 20 ppm respectively. Maximum peptide charge was set to 7 and 5  
1213 amino acids were required as minimum peptide length. A false discovery rate of 1% was set up for  
1214 both protein and peptide levels. All 4 fractions per sample were gathered and the iBAQ intensity  
1215 was used to estimate the protein abundance within a sample (Schwanhäusser et al., 2011). The  
1216 match between runs features was allowed for biological replicate only.

1217 For this large-scale proteome analysis part, the mass spectrometry data have been deposited at  
1218 the ProteomeXchange Consortium (<http://www.proteomexchange.org>) via the PRIDE partner  
1219 repository (Perez-Riverol et al., 2019; Vizcaíno et al., 2014) with the dataset identifier PXD028516

### 1221 **mtDNA content quantification**

1222 Genomic DNA was extracted using the NucleoSpin Tissue (MACHEREY-NAGEL) and quantified  
1223 with NanoQuant Plate™ (Infinite M200, TECAN). RT-qPCR was performed using the Real-Time  
1224 PCR Detection System (Applied Biosystems StepOnePlus), 20 ng of total DNA and the SYBR®  
1225 Green Master Mix (Bio-Rad). *b-Actin* was amplified as an internal, nuclear gene standard as  
1226 previously described (Cretin et al., 2021). PCR primer sequences are listed in Dataset EV4. Data  
1227 were analyzed according to the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001).

### 1229 **Mitochondrial imaging and quantification in MEFs**

1230 MEFs cells were seeded on 96-well or 384-well CellCarrier Ultra imaging plates (Perkin Elmer) 24h  
1231 before imaging. Nuclei were stained with NucBlue™ Live ReadyProbes™ Reagent (ThermoFisher  
1232 Scientific) at 1 drop per 10 mL of media. Fluorescent labeling of mitochondria was achieved using  
1233 MitoTracker DeepRed (MTDR) at 100 nM for 30 min at 37°C, 5% CO<sub>2</sub>. Cells were then washed  
1234 with regular medium and images were acquired using the Operetta CLS or Opera Phenix High-  
1235 Content Analysis systems (Perkin Elmer), with 20x Water/1.0 NA, 40x Air/0.6 NA, 40x Water/1.1  
1236 NA or 63x Water/1.15 NA. MTDR (615-645 nm) and NucBlue (355-385 nm) were excited the  
1237 appropriate LEDs (Operetta CLS) or lasers (Opera Phenix). Automatic single-cell classification of  
1238 non-training samples (i.e. unknowns) was carried out by the supervised machine-learning (ML)  
1239 module using Harmony Analysis Software (PerkinElmer) as previously described (Cretin et al.,  
1240 2021).

1242

## Analysis of oxygen consumption

1243

Oxygen consumption of intact cells or crude mitochondria was measured with the XFe96 Analyzer

1244

(Seahorse Biosciences) and High Resolution Respirometry (O2k-Fluorespirometer, Oroboros, AT),

1245

respectively. For Seahorse experiments, cells (experimentally optimized density of 20,000

1246

cells/well for MEFs and U2OS cells) were seeded onto 96-well XFe96 cell culture plates. On the

1247

following day, cells were washed and incubated with Seahorse XF Base Medium completed with 1

1248

mM Pyruvate, 2 mM Glutamine and 10 mM Glucose. Cells were washed with the Seahorse XF

1249

Base Medium and incubated for 45 min in a 37°C non-CO<sub>2</sub> incubator before starting the assay.

1250

Following basal respiration, cells were treated sequentially with: oligomycin 1 μM, CCCP 2 μM and

1251

antimycin A plus rotenone (1 μM each) (Sigma). Measurements were taken over 2-min intervals,

1252

preceded by a 1-min mixing and a 30s incubation. Three measurements were taken for the

1253

resting OCR, three for the non-phosphorylating OCR, three for the maximal OCR and three for the

1254

extramitochondrial OCR. After measurement, the XFe96 plate was washed with Phosphate-

1255

Buffered Saline (PBS) and protein was extracted with RIPA for 10 min at RT. Protein quantity in

1256

each well was then quantified by Bicinchoninic acid assay (BCA) by measuring absorbance at 562

1257

nm and used to normalize OCR measurements as previously described (Cretin et al., 2021).

1258

For O2k respirometry, isolated cardiac mitochondria were freshly isolated from adult WT and

1259

cMKO mice (8, 18 or 34 weeks) as described above. Simultaneous measurement of mitochondrial

1260

respiration and membrane potential ( $\Delta\psi$ ) was assessed by O2K-Fluorometry using a O2K-

1261

Fluorescence LED2-Module operated through the amperometric channel of the O2K. Briefly, 25 or

1262

50 μg of cardiac mitochondria were resuspended in Mir05 buffer [MgCl<sub>2</sub>-6H<sub>2</sub>O 3 mM, Lactobionic

1263

Acid 60 mM, Taurine 20 mM, KH<sub>2</sub>PO<sub>4</sub> 10 mM, Hepes-KOH 20 mM, Sucrose 110 mM, EGTA-KOH

1264

0.5 mM, BSA (1g/L)] in presence of Rhodamine 123 (RH-123) (0.66 μM). Maximal mitochondrial

1265

respiration capacity (OXPHOS) was determined under consecutive administrations of PGM

1266

[pyruvate 10 mM, glutamate 5 mM, malate 5 mM, state 2] or malate (2 mM) and palmitoyl-carnitine

1267

(10 μM) in presence of ADP (1 mM, state 3) to assess complex I-driven respiration; rotenone (0.5

1268

μM) and succinate (10 mM, state 2) for the measurement of complex II-driven respiration;

1269

antimycin A (2.5 μM), ascorbate (2 mM), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD, 0.5

1270

mM) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 2 μM) for the determination of the

1271

complex IV-driven respiration. The addition of oligomycin (Omy, 25 nM) or carboxyatractyloside

1272 (CATR, 1  $\mu$ M) was used to determine the non-phosphorylating respiration (state 4). Cytochrome c  
1273 (2  $\mu$ M) – mediated O<sub>2</sub> flux was used to evaluate the integrity of the OMM following the  
1274 mitochondria isolation. Respiratory control ratios (RCR) of state 3: state 4 were used to evaluate  
1275 the integrity of the IMM. RH-123 fluorescence quenching ( $\Delta$  fluorescence) in energized  
1276 mitochondria was used as a direct measurement of  $\Delta\psi$ .

## 1277

### 1278 **Echocardiography**

1279 Transthoracic echocardiographic acquisitions were performed by using a Vevo 3100 Imaging  
1280 System coupled to a 25-55 MHz linear-frequency transducer (MX550D, FUJIFILM VisualSonics).  
1281 Randomized WT and cMKO mice [10 to 34 weeks of age (male), 34 weeks (female) were  
1282 anesthetized and maintained with 2% isoflurane in oxygen, placed in a supine position on a 37°C  
1283 warmed pad. Limb electrodes and a rectal probe were used to monitor define (ECG) and body  
1284 temperature. Before echocardiography and the addition of the pre-warmed ultrasound gel, thorax  
1285 fur was removed using hair-removal cream. To assess the overall left ventricle (LV) size and LV  
1286 function, B- and M-Mode images were acquired in parasternal long axis view (PLAX) between 400-  
1287 500 bpm. The systolic and diastolic LV dimensions [interventricular septum thickness (IVS; mm),  
1288 LV diameter (LVD; mm), LV posterior wall thickness (LVPW; mm)] and cardiac output [ejection  
1289 fraction (% EF)] were determined by acquiring and analyzing at least 3 independent cardiac cycles  
1290 within at least 3 M-Mode images.

