1 **iPSC modeling shows uncompensated mitochondrial mediated oxidative stress underlies**

2 **early heart failure in hypoplastic left heart syndrome**

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31 **Summary**

32       Hypoplastic left heart syndrome (HLHS) is a severe congenital heart defect with 30%

33 mortality from heart failure (HF) in the first year of life, but why only some patients suffer early-

34 HF and its cause remain unknown. Modeling using induced pluripotent stem cell-derived

35 cardiomyocytes (iPSC-CM) showed early-HF patient iPSC-CM have increased apoptosis, redox

36 stress, and failed antioxidant response. This was associated with mitochondrial permeability

37 transition pore (mPTP) opening, mitochondrial hyperfusion and respiration defects. Whereas

38 iPSC-CM from patients without early-HF had hyper-elevated antioxidant response with increased

39 mitochondrial fission and mitophagy. Single cell transcriptomics showed dichotomization by HF

40 outcome, with mitochondrial dysfunction and endoplasmic reticulum (ER) stress associated with

41 early-HF. Importantly, oxidative stress and apoptosis associated with early HF were rescued by

42 sildenafil inhibition of mPTP opening or TUDCA suppression of ER stress. Together these

43 findings demonstrate a new paradigm for modeling clinical outcome in iPSC-CM, demonstrating

44 uncompensated mitochondrial oxidative stress underlies early HF in HLHS.

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46 **Keywords**

**Introduction**

Congenital heart disease is one of the most common birth defects affecting 0.5% of live births (Feinstein et al., 2012). Hypoplastic left heart syndrome (HLHS) is a severe CHD in which the left ventricle (LV) and aorta are small and nonfunctional. While survival with HLHS is made possible by staged surgical palliation that recruits the RV to become the single pumping chamber (Oster et al., 2013), there remains high morbidity and mortality. The10-year transplant free survival stands at only 39-50%(Driscoll et al., 1992; Garcia et al., 2020; Gentles et al., 1997). However, the greatest risk is in the first year of life with 30% mortality reported (Alsoufi et al., 2016; Cleves et al., 2003; Tweddell et al., 2002). While HLHS patients have complicated clinical course, the early mortality is largely associated with ventricular dysfunction with rapid progression to acute heart failure (HF) (Garcia *et al.*, 2020). Unfortunately, therapies developed for HF in adults have been ineffective for treating HF in HLHS(Hsu et al., 2010; Shaddy et al., 2007). Without insights into the underlying mechanisms driving early HF in HLHS, the clinical management of this patient population is largely empirical.

Investigations into the mechanism of HLHS-HF have been hampered by the difficulty in obtaining human heart tissue for analysis. An alternative strategy entails in vitro disease modeling using induced pluripotent stem cells (iPSC) and their differentiated derivatives such as cardiomyocytes (iPSC-CM), endothelial/endocardial cells, and other cell types. While this has been successfully deployed for investigating HLHS disease mechanisms (Gaber et al., 2013; Hrstka et al., 2017; Jiang et al., 2014a; Kobayashi et al., 2014; Miao et al., 2020; Paige et al., 2020), no studies have explored the possibility of using iPSC-CM to model disease outcome. Particularly compelling is the question as to why only some HLHS patients develop early-HF even with the same surgical palliation, and what might be the underlying cause for HF. The feasibility to model

73    HLHS HF in iPSC-CM is suggested by our previous studies of a mouse model of HLHS (Liu et

74    al., 2017). We found cell autonomous defects were associated with prenatal/neonatal lethality from

75    HF in the HLHS mutant mice. In the present study, we showed mouse iPSC-CM generated from

76    the HLHS mice replicated defects observed in the HLHS mouse heart tissue, confirming the

77    defects are cell autonomous and thus suitable for in vitro iPSC-CM modeling. Generating iPSC

78    and iPSC-CM from HLHS patients dichotomized by clinical outcome, either with or without acute

79    early-HF (Xinxiu Xu, 2018), we further investigated and demonstrated the feasibility of using

80    patient iPSC-CM to investigate HF outcome in vitro. These studies provided surprising insights

81    not only into possible causes for early HF in HLHS, but they also uncovered mechanisms that may

82    protect against early HF in HLHS patients surviving heart transplant free.

83    **RESULTS**

84    **Cell Autonomous Mitochondrial defects in the Ohia HLHS Mouse Model**

85    The Ohia HLHS mouse model exhibits mid to late gestation lethality with acute heart failure

86    characterized by severe pericardial effusion with poor cardiac contractility, this is associated with

87    decreased proliferation and increased apoptosis (Liu *et al.*, 2017). Ultrastructural analysis showed

88    the myocardium with poorly organized thin myofilaments and altered mitochondrial morphology

89    (Liu *et al.*, 2017). Dynamic changes in mitochondria morphology play an important role in the

90    developmentally regulated metabolic switch from glycolysis to oxidative phosphorylation, a

91    process that also plays a critical role in regulating cardiomyocyte differentiation (Hom et al., 2011).

92    This entails closure of the mitochondrial permeability transition pore (mPTP) and formation of a

93    mitochondrial transmembrane potential ($\Delta\Psi_m$) mediating oxidative phosphorylation. Using

94    primary cardiomyocyte explants from the E14.5 *Ohia* HLHS mouse heart, we measured the $\Delta\Psi_m$,

95    in cardiomyocytes from the right and left ventricle (RV, LV). A reduction was observed in both

96    the RV and LV cardiomyocytes, indicating failure of the mPTP to close (**Figure 1A**). However,

97    mitochondrial mass was unchanged (**Extended Data Figure S1A).**

98    To determine whether the abnormal open state of the mPTP is a cell autonomous defect, we

99    generated iPSC from *Ohi*a mutant fibroblasts and differentiated them into iPSC-CM (**Figure 1B**).

100    These *Ohia* iPSC-CM generated entirely *in vitro* showed reduced cell proliferation with lower

101    *Myh6/Myh7* ratio indicating a cardiomyocyte differentiation defect (**Figure 1C,D;Figure S1C,D**),

102    phenotypes reminiscent of those observed in the *Ohia* HLHS myocardium. Poor cardiac function

103    was also indicated by reduced beat frequency (**Figure 1E**). Mitochondrial function was assessed

104    with measurement of $\Delta\Psi_m$ and oxygen consumption rate (OCR) using the Seahorse Flux Analyzer

105    (**Figure 1F,G**). This analysis uncovered mPTP and mitochondrial respiration defects in both the

106  undifferentiated *Ohia* iPSC and iPSC-CM. While the iPSC showed lower respiratory reserve and

107  respiratory maxima (**Figure S1B**), the iPSC-CM from *Ohia* exhibited reduction in basal respiration,

108  ATP production, respiratory reserve, and respiratory maxima (**Figure 1G**; **Figure S1E**). Together

109  these findings indicate the mitochondrial dysfunction, and proliferation and differentiation defects

110  observed in the Ohia HLHS heart tissue are cell autonomous.

111  **Generating HLHS Patient iPSC-CM for Investigating Early Heart Failure**

112  The finding that *Ohia* iPSC-CM replicated defects seen in the HLHS heart tissue suggested

113  HLHS patient derived iPSC-CM may have utility for investigating acute early HF in HLHS

114  patients. For this study, we generated iPSC from 10 HLHS patients, including six >5-year old with

115  transplant free survival (Group I) (**Figure 1H;Figure S2A**), and four that died (n=3) or survived

116  (n=1) with a heart transplant at <1 year of age (Group II). In addition, we also generated iPSC

117  from 3 healthy subjects as controls. Using standard iPSC-CM differentiation protocols, iPSC-CM

118  at Day 16-20 of differentiation were generated and used for the subsequent analysis.

119  **Impaired Cardiomyocyte Differentiation and Contractile Dysfunction**

120  The iPSC-CM were found to be predominantly ventricle-like as shown by high expression of

121  the ventricular marker MYL2, but low expression of atrial marker NR2F2 (Biendarra-Tiegs et al.,

122  2019) (**Figure 1I**). The Group II iPSC-CM had fewer cardiac troponin T (cTnT) positive cells with

123  higher ratio of *MYH6* (atrial myosin heavy chain) to *MYH7* (ventricular myosin heavy chain)

124  transcripts, indicating poor differentiation (Jiang et al., 2014b) (**Figure S2E;Figure 1J**). Group II

125  iPSC-CM also showed reduced Ki67, but increased pH3 immunostaining, suggesting cell cycle

126  disturbance with possible metaphase arrest (**Figure 1K;** Figure **S2F,G**), reminiscent of findings in

127  the *Ohia* HLHS LV (Liu *et al.*, 2017). Poor cardiomyocyte differentiation was indicated by low

128  expression of cTnT (A-band) and α-actinin (Z-disc) containing myofilaments together with

129   increased myofibrillar disarray (**Figure 1L, M**). However, no change was observed for

130   cardiomyocyte cell size (**Figure S2H**).

131     Further assessment of cardiomyocyte contractile function showed the Group II iPSC-CM have

132   lower beat frequency with reduced calcium transients (**Figure 1N-P;Supplemental Video 1&2**).

133   Examination of the profile of calcium transients confirmed the majority (84~89%) of the iPSC-

134   CM are ventricle-like (Cyganek et al., 2018) (**Figure S2I**). Analysis of the cardiomyocyte

135   contractile motion by high resolution video microscopy showed reduced fractional shortening

136   accompanied by decreased contraction and relaxation rates in the Group II but not Group I iPSC-

137   CM. This was associated with reduction in the diastolic sarcomere length, but not systolic

138   sarcomere length **(Figure 1Q-S;Figure S2J,K; Supplemental video 3 )**. Together these findings

139   indicate the Group II iPSC-CM have profound differentiation defects causing impaired calcium

140   handling and poor contractile function.

141   **Mitochondrial Respiration and Transition Pore Closure Defects**

142     Various parameters of mitochondrial function were assessed in the iPSC-CM. A marked

143   decrease in mitochondrial membrane potential ($\Delta\Psi_m$) was observed in the Group II iPSC-CM,

144   suggesting abnormal mPTP opening (**Figure 2A**). OCR measurements showed mitochondrial

145   respiration defects with reduction in basal respiration, ATP production, H+ leak, respiratory

146   reserve and maximal respiratory capacity (**Figure 2B**). For Group I iPSC-CM, only respiratory

147   reserve and maximal respiratory capacity showed significant change (**Figure 2B**). These same two

148   parameters also were reduced in the undifferentiated iPSC of Group II patients, findings

149   reminiscent of the *Ohia* mouse iPSC (**Figure S2C & S1B**). Western blotting showed no change in

150   abundance of the electron transport chain (ETC) complexes **(Figure S3A,B)**.

151     Consistent with the uncoupling of oxidative phosphorylation in the Group II iPSC-CM, we

152 observed a marked increase in mitochondrial reactive oxygen species (ROS) indicated by

153 increased MitoSOX staining (**Figure 2C**) (Hom *et al.*, 2011). Also observed was a reduction in

154 nitric oxide (NO), suggesting perturbation of protein nitrosylation required for normal

155 mitochondrial respiration (**Figure S3F**). The mitochondrial respiration defects and increase in

156 ROS in the Group II iPSC-CM are accompanied by increase in apoptosis and activation of a DNA

157 damage response, findings similar to those observed in *Ohia* (Liu *et al.*, 2017) and human HLHS

158 fetal heart tissue (Gaber *et al.*, 2013) (**Figure 2D-F**). Parallel analysis of the Group I iPSC-CM

159 showed no significant change in these parameters.

160 **Perturbation of Mitochondrial Dynamics**

161 As the regulation of mitochondrial fission and fusion play important roles in metabolic and

162 redox regulation, its disturbance can contribute to cardiomyocyte death in HF (Marin-Garcia and

163 Akhmedov, 2016). Using confocal imaging, we assessed mitochondrial mass and morphology. In

164 the Group II but not Group I iPSC-CM, mitochondrial linkage constant and cluster length were

165 increased, while mitochondrial mass was unchanged, indicating increase in mitochondrial fusion

166 in the Group II iPSC-CM (**Figure 2F,G; Figure S3C-E).** Group II iPSC-CM also showed

167 decreased expression of *DNML (DRP1),* gene regulating mitochondrial fission, and increase in

168 *MFN1,* gene promoting mitochondrial fusion (**Figure 2H**). Expression of *BNIP3*/*NIX* regulating

169 mitophagy were decreased in Group II but increased in Group I iPSC-CM (**Figure 2H**). Lysosomes,

170 which are involved in mitophagy, were reduced in both Group I and II (**Figure 2I**). These findings

171 indicate the hyperfused mitochondria in the Group II iPSC-CM likely arise from altered

172 mitochondrial dynamics associated with increased mitochondrial fusion and decreased mitophagy.

173 In contrast, Group I iPSC-CM exhibited more normal mitochondrial dynamics that may be

174 accompanied by increase in mitophagy.

**Mitochondrial Respiration Defects in the Left Ventricle of HLHS Human Heart Tissue**

To explore the clinical relevance of the abnormal mitochondrial function observed in the HLHS patient derived iPSC-CM, heart tissue was obtained from HLHS patients undergoing heart transplant. Analysis of mitochondrial respiration showed basal respiration was reduced in the HLHS-LV vs. RV tissue, but such LV-RV difference was not observed in heart tissue from age-matched heart transplant patient with doxorubicin induced HF (**Figure 2J**). Analysis of two additional HLHS neonates and two neonatal control subjects the HLHS-LV being more sensitive to lower ADP, indicating possible adaptation to bioenergetic stress. However, respiration in the LV failed to increase with increasing ADP concentration, indicating the hypoplastic LV has reduced respiratory capacity (Ventura-Clapier et al., 2011) (**Figure 2K;Figure S3G**). Western blotting showed no change in ETC components (**Figure S3H,I**). These findings suggest LV specific mitochondrial respiration defects in HLHS.

**Defects in Yap-Regulated Antioxidant Response**

Activation of an antioxidant defense pathway occurs during developmental with metabolic transition to mitochondrial respiration (Perrelli et al., 2011; Tsutsui et al., 2011). This pathway is regulated by transcription factors NRF2 (Itoh et al., 1999) together with PITX2 and YAP (Tao et al., 2016). These three transcription factors play an essential role in regulating the expression of antioxidant genes that scavenges ROS to prevent oxidative stress. These transcription factors also have critical roles in regulating cardiac regeneration and repair, with YAP also shown to regulate heart organ size (Heallen et al., 2013; von Gise et al., 2012; Zhou et al., 2015). Interestingly, YAP also has been demonstrated to regulate mitochondrial fission (Huang et al., 2018).

We observed *NRF2* and *PITX2* transcripts are both reduced in Group II iPSC-CM, but in Group I iPSC-CM, *PITX2* was elevated and *NRF2* was unchanged (**Figure 3A**). In contrast, *YAP1*

198    transcripts showed no change in either Group or Group II iPSC-CM (data not shown). Analysis of

199    downstream genes in the antioxidant pathway revealed up regulation of thioredoxin (*TXN*),

200    peroxiredoxin 1 (*PRDX1*), glutathione peroxidase 1 (*GPX1*), and superoxide dismutase 2 (SOD2)

201    in the Group I iPSC-CM, but in Group II, expression was either unchanged or downregulated, such

202    as for PRDX1 (**Figure 3A**). In the Group I iPSC-CM, we also observed increased expression of

203    HIF1$\alpha$, a transcription factor regulating cell stress response to hypoxia. This was associated with

204    increased expression of *VEGF*, a known downstream transcriptional target of HIF1$\alpha$ (**Figure 3A**)

205    (Guimaraes-Camboa et al., 2015).

206    Antibody staining showed nuclear localization of NRF2/PITX2/YAP were reduced in the

207    Group II iPSC-CM, while in Group I, only PITX2 showed a modest reduction in comparison to

208    Group II and control (**Figure 3B**-**D**; **Figure S3J**). However, total YAP and β-catenin protein

209    expression levels were unchanged (**Figure S3K,L**). We further examined expression of

210    downstream target genes of YAP - NRG1 and MYC *(Artap et al., 2018)*, and observed both were

211    reduced in the Group II iPSC-CM. In contrast, the opposite was observed in Group I with *NRG1*

212    being upregulated, while *MYC* was unchanged (**Figure 3F**). However, nuclear localized β-catenin

213    and transcripts for two downstream YAP/β-catenin targets, *BIRC5* and *SNAI2*, were reduced in

214    both Groups I and II iPSC-CM (**Figure 3E,F**). Together these findings show defects in the

215    mounting of an effective antioxidant response in the Group II iPSC-CM (**Figure 3G**). In contrast,

216    in Group I, the antioxidant capacity is expanded, and may promote and support the restoration of

217    redox homeostasis.

218    **Inhibition of mPTP Opening Rescues Mitochondrial Respiration and Apoptosis**

219    The mPTP closure defect observed in the Group II iPSC-CM suggests compounds promoting

220    mPTP closure might rescue the mitochondrial defect. This was investigated using the Seahorse

221 Analyzer to screen compounds known to inhibit mPTP opening(Martel et al., 2012) or otherwise

222 modulate mitochondrial respiration. This analysis yielded 7 compounds showing some rescue of

223 mitochondrial respiration in the Group II iPSC-CM. This included fasudil, sildenafil, cyclosporin

224 A (CsA), Metformin, JP4, SS31, and Y27632 (**Figure S4A**). In contrast, treatment with ascorbic

225 acid, a general antioxidant, had no effect (Myung et al., 2013; Ye et al., 2013). Given sildenafil is

226 commonly used among CHD patients for its vasodilatory effects(Galie et al., 2005), more in-depth

227 analysis was carried out with sildenafil (**Figure 4**).

228 Titration of sildenafil showed rescue down to 0.1 μM, which restored not only $\Delta\Psi_m$ and

229 maximal mitochondrial respiration. This also reduced mitochondrial ROS to levels similar to the

230 Group I and control iPSC-CM (**Figure 4A-C; Figure S4B-D**). Sildenafil is also known to affect

231 NO production, but normal NO level was restored only at 1.0 μM concentration (**Figure**

232 **S4D**)(Prabhu et al., 2013). Cell proliferation, apoptosis (**Figure 4E,F**), and YAP nuclear

233 trafficking were rescued at ten times lower dose of 0.01 μM (**Figure 4G**). However, β-catenin

234 nuclear trafficking was not rescued (**Figure 4H; Figure S4E**). To verify that sildenafil is targeting

235 the mPTP, we further assessed treatment with BKA (bongkrekic acid) and CAT

236 (carboxyatractyloside), which activate and inhibit the mPTP, respectively. As expected, BKA but

237 not CAT rescued the mPTP defect, with opposing effects observed for maximal and basal OCR,

238 mitochondrial ROS, and YAP nuclear localization (**Figure 4I-L, Figure S4F**). Similar treatment

239 of control iPSC-CM showed repression of respiration by CAT, while BKA had no effect (**Figure**

240 **S4G**).