### 1291

### 1292 **Doxorubicin Treatment**

1293 WT and cMKO mice aged 8 weeks received a cumulative dose of 20 mg/kg of doxorubicin (Doxo,  
1294 2 intra-peritoneal injections of 10 mg/kg/saline, given at 3-day intervals; control mice received  
1295 saline injections). Transthoracic echocardiography was performed at day 14 post treatment. Body  
1296 weight was recorded every 3 days and a loss higher than 20% was considered as endpoint of the  
1297 study.

### 1298

### 1299 **Determination of serum levels of cardiac troponin I and cardiac MLC1**

1300 A mouse ELISA kit was used to compare serum levels of cardiac troponin I (cTnI, Life Diagnostics)  
1301 and cardiac myosin light chain 1 (MLC1, Life Diagnostic) in WT and cMKO male mice aged 18 and

1302 34 weeks. Blood was collected via submandibular vein puncture from non-anesthetized mice, left  
1303 for 30 min at RT and then centrifuged at 5,000 g at 4°C for 10 min then snap-frozen into liquid  
1304 nitrogen. Serum was stored at -80°C until next use. The assays to determine cTnI and MLC1  
1305 levels were performed following the exact manufacturer's instructions.

## 1307 **Histology**

1308 WT and cMKO mice aged 34 weeks were sacrificed by cervical dislocation and the hearts excised  
1309 post-mortem. The whole hearts were then fixed with Formol 4 % (VWR chemicals) overnight and  
1310 then fully dehydrated by a series of ethanol gradients. Tissues were then paraffin-embedded and  
1311 sectioned in a short view at a thickness of 4 µm on a microtome. The sections were de-paraffinized  
1312 in xylene and rehydrated followed by hematoxylin and eosin (H&E), Masson's trichrome or  
1313 Picrosirius Red staining according to the standard protocols.

## 1315 **Transmission Electron Microscopy**

1316 Transmission electron microscopy was performed on cardiac tissue from WT and cMKO mice  
1317 aged 8-10 weeks. Small pieces (1x1x1 mm) from the left ventricle posterior wall were fixed in a  
1318 37°C prewarmed mix of PHEM 1x buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM  
1319 MgCl<sub>2</sub>; pH 7.3), 2.5% glutaraldehyde and 2% PFA for 30 min, followed by an overnight fixation at  
1320 4°C. Specimens were then rinsed 3 times with PHEM 2X buffer. Samples were incubated with 1 %  
1321 osmium tetroxide (Merck) and 1.5 % ferrocyanide (Sigma Aldrich) in 0.1M PHEM. After  
1322 dehydration by a graded series of ethanol, samples were gradually infiltrated at RT with epoxy  
1323 resin and after heat polymerization, sections with a thickness of 70 nm were cut with a Leica UCT  
1324 microtome and collected on carbon, formvar coated copper grids. Sections were contrasted with  
1325 4% aqueous uranylacetate and Reynold's lead citrate. Generation of ultra-large electron  
1326 microscopy montages at a magnification of 14500x (pixel size = 6.194 nm, bin 1, US4000  
1327 Ultrascan camera) were acquired using a TECNAI F20 Transmission Electron Microscope (FEI)  
1328 with a field emission gun (FEG) as an electron source, operated at 200kV. The SerialEM  
1329 software(Mastrorade and Held, 2017; Schorb et al., 2019) was used for multi-scale mapping as  
1330 previously described(Cretin et al., 2021). The 'Align Serial Sections/Blend Montages' interface of  
1331 IMOD16 was used for blending the stacks of micrographs collected to single large images.

1332 Quantification of mitochondrial cross-sectional area was determined by tracing the perimeter of  
1333 individual mitochondria with the use of 3dmod software.

### 1334 **Primary adult cardiomyocytes isolation**

1335 Primary adult ventricular cardiomyocytes (CMs) were isolated from WT (*Myh6-Cre*<sup>+/+</sup>, *Mtfp1*<sup>LoxP/LoxP</sup>)  
1336 and *Mtfp1* KO mice (cMKO, *Myh6-Cre*<sup>Tg/+</sup>, *Mtfp1*<sup>LoxP/LoxP</sup>) mice aged 8-10 weeks to perform live-  
1337 cell imaging experiments by using the simplified Langendorff-free method as previously published  
1338 (Ackers-Johnson et al., 2016) with some modifications. Briefly, mice were weighed, injected  
1339 intraperitoneally with ketamine/xylazine (ketamine: 3 x 80 mg/kg/bw; xylazine 3 x 10 mg/kg/bw).  
1340 Once the thorax was opened, the descending aorta was cut and the heart exposed and flushed  
1341 into the right ventricle with EDTA buffer pH 7.8 (NaCl 130 mM, KCl 5 mM, NaH<sub>2</sub>PO<sub>4</sub> 0.5 mM,  
1342 HEPES 10 mM, Glucose 10 mM, 2,3-butanedione monoxime (BDM) 10 mM, Taurine 10 mM,  
1343 EDTA 5 mM). The heart was then excised, transferred into a dish containing EDTA buffer, where  
1344 the ascending aorta was clamped. Tissue digestion was performed by sequential injection of EDTA  
1345 buffer, perfusion buffer (NaCl 130 mM, KCl 5 mM, NaH<sub>2</sub>PO<sub>4</sub> 0.5 mM, HEPES 10 mM, Glucose 10  
1346 mM, BDM 10 mM, Taurine 10 mM, MgCl<sub>2</sub> 1 mM, pH 7.8) and collagenase buffer (Collagenase II  
1347 0.5 mg/mL, Collagenase 4 0.5 mg/mL, Protease XIV 0.05 mg/mL) into the left ventricle (LV)  
1348 through the apex. To control the flow of the digestion buffer the LV was perfused via a flexible  
1349 linker using an automated infusion pump (Graseby 3200). After digestion, ventricles were then  
1350 separated from the atria and ultimately dissociated by gentle pipetting. The digestion process was  
1351 then inhibited by the addition of the stop buffer (perfusion buffer containing FBS 10%). Cellular  
1352 suspension was filtered using a 100 µm pore size strainer to remove undigested debris. CMs were  
1353 then collected by gravity settling (15 min) while resuspended sequentially in 3 calcium  
1354 reintroduction buffers to gradually restore calcium levels (0.34 mM, 0.68 mM, and 1.02 mM Ca<sup>2+</sup>).  
1355 CMs yields were quantified using a hemocytometer. CMs were resuspended in prewarmed culture  
1356 media (M199 Medium, BSA 0.1%, Insulin-Transferrin-Selenium (ITS) 1x, BDM 10 mmol/l, CD lipid  
1357 1x, P/S 1x), and plated into laminin (10 µg/mL) precoated Cell Carrier Ultra-96 well (Perkin Elmer)  
1358 plates, in a humidified tissue culture incubator (37°C, 5% CO<sub>2</sub>) for at least 1 hour before  
1359 proceeding to live imaging.