241 **Single Cell Transcriptome Profiling**

242 We performed single cell RNAseq on iPSC-CM from two Group II patients, 7042 with heart

243 transplant at 11 months and patient 7052 deceased at 2 months, Group I patient 7464 surviving

244    transplant free at 7 years of age, and healthy control subject 1053. Data was obtained from 4403

245    cardiomyocytes forming 9 clusters (Stuart et al., 2019) (**Figures 5A and S5A-C**). Marker gene

246    analysis showed these cardiomyocytes were largely of ventricular identity (**Figure S5D)**. Clusters

247    0 (CM I), 1 (CM II) and 5 (CM III) comprising the majority of cells are well differentiated

248    cardiomyocytes of increasing maturation (**Figure S5E; Supplemental Spreadsheet 2**). Group II

249    vs. control comparison yielded the greatest number of DEGs (**Figure 5B**). Enrichment was

250    observed for mitochondrial related pathways in all three clusters, suggesting Group II

251    mitochondrial defects likely arise early in cardiomyocyte differentiation (**Figure 5C).** In contrast,

252    Group I vs Control yielded the fewest DEGS. These were associated with heart development and

253    muscle organ development terms in Clusters 0 and 1, and mitochondrial related terms in Cluster 5

254    (**Figure 5E**). Group II vs. Group I comparisons yielded apoptosis and cell death in Clusters 0 and

255    1, (**Figure 5D**), and tRNA modification and noncoding RNA in Cluster 5.

256       Combining DEGs from all pairwise comparisons showed the number of DEGs increased

257    with disease severity (**Figure 5F).** Only control 1053 yielded terms related to muscle and muscle

258    contraction. Group I 7464 recovered protein translation and cell cycle, and Group II 7042 yielded

259    mitochondria and mitochondrial translation. In Group II 7052, hypoxia related pathways were

260    recovered, but mitochondrial related terms were also recovered in DEGs shared with 7042 (**Figure**

261    **5F; Spreadsheet 2**). Overall, a high percentage of the DEGs were found to be mitochondrial

262    related (**Figure 5G**). Heatmap generated comprising only the mitochondrial related DEGS was

263    nearly identical to that for all DEGs (**Figure 5H vs. F**), indicating genes with the highest fold

264    change are mostly mitochondrial related. Interestingly pathways related to mitochondrial

265    translation, elongation, and termination were recovered in both 7464 (Group I) and 7042 (Group

266    II), but with only 60 DEGs in 7464, vs. 183 DEGs in 7042 (**Figure 5H;Spreadhseet 2**). Differing

267     from 7042, mitochondrial DEGs in 7052 were hypoxia related, confirming the recovery of these

268     same pathways in all DEG analysis (**Figure 5F**). In mitochondrial DEGs shared between

269     7042/7052 shared, the recovery of ATP synthesis and oxidative phosphorylation were observed,

270     suggesting bioenergetic deficits associated with Group II patients.

271          DEG analysis based on Group II vs. Group I comparison yielded further evidence of the

272     effective dichotomization of HLHS patients into two functional groups (**Figure 5I**). Profiling

273     upregulated DEGs showed the two Group II patients are similar to each other, while Group I is

274     similar to control (**Figure 5I**).  This analysis also recovered 28 genes highly expressed only in

275     patient 7052 (see region denoted by asterisk in **Figure 5I**) - 14 are related to mitochondria, hypoxia

276     and/or cell death, including EGLN3 encoding prolyl hydroxylase, an oxygen sensor that promotes

277     HIF1α degradation (**Figure 5J;Supplemental Spreadsheet 2**). Assembly of a protein interactome

278     network incorporating 26 of these genes showed pathway enrichment for hypoxia, apoptosis, and

279     oxidative stress, indicating these genes are part of a functional network contributing to early HF

280     in patient 7052 (**Figure S6;Supplemental Spreadsheet 2**).

**Molecular Chaperone Rescues Mitochondrial Respiration and Apoptosis**

282          Recovery of ER stress and UPR in Cluster 7 from the scRNAseq analysis was notable (**Figure**

283     **6A**), given ER stress can be triggered by mitochondrial dysfunction and oxidative stress. and ER

284     stress has been associated with HF (Schiattarella et al., 2019). This pathway has not been

285     investigated previously in the context of HLHS. Real time PCR analysis confirmed elevated

286     expression of genes (*XBP1,ATF4,ATF6*) associated with the three conserved ER stress pathways

287     (**Figure 6B**). All three pathways were elevated in patient 7052, and two (*ATF4 ATF6*) were

288     elevated in 7042. In contrast, Group I patient 7464 showed no change relative to control (**Figure**

289     **6B**). Similar analysis of three downstream ER stress target genes *HSPA5, DDIT3,* and *DNAJC3*

290    showed all three were upregulated in 7052, but only *DDIT3* and *DNJC3* were elevated in 7042. In

291    contrast, all three genes were down regulated in Group I patient 7464 (**Figure 6B**). We noted these

292    same cell stress related genes were also up regulated in the *Ohia* HLHS heart tissue, consistent

293    with their severe HF phenotype (Liu *et al.*, 2017). To assess the potential functional impact of UPR

294    on the HLHS iPSC-CM, we treated the iPSC-CM with Tauroursodeoxycholic acid (TUDCA), a

295    molecular chaperone known to promote protein folding and suppress ER-stress. TUDCA treatment

296    promoted mPTP closure, reduced mitochondrial ROS, and rescued NO production in the Group II

297    iPSC-CM **(Figure 6C-E)**. TUDCA also rescued YAP nuclear translocation, restored

298    cardiomyocyte proliferation and blocked apoptosis (**Figure 6F-H**). These findings suggest ER

299    stress and UPR may contribute to the early HF in Group II patients.

300    **Enrichment of variants associated with mitochondrial metabolism**

301         Given HLHS is well described as having a genetic etiology, we further investigated the whole

302    exome sequencing data available for 6 of our 10 HLHS patients (Group II 7042, Group I

303    7131,7400,7438,7434,7464). High impact variants comprising unique loss-of-function variants

304    were recovered (**Figure S7A;Supplemental Spreadsheet 3**). Using Webgestalt/KEGG pathway

305    enrichment analysis, four genes were identified as significantly associated with metabolic

306    pathways (*OXA1L,NNMT,NEU3,ALDH7A1*) (**Supplemental Spreadsheet 3**). An protein-protein

307    interactome (PPI) was constructed using these four genes to explore connections to Hippo

308    signaling, a pathway that plays a critical role in regulating YAP degradation and nuclear

309    translocation (Meng et al., 2016). The interactome showed enrichment for Hippo and Wnt

310    signaling, and also heart development and many mitochondrial-related terms, including regulation

311    of mitochondrial membrane permeability (**Fig7A,B;Supplemental Spreadsheet 3**).

312         Building on this finding, we interrogated the WES data from another 41 HLHS patients

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313    comprising 19 patients who died or had heart transplant (unfavorable outcome) and 22 HLHS

314    patients surviving transplant-free beyond 5 years of age (favorable outcome). Interrogating for

315    unique loss-of-function variants or predicted damaging missense or splice variants yielded

316    159 genes from the unfavorable outcome group and 194 genes from the favorable group (**Figure**

317    **S7A;**S**upplemental Spreadsheet 3**). Rendering these genes in a network plot using Metascape

318    recovered terms such as "Ion channel transport", "Mitochondrial gene expression", and

319    "Mitochondrial translation" in association with genes from the unfavorable group, while "lipid

320    location, response to IL-17" were associated with the favorable group (**Figure 7C;**S**upplemental**

321    **Spreadsheet 3)**. Some pathways were shared by both groups such as calcium signaling, MAPK

322    signaling, and nervous system development. Examining the genes recovered for intersection with

323    an expanded MitoCarta-related inventory of mitochondrial genes yielded 19 genes from the

324    unfavorable and 11 from favorable group (Calvo et al., 2012; Pagliarini et al., 2008)

325    (S**upplemental Spreadsheet 3**). ToppGene analysis of these overlapping genes recovered from

326    the unfavorable group yielded multiple mitochondrial related pathways, including mitochondrial

327    translation (**Figure 7D**). Most of these genes are highly expressed in cardiomyocytes of the human

328    fetal heart (Cui et al., 2019), supporting a role in HLHS pathogenesis (**Figure 7E**).

329 **DISCUSSION**

330     Our objective in this study was to investigate why only some HLHS patients suffer early-HF

331 and the possible cause of early-HF. Analysis of iPSC-CM from our HLHS mouse model and

332 HLHS patients revealed both have cell autonomous defects involving failure in mPTP closure.

333 Thus, mitochondrial defects seen in vivo in the HLHS mouse heart were replicated in the mouse

334 iPSC-CM. This was associated with defects in mitochondrial respiration and poor cardiomyocyte

335 differentiation. The mitochondrial defects observed in the mouse heart and iPSC-CM were

336 replicated in iPSC-CM of HLHS patients with early HF, suggesting a common cell autonomous

337 mechanism involving mitochondrial defects underlying early-HF in HLHS.

338     For these studies, we selected HLHS patients with extreme phenotype comprising death or

339 surviving with heart transplant at less than one year of age (Group II), as the first year of life poses

340 the greatest risk with 30% mortality reported (Oster *et al.*, 2013). For comparison, HLHS patients

341 surviving transplant free at more than 5 years of age were recruited (Group I). Using these two

342 HLHS group comparisons and control subjects, we interrogated a myriad of parameters such as

343 cardiomyocyte differentiation, myocyte contractility and calcium handling, mPTP closure,

344 mitochondrial dynamics, respiration, and regulation of the antioxidant pathway. From this

345 comprehensive analysis, we showed the iPSC-CM from the Group II patients closely resembled

346 each other, while the Group I patients were more similar to control. This was further corroborated

347 with scRNASeq analysis, which showed transcriptome profiles of the two Group II patients are

348 similar to each other, but very different from Group I patient 7464.

349     In the Group II iPSC-CM, we uncovered severe oxidative stress arising from

350 mitochondrial dysfunction. This is associated with mPTP closure defect with altered mitochondrial

351 dynamics and reduced mitophagy. When combined with a failed antioxidant response, this would

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352    exacerbate the redox stress to enhance apoptosis and increase DNA damage. Also observed were

353    severe defects in cardiomyocyte differentiation with poor myocyte function. Cardiomyocyte

354    differentiation and maturation are known to be regulated by mPTP closure (Hom *et al.*, 2011) and

355    a metabolic switch to mitochondrial respiration (Mills et al., 2017; Nakano et al., 2017) . We note

356    skeletal myoblast differentiation is also regulated by a similar metabolic transition(Fortini et al.,

357    2016a). Moreover, this skeletal myoblast metabolic transition was shown to be modulated by

358    mitophagy(Fortini et al., 2016b).  Also observed in the Group II iPSC-CM is the up regulation of

359    ER stress pathways. This likely occurs secondary to the mitochondrial associated increase in ROS,

360    exacerbating the oxidative stress induced apoptosis.   Recent studies have in fact shown an

361    important role for ER stress and UPR in HF (Schiattarella *et al.*, 2019). Of significant interest from

362    a therapeutic standpoint, apoptosis in the Group II iPSC-CM can be rescued using sildenafil (Ascah

363    et al., 2011) to inhibit mPTP opening or TUDCA to suppress UPR. This was associated with the

364    reduction of mitochondrial ROS, recovery of mitochondrial respiration, and restoration of YAP

365    nuclear translocation. Together these findings support mitochondrial mediated oxidative stress as

366    underlying the acute early-HF in HLHS.  The scRNAseq analysis further suggests this may involve

367    defects in the HIF1α pathway, altered mitochondrial translation, and bioenergetic deficits, findings

368    that will need to be further investigated in future studies.

369         In contrast to Group II iPSC-CM, the Group I iPSC-CM show similarities to that of control

370    with near normal mitochondrial respiration and normal mitochondrial dynamics without oxidative

371    stress nor increase in apoptosis. Nevertheless, the Group I iPSC-CM have reduced mitochondrial

372    respiratory reserve and reduced maximal respiration, indicating an overall reduction in total

373    respiratory capacity. Importantly, nuclear localization of NRF2, YAP1, PITX2 was maintained,

374    albeit with some reduction observed for PITX2. This was associated with striking gene expression

375     changes that included elevated expression of many antioxidant genes, and the elevated expression

376     of HIF1α and its downstream target genes. Genes regulating mitophagy were also elevated, while

377     *MFN1*, gene regulating mitochondrial fusion was down regulated. Significantly, key mediators of

378     all three ER stress pathways were downregulated. Together these findings suggest the maintenance

379     of mitochondrial dynamics in conjunction with the suppression of oxidative and ER stress by a

380     vigorous NRF2/YAP/PITX2 mediated antioxidant response may provide protection from early-

381     HF in Group I patients. As Group I iPSC-CM also showed better differentiation with improved

382     myocyte contractile function, these factors also may contribute to improved clinical outcome.

383        The WES sequencing analysis showed pathogenic variants in HLHS patients with

384     unfavorable outcome are enriched for genes in mitochondrial related pathways. While the genetic

385     causes for HLHS remains largely unknown, pathogenic variants in mitochondrial related pathways

386     may contribute to the pathogenesis of HLHS or they may act as genetic modifiers affecting clinical

387     outcome. It is worth noting *Sap130*, one of the two genes causing HLHS in the *Ohia* mouse model

388     is known to regulate genes involved in mitochondrial metabolism via the Sin3A complex (Pile et

389     al., 2003), suggesting the developmental etiology of HLHS may involve the disturbance of

390     mitochondrial metabolism (Liu *et al.*, 2017). We note there is mounting evidence of the integral

391     role for metabolism and mitochondrial respiration in the regulation of a wide range of

392     developmental processes (Mills *et al.*, 2017).

393        In summary, our findings point to the common involvement of mitochondrial dysfunction

394     in HLHS regardless of HF outcomes. This is supported by another study that also reported

395     mitochondrial defects in HLHS iPSC-CM (Paige *et al.*, 2020). With the outcome-based iPSC-

396     modeling, we showed the mitochondrial dysfunction and oxidative stress underlie the early HF in

397     HLHS, while a hyper-elevated antioxidant response may provide protection from oxidative and

398  ER stress to prevent early HF. Together these findings suggest early HF is the result of

399  uncompensated mitochondrial mediated oxidative stress. The observed altered regulation of YAP1

400  suggests the tantalizing possibility that the mitochondrial defects also may contribute to the LV

401  hypoplasia in HLHS, a question that warrants further studies.

402  　　We also showed possible therapeutic intervention with the targeting of mPTP closure with

403  sildenafil or suppression of UPR with TUDCA. We note Sildenafil is already being used

404  empirically to threat HF associated with pulmonary hypertension(Guglin et al., 2016). Suppression

405  of UPR, such as with TUDCA, may be another therapeutic path. TUDCA is currently in clinical

406  trial for amyotrophic lateral sclerosis(Elia et al., 2016). Providing antioxidant might be another

407  therapeutic course, although we found ascorbic acid did not rescue mitochondrial defects in the

408  Group II iPSC-CM. Overall, our iPSC modeling has yielded new insights into the underlying

409  causes for early HF in HLHS and suggest new evidence-based therapies that will need to be further

410  investigated. These findings suggest a new paradigm for modeling clinical outcome using patient

411  stratified iPSC.

412  **Limitations of the Study**

413  　　One limitation of our study is the inclusion of iPSC-CM from only 10 patients. However,

414  this compares favorably to other studies that typically include iPSC from only one to three patients,

415  and no study had controlled for outcome(Gaber *et al.*, 2013; Hrstka *et al.*, 2017; Jiang *et al.*, 2014a;

416  Kobayashi *et al.*, 2014; Miao *et al.*, 2020; Paige *et al.*, 2020). Nevertheless, the generalizability of

417  our findings will require future confirmation with analysis of iPSC-CM from additional patients.

418  As our study was focused on acute early-HF in patients less than one year old, the relevance of

419  these findings to HF in older HLHS patients will require further studies. While additional factors

420  may contribute to HF in older patients, the involvement of mitochondrial dysfunction is likely.

421    This is suggested by the recovery of mitochondrial-related pathogenic variants in the expanded

422    WES analysis of 41 HLHS patients that included older patients with heart transplant.

423      **FIGURE LEGENDS**

424      **Figure 1. Mouse and human HLHS iPSC and iPSC-CM show differentiation and functional**

425      **defects.**

426      (A)      Mitochondrial transmembrane potential ($\Delta\Psi$m) was measured with TMRE/Mitotracker

427      Green in CM from E13.5 wildtype (WT) mouse embryo (n=3) left ventricle (WT-LV; n=124 CMs)

428      and right ventricle (RV; n=86 CMs), and E13.5 *Ohia* HLHS mutant (n=3), LV (n=105 CMs) and

429      RV (n=118 CMs)

430      (B)      Mouse iPSC were generated from WT and HLHS mouse embryonic fibroblasts (two

431      independent lines eacg) and further differentiated into iPSC-CM.

432      (C)      Ki67 quantification showed reduced proliferation of the HLHS (n=1800) vs. WT (n=2800)

433      iPSC-CM.

434      (D)      *Myh6*/*Myh7* transcript ratio is decreased in the *Ohia* HLHS iPSC-CM vs. WT, indicating a

435      maturation defect. WT n=6, and HLHS n=5.

436      (E)       Beat frequency of *Ohia* HLHS iPSC-CM (n=17 clusters quantified) was reduced

437      compared to WT (n=38 myocyte clusters quantified).

438      (F)      The mitochondrial membrane potential ($\Delta\Psi$m) was reduced in the mouse HLHS iPSC-CM

439      (n=43) compared to WT (n=56).

440      (G)      Mitochondrial respiration parameters were obtained from Seahorse Analyzer oxygen

441      consumption rate (OCR) measurements (n=3 independent experiments).

442      (H)      Human iPSC-CM were generated from HLHS patients and controls. Group I comprises

443      patients with transplant free survival >5 years old. Group II are patients who died or survived with

444     heart within one year of age. Controls are healthy subjects without disease. Functional assessments

445     were conducted on 18-22 days of iPSC-CM differentiation.

446     (I)     qPCR for atrial (NR2F2) and ventricle MYL7) marker genes show the iPSC-CM are

447     ventricle-type.