1362

## Cell death assay

1363

CMs isolated from WT and cMKO mice (8-10 weeks) labelled with NucBlue™ Live ReadyProbes™

1364

Reagent (ThermoFisher Scientific) and Tetramethylrhodamine Ethyl Ester Perchlorate (TMRE; 50

1365

nM) for 20 min at 37°C, 5% CO<sub>2</sub>. Supervised machine learning (Harmony 4.9, Perkin Elmer) using

1366

TMRE intensity, cell geometry (rod- shape, area, length) and nuclei number as read-outs enabled

1367

the on-the-fly detection, imaging, and quantification of healthy CMs from dead and dying CMs and

1368

non-myocytes. Live-cell imaging was performed in the presence or absence of a cell death stimuli:

1369

H<sub>2</sub>O<sub>2</sub> (25 μM), or doxorubicin (Doxo, 60 μM,) or carbonyl cyanide m-chlorophenyl hydrazine

1370

(CCCP, 10 μM). Image acquisition was performed using the Operetta CLS High-Content Analysis

1371

system (Perkin Elmer) with a 5x air objective.

1372

WT, *Mtfp1*<sup>-/-</sup>, *Ppif*<sup>-/-</sup> and *Mtfp1*<sup>-/-</sup>*Ppif*<sup>-/-</sup> MEFs were plated in Cell Carrier Ultra-96 well (Perkin Elmer)

1373

and incubated 24h with regular media. Cells were then stained with NucBlue™ Live

1374

ReadyProbes™ to visualize nuclei. CellEvent Caspase 3/7 Green (CE) was added (1 drop per 1

1375

mL of media) to visualize the activation of caspases 3/7 in cells undergoing to apoptosis.

1376

Propidium Iodide (PI, 1:500) was added to visualize dead cells. Cell death was induced with

1377

actinomycin D (1.5 μM) alone, or in association with ABT-737 (10 μM), an inhibitor of the Bcl-2

1378

family proteins, or doxorubicin hydrochloride (DOXO, 1.5 μM), or staurosporine (1 μM) or hydrogen

1379

peroxide H<sub>2</sub>O<sub>2</sub> (500 μM). The pan-caspase inhibitor Q-VD-OPh (qVD, 20 μM) and cyclophilin D

1380

inhibitor cyclosporin A (CsA, 2 μM), were added where indicated. Image acquisition was performed

1381

using the Operetta CLS High-Content Analysis system (Perkin Elmer) with a 20x air objective. CE

1382

or PI positive nuclei number were automatically counted by using Harmony 4.9 software.

1383

1384

## Field-stimulated cardiomyocytes

1385

Adult ventricular cardiomyocytes (CMs) from WT and cMKO mice (8-10 weeks) were isolated with

1386

a Langendorff system as previously described (Chen et al., 2012; Nickel et al., 2015) and paced by

1387

electrical field stimulation at 37°C using a customized IonOptix system. CMs were exposed to a

1388

protocol that simulates a physiological workload increase by first pacing cells at 0.5 Hz in Normal

1389

Tyrode's solution pH 7.4 [NT; NaCl 130 mM, KCl 5 mM, MgCl<sub>2</sub> 1 mM, CaCl<sub>2</sub> 1 mM, Na-HEPES 10

1390

mM, glucose 10 mM, sodium pyruvate 2 mM and ascorbic acid 0.3 mM]. The β-adrenergic receptor

1391

agonist isoproterenol (30 nM) was then washed in for 1 min and then the stimulation rate was

1392 increased to 5 Hz for 3 min. After this time, stimulation rate was set back to the initial 0.5 Hz, and  
1393 isoproterenol washed out. During the measurements of sarcomere length and contraction, the  
1394 system detection was combined to recordings of the autofluorescence of NAD(P)H/NAD(P)<sup>+</sup> and  
1395 FADH<sub>2</sub>/FAD by alternately exciting cells at wavelengths ( $\lambda_{exc}$ ) of 340 and 485 nm and collecting  
1396 emission ( $\lambda_{em}$ ) at 450 and 525 nm for NAD(P)H and FAD<sup>+</sup>, respectively. Calibration was performed  
1397 inducing maximal oxidation and reduction of NAD(P)H/FADH<sub>2</sub> with FCCP (5  $\mu$ mol/L) and cyanide  
1398 (4 mmol/L), respectively.

#### 1400 **Intracellular Calcium measurement**

1401 LV cardiac myocytes were isolated by enzymatic digestion and were paced by electrical field  
1402 stimulation at 37°C using a customized IonOptix system as described previously (Kohlhaas and  
1403 Maack, 2010; Kohlhaas et al., 2010). [Ca<sup>2+</sup>]<sub>c</sub> was measured by incubating cells with indo-1 AM (5  
1404  $\mu$ mol/L) for 20 min at 25°C ( $\lambda_{exc}$ =340 nm,  $\lambda_{em}$ =405/485 nm). The bath solution contains (mM):  
1405 130 NaCl; 5 KCl; 1 MgCl<sub>2</sub>; 10 sodium HEPES; 2 sodium pyruvate; 0.3 ascorbic acid; 10 glucose  
1406 and was adjusted at pH 7.4 at 37°C; Isoproterenol was used at 3x10<sup>-8</sup> M. The Ca<sup>2+</sup> transients were  
1407 analysed with the IONWIZRD software from IonOptix. Data were collected and statistically  
1408 analysed using paired or unpaired tTest or 2way ANOVA & Bonferroni's Multiple Comparison Test.

#### 1410 **Mitochondrial swelling and mPTP opening**

1411 Calcium-induced mitochondrial permeability transition pore (mPTP) opening was determined in  
1412 freshly isolated cardiac mitochondria from WT and cMKO mice aged 8-10 weeks as previously  
1413 described (Palmeira CM, 2018). Mitochondria were suspended in Ca<sup>2+</sup> uptake buffer pH 7.4 (120  
1414 mM KCl, 5 mM MOPS, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM Glutamate, 5 mM Malate *plus* cComplete™, EDTA-  
1415 free Protease Inhibitor Cocktail (Roche) at a concentration of 0.5 mg/mL and stimulated by the  
1416 addition of a single pulse of 120  $\mu$ M CaCl<sub>2</sub>.

1417 mPTP opening was assessed in mitochondria of WT, *Mtfp1*<sup>-/-</sup>, *Ppif*<sup>-/-</sup> and *Mtfp1*<sup>-/-</sup>*Ppif*<sup>-/-</sup> MEFs as  
1418 previously described (Karch et al., 2013) at a concentration of 1 mg/mL in mitochondrial swelling  
1419 buffer (120 mM KCl, 10 mM Tris pH 7.4, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 7 mM pyruvate, 1 mM malate, and 10  $\mu$ M  
1420 EDTA). Swelling was induced by pulsing mitochondria with 250  $\mu$ M CaCl<sub>2</sub> in EDTA-free buffer.

1421 The absorbance (540 nm) was measured at intervals of ~20 seconds at 37°C using the microplate  
1422 reader Infinite® M Plex (Tecan). Cyclosporin A (CsA, 1 µM Sigma) was used as a control to inhibit  
1423 mPTP-dependent mitochondrial swelling.

1424

#### 1425 **BN-PAGE of cardiac mitochondria**

1426 BN-PAGE was performed as described previously (Wittig et al., 2006) with some modifications.  
1427 Briefly, cardiac mitochondria were isolated from WT and cMKO mice at 8-10 weeks and solubilized  
1428 with sample buffer (30 mM HEPES, 150 mM Potassium Acetate, 12% glycerol, 2 mM acid 6-  
1429 aminohexanoic, 1 mM disodium EDTA) containing digitonin 6% (w/w). Lysates were incubated for  
1430 1h at 4°C and then centrifuged at 30,000 g for 20 minutes. Supernatants were then mixed with  
1431 G250 sample additive 1% (Invitrogen, BN2004) and resolved on 3-12% Bis-Tris Gels (1.0 mm)  
1432 (Invitrogen, BN2011BX10) using the anode running buffer (Invitrogen, BN2001) and Cathode  
1433 Buffer Additive added to the anode buffer (Invitrogen, BN2002). Gels were then subjected to a  
1434 semi-dry transfer using PVDF membranes (GE, 10600023). Membranes were washed with  
1435 methanol and then blocked for at least 1h with 5% (w/v) semi-skimmed dry milk dissolved in Tris-  
1436 buffered saline Tween 0.1% (TBST), incubated overnight at 4°C with primary antibodies dissolved  
1437 1:1.000 in 2% (w/v) Bovine Serum Albumin (BSA), 0.1% TBST. The next day membranes were  
1438 incubated in secondary antibodies conjugated to horseradish peroxidase (HRP) at room  
1439 temperature for 2h (diluted 1:10,000 in BSA 2% TBST 0.1%). Finally, membranes were incubated  
1440 in Clarity™ Western ECL Substrate (Bio-Rad) for 2 min and luminescence was detected using the  
1441 ChemiDoc® Gel Imaging System. Primary antibodies are listed in Dataset EV4.

1442

#### 1443 **Co-Immunoprecipitation assay**

1444 500 µg of cardiac crude mitochondria were freshly isolated from heart tissue of cardiomyocyte  
1445 specific Flag-MTFP1 Knock-In (KI) mice (*Myh6-Cre<sup>tg/+</sup>Mtfp1<sup>+/+</sup>, CAG<sup>tg/+</sup>*) and WT mice (*Myh6-  
1446 Cre<sup>+/+</sup>Mtfp1<sup>+/+</sup>, CAG<sup>tg/+</sup>*) as described above. Mitochondria were lysed in IP buffer (20 mM HEPES-  
1447 KOH pH 7.5, 150 mM NaCl, 0.25% Triton X-100, protease inhibitor cocktail) on ice for 20 min and  
1448 then centrifugated at 10000 g, 4°C for 15 min. Supernatant obtained by centrifugation was then  
1449 incubated with 20 µL of anti-FLAG magnetic beads (Sigma M8823) for 2 hours at 4°C. The  
1450 immunocomplexes were then washed with IP buffer without Triton X-100 and eluted with Laemmli

1451 Sample Buffer 2x at 95°C for 5 min. Protein were stacked in a 15 % SDS-PAGE gel with a 10 min  
1452 long migration at 80 V. Proteins were fixed in gel and migration was visualized using the Instant  
1453 Blue stain (Expedeon). Bands were excised for digestion. Gel bands were washed twice in  
1454 Ammonium bicarbonate (AmBi) 50 mM, once with AmBi 50 mM / ACN 50 % and once with 100%  
1455 ANC. Gel band were incubated for 30 min at 56°C in 5 mM dithiothreitol (DTT) solution for  
1456 reduction. Gel bands were washed in AmBi 50 mM and then in 100% ACN. Alkylation was  
1457 performed at room temp in the dark by incubation of the gel bands in Iodocateamide 55 mM  
1458 solution. Gel bands were washed twice in AmBi 50mM and in 100% ACN. 600 ng of trypsin were  
1459 added for a 8h digestion at 37°C. Peptides were extracted by collecting 3 washes of the gel bands  
1460 using AmBi 50 mM / 50 % ACN and 5 % FA. Peptides clean up and desalting was done using  
1461 Stage tips (2 disc Empore C18 discs stacked in a P200 tip).  
1462 LC-MS/SM analysis of digested peptides was performed on an Orbitrap Q Exactive HF mass  
1463 spectrometer (Thermo Fisher Scientific, Bremen) coupled to an EASY-nLC 1200 (Thermo Fisher  
1464 Scientific). A home-made column was used for peptide separation (C<sub>18</sub> 30 cm capillary column  
1465 picotip silica emitter tip (75 µm diameter filled with 1.9 µm Reprosil-Pur Basic C<sub>18</sub>-HD resin, (Dr.  
1466 Maisch GmbH, Ammerbuch-Entringen, Germany)). It was equilibrated and peptide were loaded in  
1467 solvent A (0.1 % FA) at 900 bars. Peptides were separated at 250 nl.min<sup>-1</sup>. Peptides were eluted  
1468 using a gradient of solvent B (ACN, 0.1 % FA) from 3% to 26% in 105 min, 26% to 48% in 20 min  
1469 (total length of the chromatographic run was 145 min including high ACN level step and column  
1470 regeneration). Mass spectra were acquired in data-dependent acquisition mode with the XCalibur  
1471 2.2 software (Thermo Fisher Scientific, Bremen) with automatic switching between MS and MS/MS  
1472 scans using a top 12 method. MS spectra were acquired at a resolution of 60000 (at *m/z* 400) with  
1473 a target value of  $3 \times 10^6$  ions. The scan range was limited from 400 to 1700 *m/z*. Peptide  
1474 fragmentation was performed using HCD with the energy set at 26 NCE. Intensity threshold for  
1475 ions selection was set at  $1 \times 10^5$  ions with charge exclusion of  $z = 1$  and  $z > 7$ . The MS/MS spectra  
1476 were acquired at a resolution of 15000 (at *m/z* 400). Isolation window was set at 1.6 Th. Dynamic  
1477 exclusion was employed within 30 s.

#### 1478 Data Processing

1479 Data were searched using MaxQuant (version 1.6.6.0) [1,2] using the Andromeda search engine  
1480 [3] against a reference proteome of *Mus musculus* (53449 entries, downloaded from Uniprot the

1481 24<sup>th</sup> of July 2018). A modified sequence of the protein MTP18 with a Flag tag in its N-ter part was  
1482 also searched.

1483 The following search parameters were applied: carbamidomethylation of cysteines was set as a  
1484 fixed modification, oxidation of methionine and protein N-terminal acetylation were set as variable  
1485 modifications. The mass tolerances in MS and MS/MS were set to 5 ppm and 20 ppm respectively.  
1486 Maximum peptide charge was set to 7 and 5 amino acids were required as minimum peptide  
1487 length. A false discovery rate of 1% was set up for both protein and peptide levels. The iBAQ  
1488 intensity was used to estimate the protein abundance within a sample. The match between runs  
1489 features was allowed for biological replicate only.