448     (J) *MYH6/MYH7* transcript ratio is increased in Group II iPSC-CM, indicating cardiomyocyte

449     maturation defect. Note this ratio is reversed in mice vs. human, as the major ventricular myosin

450     heavy chain in mice is *Myh6*, and *MYH7* in human.

451     (K) Ki67 immunostaining showed decreased proliferation in Group II iPSC-CM.

452     (L, M) Quantification of myofibril organization showed myofibrillar disarray (L) in cTnT (Green)

453     and α-actinin (Red) positive human iPSC-CM (M).

454     (N-P) Beat frequency (N), and visualization (O) and quantification of calcium transients (P) in

455     iPSC-CM showed functional deficits in Group II iPSC-CM.

456     (Q-S) Quantification of contractile function in individual iPSC-CM showed decreased fractional

457     shortening (Q), contraction rate (R) and relaxation rate (S) in Group II iPSC-CM.

458     Data shown are mean±SEM using Student's t-test or ANOVA. For box plots, median/min/max are

459     shown with Kruskal-Wallis statistics. Number of Control, Group I, Group II subjects analyzed (H-

460     S): (I) n=3,3,3 subjects. (J) n=3,5,3 subjects. (K, L,N) n=3,6, 3 subjects. (P) n=3,6,4 subjects. (Q-

461     S) n=3,4,4 with n=17,23,38 cardiomyocytes respectively.

462

463 **Figure 2. Mitochondrial dynamics and respiration defects in HLHS iPSC-CM.**

464 (A) Measurement of $\Delta\Psi m$ in human iPSC-CM using TMRE and MTG staining.

465 (B) Seahorse Analyzer OCR measurement showed mitochondrial respiration defects in the HLHS

466 iPSC-CM.

467 (C) MitoSOX staining show elevated mitochondrial reactive oxygen species (ROS) in Group II

468 HLHS iPSC-CM

469 (D,E) TUNEL labeling and $\gamma$-H2AX staining show increased apoptosis (D) and DNA damage (E)

470 in Group II iPSC-CM.

471 (F,G) Mitotracker red staining showed increased linkage constant (see Methods), indicating

472 hyperfused mitochondria in Group II iPSC-CM

473 (H) qPCR of key genes regulating mitophagy and mitochondrial dynamics.

474 (I) LysoTracker Deep Red-staining of lysosomes showed lysosome reduction in Group II iPSC-

475 CM.

476 (J) Basal OCR (>3 duplicates) of explanted heart tissue from two 19-year-old HLHS patients and

477 a 15 year old cardiomyopathy patient with doxorubicin cardiotoxity were assessed using the

478 Seahorse Analyzer

479 (K). Respiratory control ratio (RCR) was obtained for 2 Control and 2 HLHS neonatal patient

480 using cell extracts from explanted heart tissue. $V_{max}$ was measured using succinate as a substrate

481 and two concentrations of ADP.

482 Bar graphs show mean±SEM with ANOVA. Number of Control, Group I, Group II subjects

483 analyzed respectively in (A-I): (A, D) n=3,6,3 subjects. (B, C,F) n=3,5,3 subjects,(E) n=3,4,3

484 subjects. (H) n=3,3,3 subjects (I) n=3, 4, 3 subjects.

485 **Figure 3. HLHS iPSC-CM with failed antioxidant response show cytoplasmic localization of**

486 **NRF2, YAP1, and PITX2**

487 (A) qPCR showed key antioxidant genes and HIF pathway are up regulated in Group I iPSC-CM,

488 and either unchanged or downregulated in Group II iPSC-CM.

489 (B-E) Immunostaining show defect in NRF2, PITX2, YAP and β-catenin nuclear localization in

490 Group II iPSC-CM (see FigS6 G). N/C = nuclear to cytoplasmic ratio.

491 (F) qPCR quantification of YAP and YAP/β-actinin downstream target genes.

492 (G) Diagram summarizing HLHS associated defects in mitochondrial dynamics with elevated ROS

493 and failed antioxidant response with failure in NRF2, YAP, and YAP/PITX2 nuclear translocation.

494 (A, F) show mean±SEM with one-way ANOVA. Box plot with median/min/max shown with

495 Kruskal-Wallis statistics. n=3 control, 3 Group I, 3 Group II subjects.

496 Number of Control, Group I, and Group II subjects analyzed: (B, C) n=130, 117, 64 CM. (D) n=90,

497 78, 79 CM. (E) n=44, 15, 17 CM

498 **Figure 4. Inhibition of mitochondrial membrane permeability rescues mitochondrial**

499 **respiration and apoptosis.**

500 (A-H) Sildenafil (Sil) rescued Group II iPSC-CM including $\Delta\Psi$m (A), maximum OCR (B),

501 mitochondrial ROS (C), NO level (D), cell proliferation (E), apoptosis (F), and YAP (G) and β-

502 catenine (H) nuclear localization.

503 (I-L) Treatment with bongkrekic acid (BKA), but not carboxyatractyloside (CAT) rescued $\Delta\Psi$m

504 (I), maximum OCR (J), mitochondrial ROS (K) and YAP nuclear localization (L).

505 Bar graphs show mean±SEM, analyzed by one-way ANOVA, and box plot with median/min/max

506 analyzed by Kruskal-Wallis. n≥3 independent repeats. Subjects analyzed: Control n=3, Group I

507 n=4 or 5, and Group II, n=3 or 4.

508 **Figure 5. Single cell RNAseq showed mitochondrial pathways associated with early-HF.**

509 (A)    Single cell RNAseq data yielded 9 distinct clusters (See Figure S5E).  Clusters 0,1, and 5

510 are comprised of well differentiated cardiomyocytes of increasing maturation. Clusters 4 and 6

511 correspond to proliferating cardiomyocytes at G2/M and S phase (also see Figure S5E). Cluster 2

512 include cardiomyocytes undergoing apoptosis, while Cluster 7 exhibit evidence of ER stress with

513 UPR (Figure S5E). In Clusters 3 and 8, enrichment for oxidative phosphorylation and genes related

514 to hypertrophic cardiomyopathy are observed (see Spreadsheet 2).

515 (B)    DEGs recovered in each cluster with pairwise comparisons.

516 (C-E) Pathway enrichment of with DEGs in pairwise patient group comparisons in Clusters 0, 1

517 and 5.

518 (F). Heat map of DEGs from all pairwise comparisons for Clusters 0,1, and 5 and the top Biological

519 Processes recovered.

520 (G). Percentage of DEGs that are mitochondrial related is shown for each group in F. The n

521 represents number of mitochondrial DEGs observed.

522 (H). Heat map of mitochondrial related DEGs in clusters 0, 1 and 5 and the top Biological

523 Processes recovered.

524 (F, H) Color scale showed relative maximum and minimum value.  Grayscale showed the adjust p

525 value (-log10FDR) of each GO term and circle size showed the count of genes in each GO term.

526 (I) Hierarchical clustering based on DEGs from Group II (7052 and 7042) vs. Group I (7464).

527 Asterisk (∗) denotes region with 28 DEGs upregulated only in 7052 (Spreadsheet 2).

528 (J) Violin plot showing transcript expression for 6 of the 28 DEGs from panel I.

529 **Figure 6. ER stress in the iPSC-CM and its suppression rescued mPTP closure and apoptosis.**

530 (A)     ER stress was recovered as top pathway in Cluster 7.

531 (B)     Real time PCR confirmed elevated expression of ER stress marker genes in Group II

532 (7042,7052) iPSC-CM.

533 (C-H) Treatment with molecular chaperone TUDCA, an ER stress inhibitor, rescued $\Delta\Psi$m (C),

534 ROS (D), NO level (E). YAP nuclear localization (F), and restored cell proliferation (G) and

535 suppressed apoptosis (H)

536 Bar graphs show mean±SEM with Student's t-test. Box plots show median and minimum-

537 maximum, with Mann-Whitney statistical test. (B) n=3 independent repeats for each sample. (C-

538 H) n≥3 independent repeats for each bar. Subjects analyzed: Control n=3, Group I n=4 or 5, and

539 Group II, n=2 (7042 and 7052).

540     **Figure 7. Damaging variants in mitochondrial and Hippo related pathways**

541     (A,B) Protein-protein interactome constructed with four genes (*OXA1L, NNMT, NEU3, ALDH7A;*

542     blue triangle nodes) to explore interconnections with the Hippo pathway recovered many genes

543     related to Hippo and Wnt signaling and also mitochondrial and cardiac related genes

544     (C) Pathway enrichment rendered using Metascape comprising genes recovered with extreme

545     unique variants from 41 HLHS patients. Size of the collective node slice represents percentage of

546     genes originating from the corresponding gene list.

547     (D) Pathway enrichment related to the 19 mitochondrial genes recovered from the unfavorable

548     outcome HLHS patients that intersected with the MitoCarta inventory of mitochondrial genes (see

549     overlapping genes in Supplementary Spreadsheet 3).