1490 For this Affinity Purification Mass Spectrometry analysis part, the mass spectrometry data have  
1491 been deposited at the ProteomeXchange Consortium (<http://www.proteomexchange.org>) via the  
1492 PRIDE partner repository(Perez-Riverol et al., 2019; Vizcaino et al., 2014) with the dataset  
1493 identifier PXD028529

#### 1494 Data analysis

1495 Quantitative analysis was based on pairwise comparison of intensities. Values were log-  
1496 transformed (log<sub>2</sub>). Reverse hits and potential contaminant were removed from the analysis.  
1497 Proteins with at least 2 peptides (including one unique peptide) were kept for further statistics.  
1498 Intensities values were normalized by median centering within conditions (normalizedD function of  
1499 the R package DAPAR). Remaining proteins without any iBAQ value in one of both conditions  
1500 have been considered as proteins quantitatively present in a condition and absent in the other.  
1501 They have therefore been set aside and considered as differentially abundant proteins. Next,  
1502 missing values were imputed using the impute.MLE function of the R package [imp4p](#). Statistical  
1503 testing was conducted using a limma t-test thanks to the R package limma(Pounds and Cheng,  
1504 2006). An adaptive Benjamini-Hochberg procedure was applied on the resulting p-values thanks to  
1505 the function adjust.p of R package cp4p(Smyth, 2004) using the robust method previously  
1506 described(Giai Gianetto et al., 2016) to estimate the proportion of true null hypotheses among the  
1507 set of statistical tests. The proteins associated to an adjusted p-value inferior to a FDR level of 1%  
1508 have been considered as significantly differentially abundant proteins.

#### 1509 **Statistical Analyses**

1510 Experiments were repeated at least three times and quantitative analyses were conducted blindly.  
1511 Randomization of groups (e.g., different genotypes) was performed when simultaneous, parallel  
1512 measurements were not performed (e.g., Oroboros, CM isolation). For high-throughput  
1513 measurements (e.g., mitochondrial morphology, cell death), all groups were measured in parallel  
1514 to reduce experimental bias. Statistical analyses were performed using GraphPad Prism 9  
1515 software. Data are presented as mean  $\pm$  SD or SEM where indicated. The statistical tests used,  
1516 and value of experiment replicates are described in the figure legends. Comparisons between two  
1517 groups were performed by unpaired two-tailed T test. To compare more than two groups or groups  
1518 at multiple time points 1-way ANOVA or 2-way ANOVA was applied. Tests were considered  
1519 significant at p-value  $< 0.05$  (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

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1536

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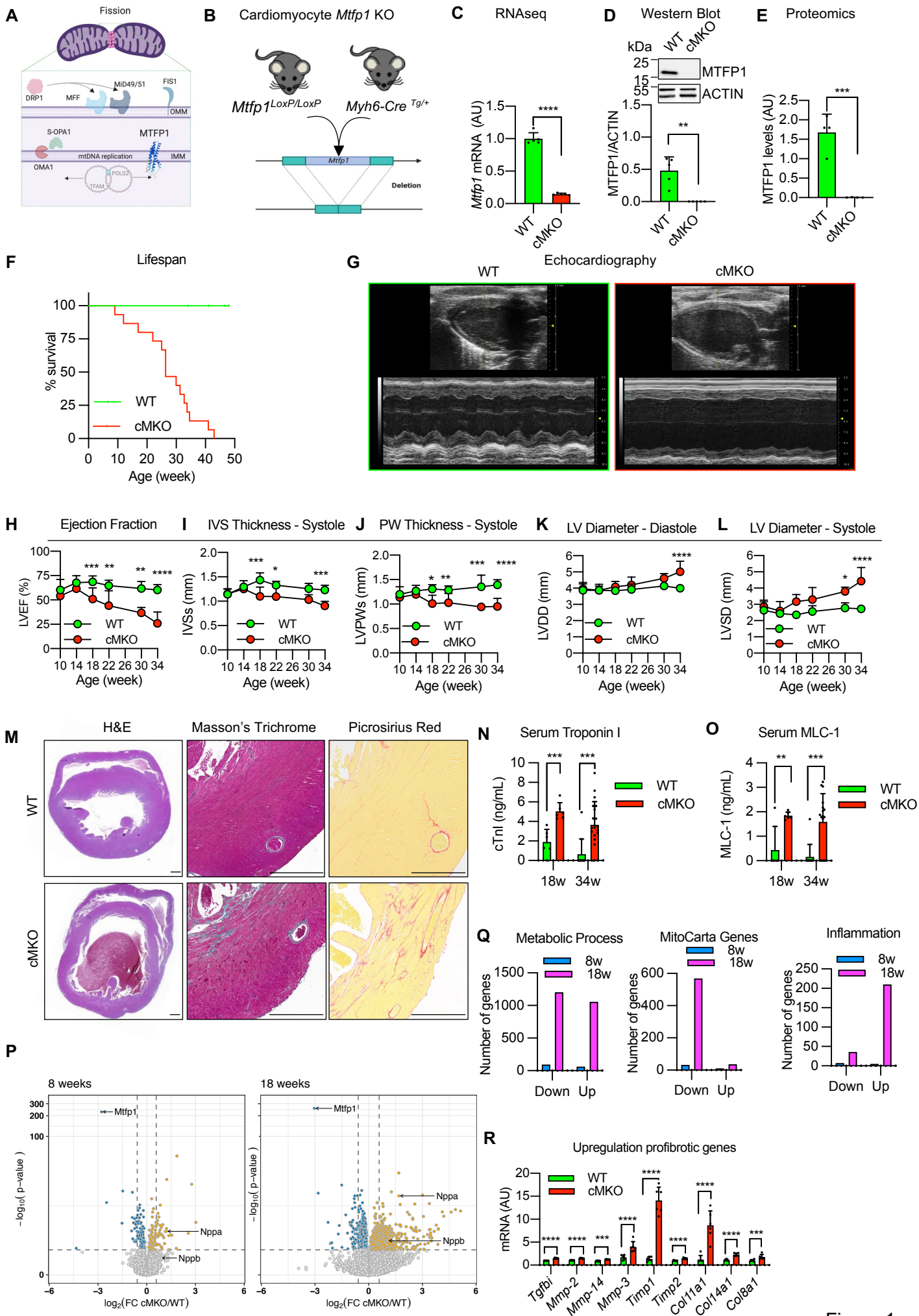


Figure 1

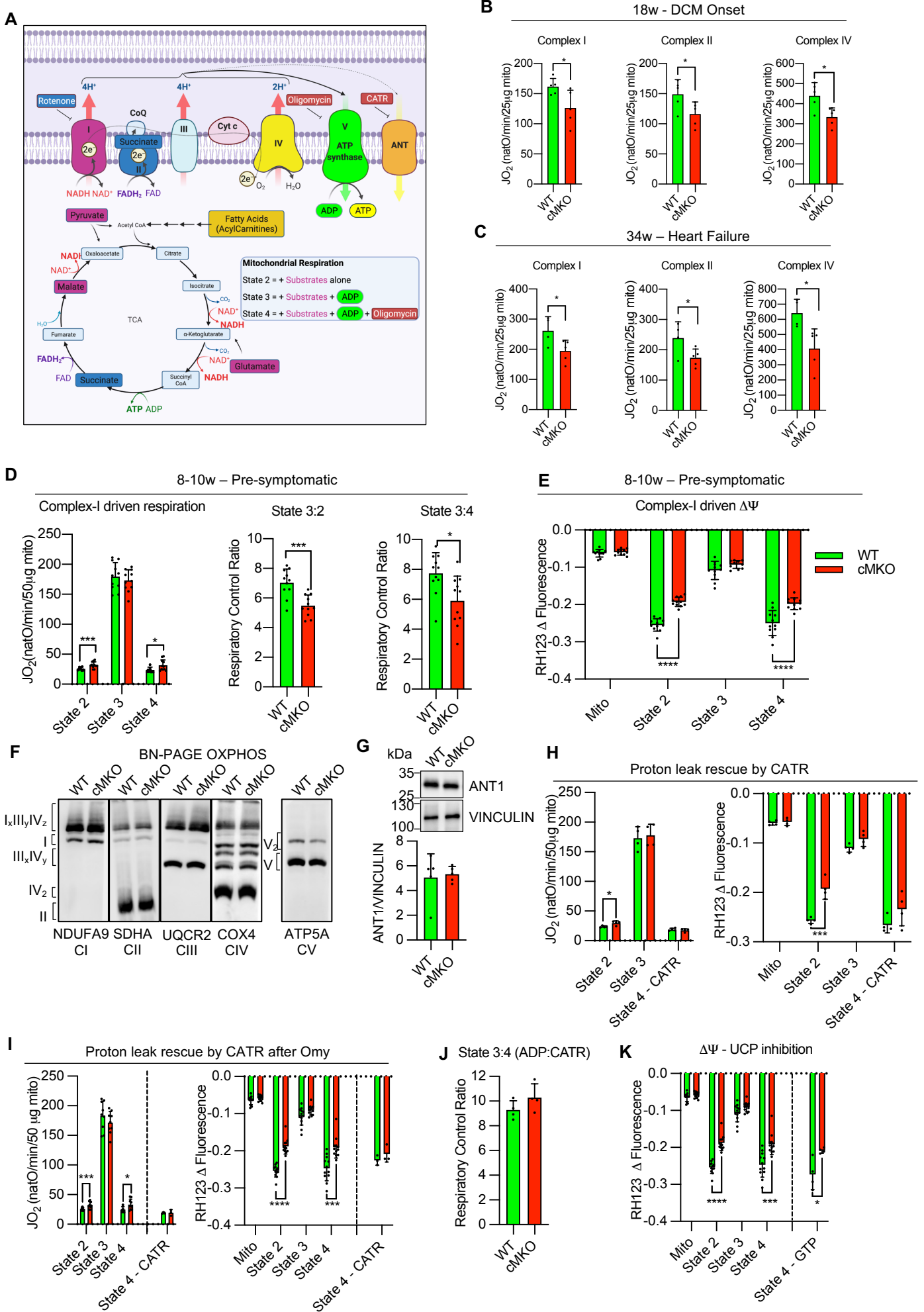


Figure 2

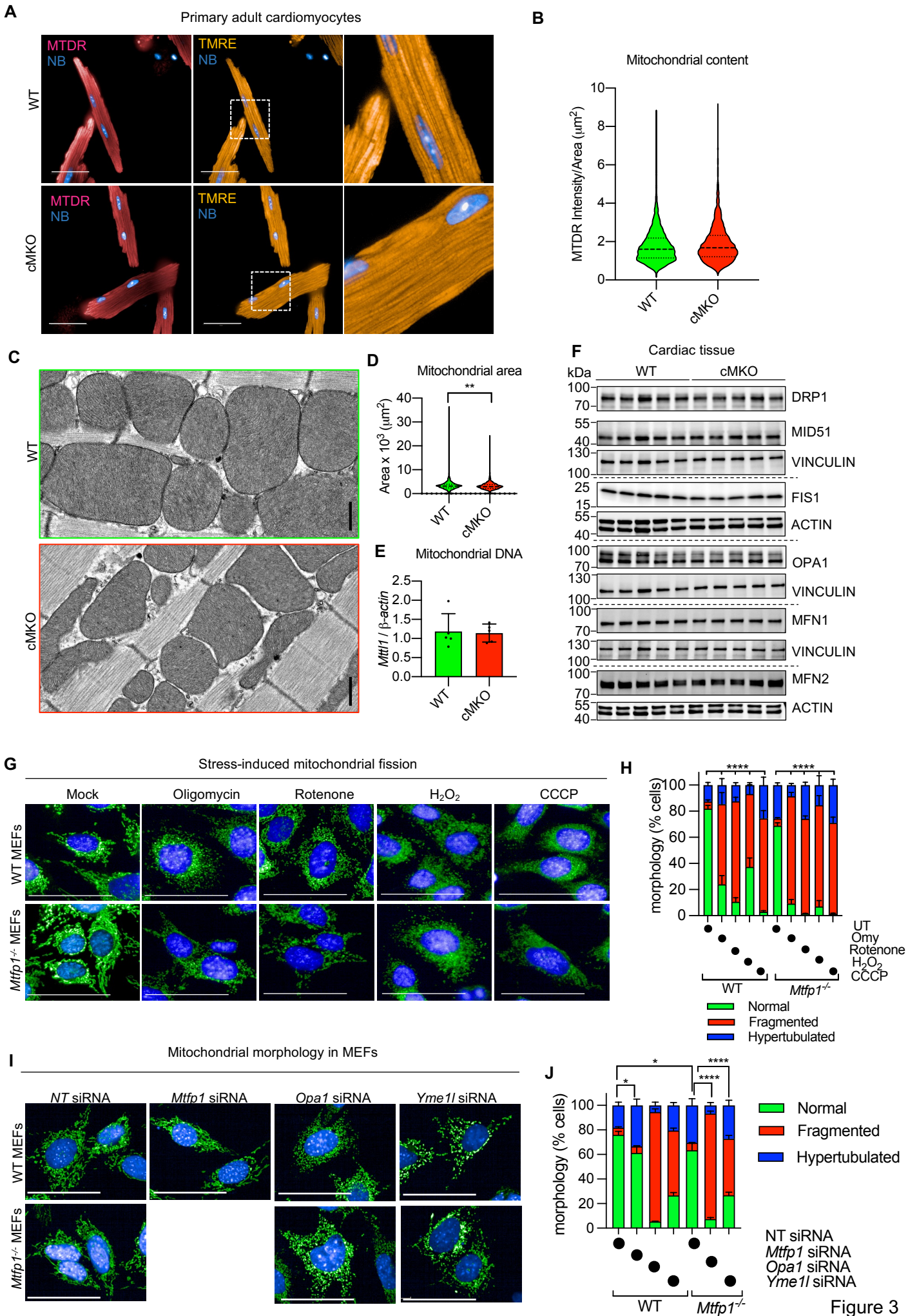