550     (E) Mitochondrial transcript expression in cardiomyocytes from human heart tissue at 5-15 weeks

551     gestation from scRNAseq data of Cui, et al (Cui *et al.*, 2019). Size of the circle corresponds to

552     percentage of cells expressing the gene (Exp%) and the color show average expression values with

553     Z-transform.

564 **AUTHOR CONTRIBUTIONS**

565 Study design: C.W.L. and X.X; miPSC and hiPSC reprogramming: iPSC-CM differentiation,

566 cardiomyocyte proliferation and apoptosis, cardiomyocyte and mitochondrial function

567 measurements, drug screening and data analysis: X.X; Single cell RNAseq and data analysis:

568 X.X,K.J,B.A,H.Y.,C.W.L.,A.S.B,D.K; human patient clinical data analysis: J.I.L,P.A; recruitment

569 of subjects and human tissue sample collection: C.W.L.,J.I.L.,P.A.,G.B, G.A.P; human heart tissue

570 analysis: G.B,G.A.P.,X.X.; Seahorse measurement support: S.S.S; mouse fetal ultrasound imaging

571 and mouse phenotyping: X.L.,X.X.; mitochondrial staining support and analysis:

572 X.X.,T.N.F.G.A.P; iPSC-CM sarcomere video analysis: P.N, J.C, C.K.K; human exome

573 sequencing analysis: W.Z; protein network analysis, K.B.K, M.K.G.; statistics: X.X; manuscript

574 preparation: C.W.L, X.X, A.S.B, K.J, B.A, G.A.P., J.I.L, R.A.D, M.T., M.K.G., W.Z and T.N.F.

575

576 **COMPETING INTERESTS STATEMENT**

577 The authors declare no competing financial interests.

578

579    **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

580    **Mouse Strain**

581    E13.5-E14.5 Ohia HLHS mouse (*Sap130m/m;Pcdha9m/m*) or CRISPR HLHS mouse

582    (*Sap130m/m;Pcdha9m/m*) and littermate controls were used for primary cardiomyocytes explants

583    from heart tissue. Mouse embryo fibroblasts used for mouse iPSC generation were generated from

584    E14.5 – 17.5 mouse embryos (**See Supplemental Spreadsheet1**). All mice were housed, treated,

585    and handled in accordance with the guidelines set forth by the University of Pittsburgh Institutional

586    Animal Care and Use Committee and the National Institutes of Health's Guide for the Care and

587    Use of Laboratory Animals.

588    **Human Blood, Cells, and Surgical Tissue**

589    Cells, heart tissue and blood were obtained from HLHS patients recruited from Children's

590    Hospital of Pittsburgh of UPMC with informed consent under a human study protocol approved

591    by the University of Pittsburgh Institutional Review Board (Supplemental Spreadsheet 1). For

592    infants and minors, informed consent was obtained from the legal guardian. Some human heart

593    tissues were obtained from the Molecular Atlas of Lung Development Program (LungMAP)

594    Consortium distributed by Human Tissue Core (HTC). Donor tissue was supplied through the

595    United Network for Organ Sharing for Western blot and isolated mitochondrial OCR

596    measurements.

597    **METHOD DETAILS**

598    **Production of patient iPS cells**

599    Mouse embryonic fibroblasts were reprogrammed using the CytoTune-iPS Sendai

600    Reprogramming kit(Fusaki et al., 2009). Human fibroblasts or lymphoblastoid cells were

601    transfected with four episomal plasmids(Okita et al., 2011) using electroporation. iPSCs clones

602    were identified by immunofluorescent staining of pluripotency marker Oct4 and Nanog and qPCR

603    analysis of stem cell markers **(Supplemental Figure S2B)**(Xu et al., 2013). All antibody and

604    primer sequence information are provided in **Supplemental Spreadsheet 1**

605    Several independent iPSC clones were isolated for four of the HLHS patients, and analysis

606    conducted with these independent clones generally yielded similar results (see **Figure S2D**). While

607    independent iPSC clones from one subject are often used to demonstrate reproducibility of

608    findings, one study using transcriptome profiling showed the importance of using iPSCs of

609    different parental origin rather than multiple sister iPSC clones to distinguish disease-associated

610    mechanisms from genetic background effects in disease modeling(Schuster et al., 2015).

611    **Production of iPS derived cardiomyocytes**

612    The iPSC cells were seeded on BD Matrigel pre-coated plates for 2-3 days under mTESR1

613    media then switched to CDM3 media consisting of RPMI 1640, BSA, 213 μg/ml Vitamin C

614    (Ascorbic acid) and 6 μM CHIR99021(Burridge et al., 2014). After 2 days the media was replaced

615    with CDM3 Media containing RPMI 1640, BSA, 10 μM XAV939, 213 μg/lm Vitamin C, and

616    BSA. Finally, ~14 days after initiating reprograming, beating cells are observed and further

617    analyzed in the following days (Day18-22**).**

618    **Immunofluorescence Staining**

619    Cells were fixed with 4% paraformaldehyde with 0.1% Triton X-10, followed by blocking in

620    5% goat serum, then staining overnight with primary antibody in 0.5% bovine serum

621    albumin/phosphate-buffered saline (BSA/PBS). After washing in PBS, incubation with secondary

622    antibody was performed in 0.5% BSA/PBS for 1 hour at room temperature and nuclei were stained

623    with 2 μg/ml Hoechst 33342 (Life Technologies). Images were acquired using the Leica SP8

624    confocal or Leica DMI6000-SD microscopes. Antibody information in **Supplemental**

625 **Spreadsheet 1**

626 **Analysis of mitochondrial calcium transients**

627    iPSC-CMs cultured in chamber slides were loaded with 1 μM Rhod-2 (Molecular Probes, Life

628 Technologies, Carlsbad, CA, USA) in Hank's balanced salts modified buffer (HBSS, pH 7.4) for

629 15 minutes at 37°C and washed twice for 15 minutes in HBSS. The slides were placed on a

630 temperature-regulated microscope stage and kept at 37°C. Fluorescence images were acquired

631 using the ImageJ time series analyzer package (NIH, Bethesda, MD; Version: 2.0.0-rc-69/1.52K)

632 together with Leica DMI6000-SD fluorescence microscope. The data shown represent the average

633 of Rhod-2 intensity for 3 controls and 10 HLHS patients iPS-CMs from three independent

634 experiments.

635 **Analysis of sarcomere contractility in iPSC-CM**

636    Single iPSC-CM cell videos were collected by Leica DMI 3000B microscope and videos of

637 human iPSC-CM (200 Hz) containing striated sarcomere were analyzed using a custom MATLAB

638 code (available upon request) written to apply the fast Fourier transform (FFT) algorithm to each

639 frame (approximately 1400 frames per video) to compute the spatial frequencies of the sarcomeres.

640 The frequency ($f_0$) of the highest amplitude peak of the FFT within a user defined range was

641 identified, and sarcomere length (L) in each frame was calculated by taking the reciprocal of $f_0$,

642 $L = \frac{1}{f_0}$. The user defined range was determined and optimized to ensure that the maximum and

643 minimum measured sarcomere lengths always occurred within this range. The sarcomere length

644 in units of pixels was then converted to units of micrometers, $1\mu m = 4.58$ pixels (100X

645 magnification video), and plotted as a function of time (seconds). From the plots of sarcomere

646 length versus time, the point of maximum sarcomere length immediately before contraction ($t_1$,

647 max), minimum sarcomere length (Systolic length) during contraction ($t_2$, min), and maximum

33

648    sarcomere length (Diastolic length) immediately after contraction ($t_3$, max) were identified for each

649    contraction. Fractional shortening (FS) was calculated as $FS = \frac{max-min}{max}$, contraction rate (CR)

650    was calculated as $CR = \frac{max-min}{t_2-t_1}$, and relaxation rate (RR) was calculated as $RR = \frac{max-min}{t_3-t_2}$.

651    **RNA extraction, real-time PCR, and transcript splicing analysis**

652    Total RNA was isolated using the miRNeasy micro-Kit (QIAGEN) with on-column DNase I

653    digestion (QIAGEN). cDNA was prepared with high-capacity RNA to cDNA kit (Applied

654    Biosystems). Real-time PCR was conducted using 7900HT Fast Real Time PCR System. All

655    primer sequence information is provided in **Supplemental Spreadsheet 1**

656    **Seahorse Analyzer analysis of oxygen consumption rate**

657    For cell oxygen consumption rate (OCR) quantification, 20,000 iPS-CMs or 20000 iPSCs were

658    seeded into each well of a Seahorse XFe96 cell culture plate and cultured for 2 days for adherence

659    to the culture plate. On day of measurement, the medium was changed to pre-warmed Seahorse

660    assay medium, and OCR determined using the Seahorse XF Cell Mito Stress Kit (Agilent). Basal

661    respiration was measured in unstimulated cells. Afterwards, oligomycin (1 μM) was added to

662    quantify respiration coupled to ATP production and proton leak followed by carbonyl cyanide-4-

663    (trifluoromethoxy)-phenylhydrazone (FCCP; 1 μM) injection to assess maximal cellular

664    respiration (respiratory capacity). Finally, antimycin A (1 μM) and rotenone (1 μM) were used to

665    assess non-mitochondrial respiration. For mouse and human heart tissue OCR quantification, 2mm

666    X 2mm heart tissue pieces were seeded into each well of a Seahorse XF24 islet capture microplate

667    and OCR were measured using the same Seahorse XF Cell Mito Stress Kit to obtain the basal

668    respiration rate in unstimulated cells during two cycles of measurement.

669    **Analysis of inner mitochondrial transmembrane potential**

670    Embryonic left and right ventricles were dissociated with papain to generate primary

671    cardiomyocytes for live imaging as previously described (Hom *et al.*, 2011). Briefly, this entailed

672    loading live explanted cardiomyocytes, iPSC, and iPSC-CM for 35 minutes with

673    tetramethylrhodamine ethyl ester (TMRE, 20 nM, Invitrogen, Cat# T-669) and Mito Tracker Green

674    (MTG, 200 nM, Invitrogen, Cat# M-7514) in Hepes-Tyrode's buffer, washed, and equilibrated for

675    20 minutes in the same buffer. The live cells were then imaged using epifluorescence microscopy.