Figure 3



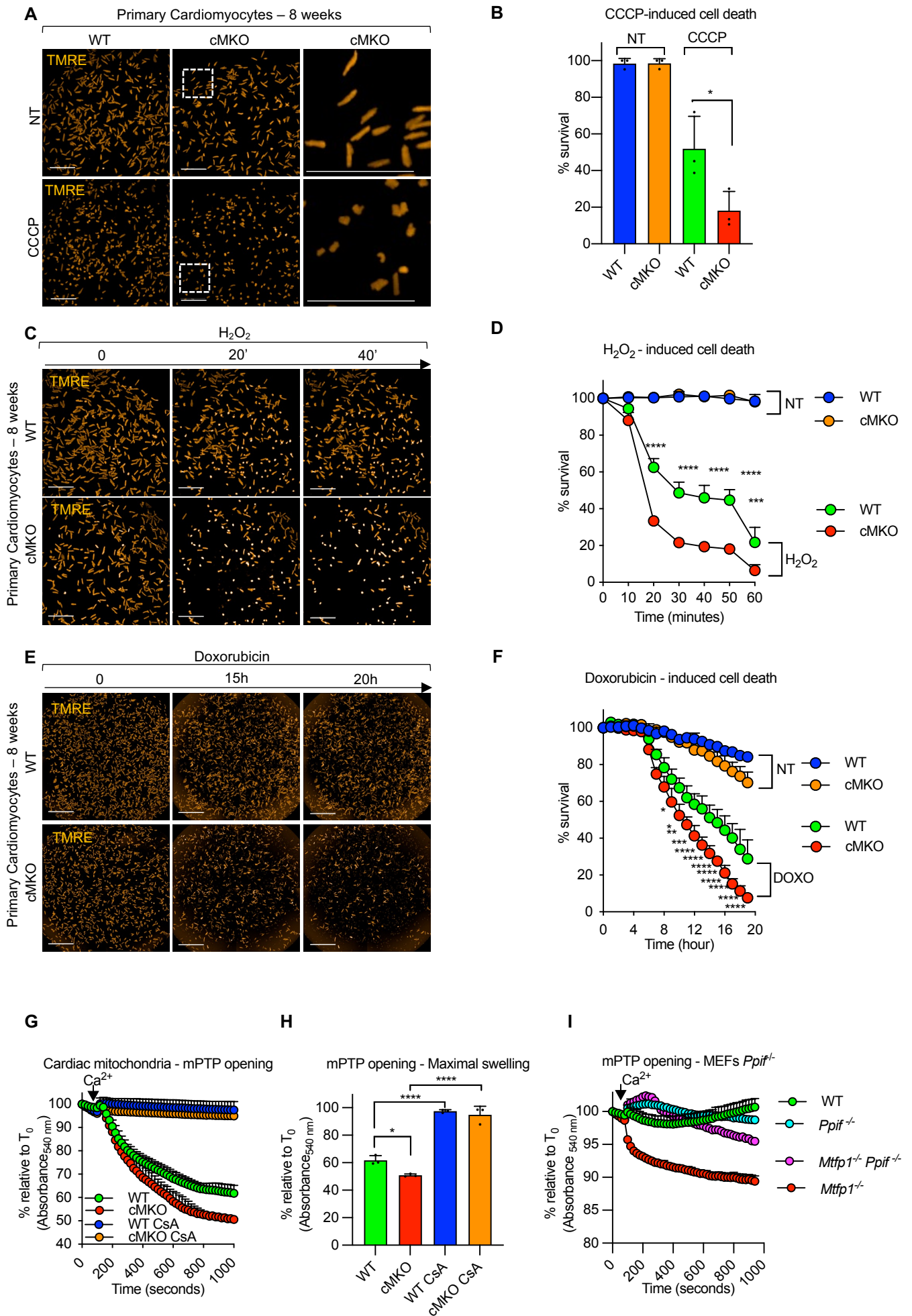


Figure 4

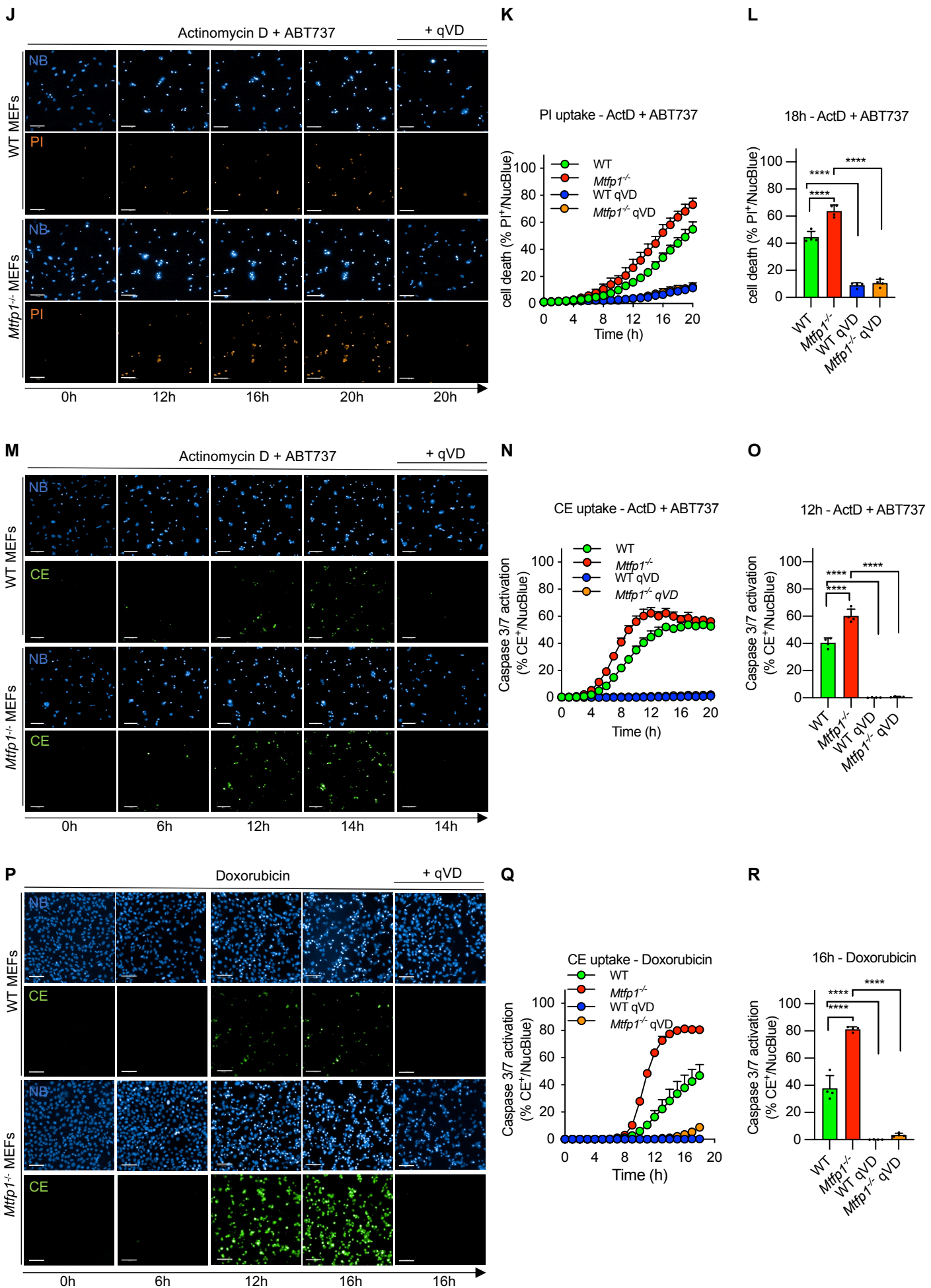


Figure 4 cont