676    Mitochondrial membrane potential ($\Delta\psi_m$) was quantified as the ratio of TMRE to MTG intensity

677    (Galmiche et al., 2011).

**Mitochondrial network analysis**

679    MitoTracker Red (100 μM) was loaded into live cells following manufacturer's

680    recommendations, and then cells were fixed in 4% paraformaldehyde/PBS at 37°C for 15 minutes.

681    Cells were permeabilized in 0.2% Triton X-100/PBS for 10 minutes and then immunostained for

682    cTnT while DNA was labeled with Hoechst. Mitochondria were imaged using a Leica SP8

683    confocal with a 40x / 1.3NA objective. Acquisition settings and deconvolution were done with the

684    guidance of SVI Huygens software, and images were post-processed in ImageJ (NIH, Bethesda,

685    MD; Version: 2.0.0-rc-69/1.52K) with unsharp mask (radius 2; mask weight 0.7), background

686    subtraction, and the tubeness filter (sigma = 0.25 microns) to highlight mitochondrial filaments.

687    Mitochondria were segmented with Skeletonize 2D/3D. Mitochondrial networks were then

688    analyzed ("Analyze Skeleton") using BoneJ. Clusters with 20 branches or more were used for

689    measuring average branch length and linkage statistics. To quantify the degree of mitochondrial

690    consolidation, clusters were ranked from most to least branches (see graphs) and a mono-

691    exponential decay curve is fit to the resulting data. The curve's decay constant is then inverted so

692    that higher values reflect more-linked mitochondrial networks.

693



694
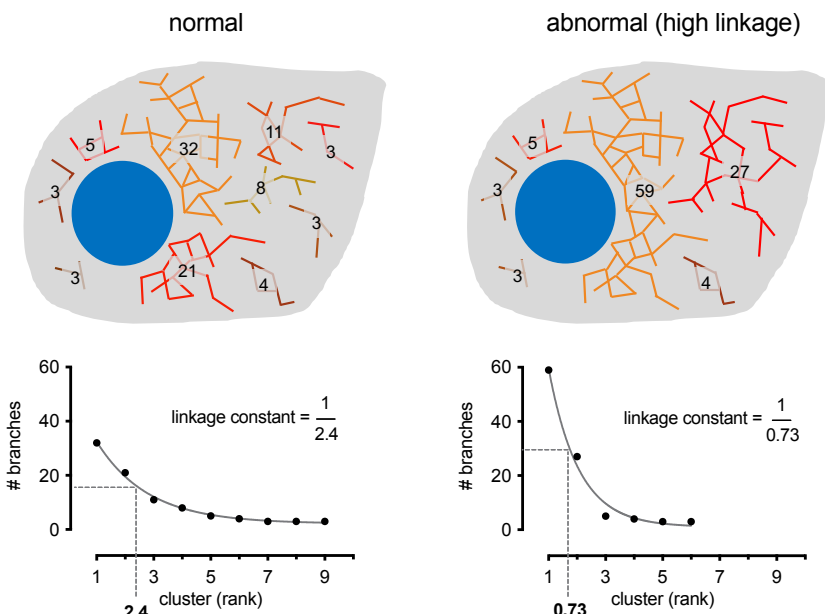
**Mitochondrial DNA copy number assays**

DNA was extracted from human iPSC-CM. qPCR was performed and mitochondrial DNA copy number was determined by normalizing results from primers targeted to mtDNA-tRNA-Leu (Forward: 5'- CAC CCA AGA ACA GGG TTT GT-3' and Reverse: 5′- TGGCCATGG GTA TGT TGT TA -3′) against results from primers targeted to nuclear B2-microglobulin (Forward: 5'- TGC TGT CTC CAT GTT TGA TGT ATC T-3' and Reverse: TCT CTG CTC CCC ACC TCT AAG T-3'(Rooney et al., 2015).

**Reactive oxygen species, nitric oxide and lysosome measurements**

To quantify reactive oxygen species (ROS), nitric oxide level and lysosome abundance, iPSC-CMs were incubated at 37 °C for 30 minutes with 5 μM MitoSOX, 5 μM DAF-FM diacetate and 1 μM LysoTracker Red DND-99 (Life Technologies), respectively. For NO measurement, additional 15–30 minutes incubation could complete de-esterification of the intracellular diacetates. CD172a(SIRPα/β) was used as a human iPSC-CM marker (Dubois et al., 2011). Live cell

708 fluorescent imaging was conducted using the Leica DMI6000-SD microscope.

**Human heart tissue and iPSC-CM Western blotting**

710 LV and RV tissue or iPSC-CM were homogenized and processed for Western blotting using a

711 ChemiDoc (Biorad) with Image J image processing (Beutner et al., 2017; Beutner et al., 2014).

712 Antibodies from Abcam and BioRad were used and included: OXPHOS Rodent Cocktail

713 (ab110413), AC (#154856), Starbright 700 (anti-mouse), Starbright 520 (anti-rabbit).

**Isolation of mitochondria and oxygen consumption assay**

715 Mitochondria where isolated on ice from fresh or frozen tissue (~140 mg) in isolation medium by

716 homogenization and differential centrifugation and resuspended in EGTA/EDTA-free isolation

717 (Beutner *et al.*, 2017; Beutner *et al.*, 2014). Oxygen consumption was measured at room

718 temperature in respiration medium with a Clark oxygen electrode (Hansatech) using published

719 protocols. Cytochrome c (50µM) and atractyloside (100µM) were used to test mitochondrial

720 membrane integrity. Substrate-mediated respiration (state 2 or V0), maximal respiration (state 3

721 or Vmax), and RCR (Vmax over V0) were calculated.

**Single-cell RNA sequencing**

723  Previous study proved there is no significance difference between iPSC-CM from day 21 and

724 day 30(funakoshi et al., 2018), hence, the iPSC-CM differentiated at day 22 were choose as

725 scRNAseq samples. The iPSC-CM from three patients and one control was prepared for single cell

726 RNAseq. The iPSC-CMs were disaggregated using cold active protease [10 mg/ml Bacillus

727 Licheniformis protease; Creative Enzymes NATE0633) and 125 U/ml DNase (Applichem, A3778)

728 incubated on ice with trituration 5-7 minutes, then 5% bovine serum albumin (BSA) was added,

729 and cells were filtered by 100 µm cell strainer and the cells pelleted, then re-suspended in 200ul

730 PBS/BSA. Trypan blue exclusion was used to quantify cell viability, and the volume was adjusted

731    to 200,000 cells/ml for 10X chromium single-cell RNA-seq. Pair-end library preparation was

732    carried out using the V3 version (10X Genomics). Single-cell droplet libraries from ~10K cells

733    from each suspension were generated using the 10X Genomics Chromium controller with the

734    Chromium Single Cell 3' GEM Library and Gel Bead Kit v.3 and the Chromium Chip B Single

735    Cell kit (1 GEMs per sample, expected recovery ~6k cells per GEM). All samples were barcoded

736    with the Chromium i7 Multiplex Kit. All libraries were pooled and sequenced across two lanes of

737    a HiSeq4000, 150bp paired end reads with a target coverage of 20k fragments per cell. All samples

738    were uniquely indexed, mixed, and evenly distributed into the Illumina HiSeq 4000 for sequencing.

739    **Single-cell RNA-Sequencing Data Analysis**

740    Single-cell sequencing data was processed using the Cell Ranger (version 3.1.0) count pipeline

741    using the human reference genome GRCh38 and annotations from Ensembl (version 93). Quality

742    control and filtering were performed using scater (McCarthy et al., 2017) (v1.18.6). For each

743    sample, cells with library size less than 500, number of detected genes less than 300 or greater than

744    6,000, or mitochondrial percentage greater than 4 times the median absolute deviation (MAD)

745    from the median value were excluded. Additionally, top 3% cells ranked by the doublet score

746    (hybrid) calculated using the scds R package (Bais and Kostka, 2020) (v1.6.9) were excluded.

747    Only non-ribosomal genes with at least 1 count in $\geq 5$ cells were considered. We adapted the

748    approach of Kannan et al. [https://doi.org/10.1101/2020.04.02.022632] for cell type classification

749    using SingleCellNet(Tan and Cahan, 2019) (v0.1.0) and further limited to cells classified as

750    "cardiac muscle cells" yielding a data for 13,954 genes across 8,094 cells. We performed

751    downstream analyses using the Seurat package (Stuart *et al.*, 2019). To focus on high-quality CMs,

752    we further removed cells with total library size less than 1,400 or number of detected genes $\leq 800$,

753     or percentage of mitochondrial gene counts greater than 20%. This yielded a final set of 877, 1,718,

754     1,434, 374 cells for 1053, 7042, 7052 and 7464, respectively, for downstream analysis.

755         We normalized total count per cell to 10,000 and find top 2000 highly variable genes in each

756     sample. Integration of cells from different samples and batch correction were performed using

757     IntegrateData function in Standard procedure of Seurat 3. Scaled data after integration was used

758     for principal component analysis (PCA) and top 30 dimensions were used for neighbor detection

759     and Louvain clustering (resolution = 0.5). UMAP was drawn for the visualization of single-cell

760     data in reduced dimensions.