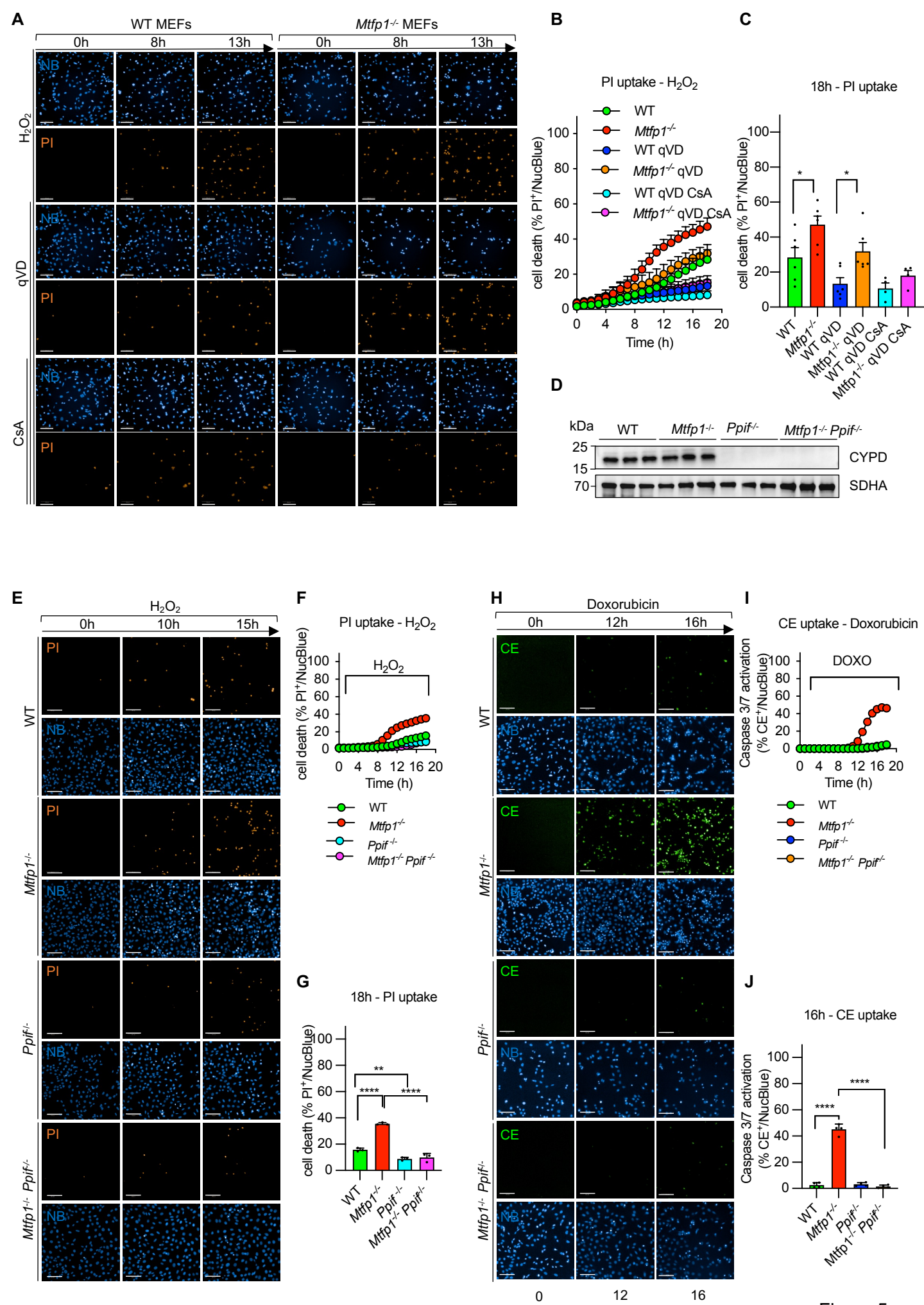


Figure 5

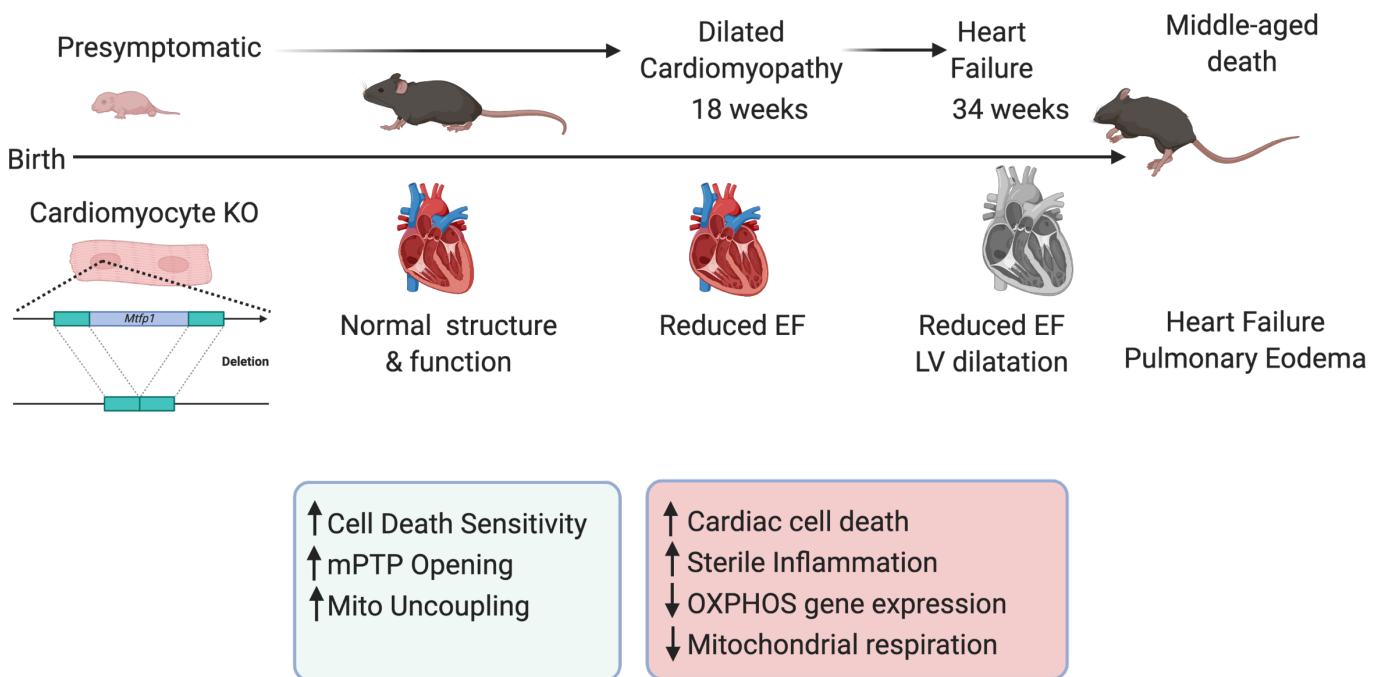


Figure 6