761         Differentially expression analysis was conducted using student t-test in Scanpy(Wolf et al.,

762     2018). We compared differentially expressed genes of clusters and sample groups, as well as

763     samples and sample groups per cluster (Figure 5C-F,G, S5F). Genes with FDR < 0.05 in tests were

764     selected as DEGs. ToppGene(Chen et al., 2009) was used for gene enrichment analysis and Gene

765     Ontology (Biological Process) terms and coexpression of MSigDB were used for annotations of

766     gene lists. The strength of associations was represented by -log10(FDRToppGene) (Figure 5C-

767     F,G). Gene modules of cardiomyocyte clusters were generated using 200 most significantly

768     upregulated genes (Figure S5E) and their top enriched Gene Ontology (Biological Process) terms

769     were used for annotating cluster identities. Similarity between these clusters were evaluated using

770     Pearson correlation of genes in gene modules. Cell cycle scores were calculated in Seurat using

771     CellCycleScoring function and cell cycle phases were inferred accordingly (Figure S5C).

772     **Whole exome sequencing analysis**

773         Whole-exome capture was carried out on 6 Caucasian HLHS subjects with iPSC and 41 HLHS

774     subjects (including the 6 HLHS subjects) at BGI Americas. Genomic DNA from venous blood

775     was captured with Agilent V4 Exome Capture kit. Sequencing was performed on the Illumina

776    HiSeq2000 platform with 100 paired-end reads, or the Illumina HiSeq4000 with 150 paired-end

777    reads at 100× coverage. Sequence reads were mapped to the reference genome (hg19) with BWA-

778    MEM(Arakawa et al., 2010) and further processed using the GATK(McKenna et al., 2010) Best

779    Practices workflows, which include duplication marking, and base quality recalibration. Single

780    nucleotide variants (SNVs) and small indels (InDels) were detected using GATK haplotypeCaller

781    and annotated by Annovar(Wang et al., 2010).  High quality variants were recovered that: 1)

782    passed GATK Variant Score Quality Recalibration (VSQR); 2) have minimum 5 supported reads;

783    3) have genotype quality ≥ 20 or 60 for SNVs or InDels, respectively; 4) SNVs or InDels not

784    within 10bp or 5bp of an indel, respectively.

785    Variants with minor allele frequency (MAF) was less than 0.01 in GnomAD exome

786    (Karczewski et al., 2020)or Kaviar database were retained for downstream analyses. Only loss-of-

787    function (LoF) mutations (nonsense, canonical splice-site, frameshift indels, and start loss), likely

788    damaging missense variants (D-Mis) and non-frameshift indels were considered potentially

789    damaging. Missense variants were considered likely damaging if it was predicted to be damaging

790    by at least three out of nine prediction scores available via dbNSFP v3.5a (Liu et al., 2016). All

791    filter processes are shown in **Figure S7.**

792    **Functional enrichment and interactomes analysis**

793    Webgestalt KEGG pathway analysis (http://webgestalt.org/) was performed for unique LoF

794    variants from 6 HLHS cohort. The interactomes of the four genes harboring unique variants in the

795    HLHS unfavorable patient was assembled by including their protein-protein interactions (PPIs)

796    collected from BioGRID (Stark et al., 2011) and HPRD (Prasad et al., 2009), and novel PPIs

797    predicted by High-precision PPI Prediction (HiPPIP) model (Ganapathiraju et al., 2016) focusing

798    on short path connections to the Hippo pathway. Hippo pathway genes were extracted from KEGG

799     (Kanehisa et al., 2008). Enrichment Analysis Tool available on Gene Ontology (GO) website

800     which uses PANTHER (Ahmad et al., 2013) on the backend, was used to find biological process

801     terms associated with the interactome genes with statistical significance. It computes fold

802     enrichment of the genes in the input list over the expected value. Fold enrichment>1 and fold

803     enrichment<1 showed that the annotation is overrepresented and underrepresented in the list

804     respectively. It presents p-value determined by Fisher's exact test with FDR correction, and a cut-

805     off of 0.05 was used to select significantly enriched annotations.

806     **QUANTIFICATION AND STATISTICAL ANALYSIS**

807     Standard statistical analyses were performed using GraphPad Prism 9. D'Agostino & Pearson

808     normality test and Shapiro-Wilk normality test were used to test if the data had a Gaussian

809     distribution. For Gaussian distribution, data are presented as bar graphs and expressed as

810     mean±SEM, either Unpaired t-test (Two- tailed) or One-way ANOVA (FDR B&Y correction were

811     used for multiple comparisons) were applied. Data without Gaussian distribution showed by box

812     plot (Line at median and minimum-maximum were represented by the top /bottom of box), either

813     non-parametric Mann-Whitney test (Two-tailed) or Kruskal-Wallis tests (FDR B&Y correction

814     were used for multiple comparisons) were used. The experiments were not randomized. The inves-

815     tigators were not blinded to allocation during experiments and outcome assessment.

816

817    **SUPPLEMENTAL INFORMATION**

818    **Supplemental Figure 1: Mitochondrial defects in the HLHS mouse heart tissue and HLHS**

819    **mouse iPSC-CM.** Related to Figure 1.

820    **Supplemental Figure 2: Generating iPSC and iPSC-CM from HLHS patients and control**

821    **subjects** Related to Figure 1.

822    **Supplemental Figure 3: Mitochondrial defects and altered Hippo signaling** Related to Figure

823    2&3.

824    **Supplemental Figure 4: Inhibition of the mitochondrial permeability transition pore rescues**

825    **mitochondrial respiration and YAP1 nuclear localization.** Related to Figure 4.

826    **Supplemental Figure 5: Analysis of HLHS patient iPSC-CM using single-cell RNAseq**

827    Related to Figure 5.

828    **Supplemental Figure 6: Protein-Protein Interactome of Genes Highly Expressed Only in**

829    **Patient 7052.** Related to Figure 5.

830    **Supplemental Figure 7: Whole exome sequencing analysis of pathogenic variants show**

831    **enrichment for metabolic-mitochondrial pathways in Group II HLHS patients** Related to

832    Figure 7.

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840 **Supplemental Spreadsheet 1: Patient information, iPSC production, antibodies, and primer**

841 **sequences. Related to STAR Methods.**

842 1. miPSCs generated and used in this study

843 2. HLHS patient medical history

844 3. Patient iPSCs reprogramming

845 4. Human and mouse primer sequences.

846 **Supplemental Spreadsheet 2: Single cell RNAseq related information. Related to Figure5,6.**

847 1. Marker gene list for C0-8 (Figure S5 E)

848 2.1-2.9. GO enrichment analysis for Cluster 0-8 (Figure S5 E)

849 2.10. HCM related genes in Clusters C3 and C8 (Figure 5 A)

850 3.1. DEG No. in Each Cluster (Figure 5 B)

851 3.2-3.10. GO enrichment analysis of DEG in C0/C1/C5 under different comparisons (Figure 5 C-

852 E)

853 4.1 All DEGs in Figure 5F

854 4.2 Toppgene of Control 1053 DEGs (Figure 5F)

855 4.3 Toppgene of Patient 7464 DEG (Figure 5F)

856 4.4 Toppgene of Group II 7042/7052 Shared DEG (Figure 5F)

857 4.5 Toppgene of Patient 7042 DEG (Figure 5F)

858 4.6 Toppgene of Patient 7052 DEG (Figure 5F)

859 5.1. Mitochondrial DEGs (Figure 5H)

860 5.2 Toppgene of Control 1053 Mitochondrial DEGs (Figure 5H)

861 5.3. Toppgene analysis of Patient 7464 Mitochondrial – DEGs (Figure 5H)

862 5.4. Toppgene of Group II Mitochondrial-DEG (Figure 5H)

863    5.5. Toppgene of Patient 7042 Mitochondrial-DEG (Figure 5H)

864    5.6. Toppgene of Patient 7052 Mitochondrial-DEG (Figure 5H)

865    6. Patient 7052 - 28 upregulated DEGs (Figure 5G)

866    7.1. Protein-Protein Interactome network genes (Figure S6A)

867    7.2. PPI BiNGO Biological Process Pathway Enrichment (Figure 5I)

868    8.1-8.2. GO enrichment analysis of DEG of Group II VS Control in C7 (Figure 6A)

869    **Supplemental Spreadsheet 3: Whole exome sequencing and interactome analysis related**

870    **information. Related to Figure7.**

871    1. Description

872    2. Unique LoF genes from Group II patient (Figure 7A)

873    3. LoF (loss of function) variants from Group II patient (Figure 7A)

874    4. Webgestalt/KEGG pathway enrichment of unique LoF genes from Group II patient (*OXA1L,*

875    *NNMT, NEU3, ALDH7A1*) (Figure 7A)

876    5. Protein-protein interactome GO Biological Processes

877    6. GO Biological Processes in Figure 7B

878    7. Genes in GO Biological Processes in Figure 7B.

879    8. Unique gene with variants in 41 HLHS cohort (Figure 7C)

880    9. Extreme variant list in 41 HLHS cohort (Figure 7C)

881    10. Metascape-GoEnriched (Figure 7C)

882    11. Mitochondrial gene list (Figure 7D)

883    12. Damaging variants in mitochondrial-related genes (Figure 7D)

884    13. Overlapping mitochondrial and unique genes in 41 HLHS cohort (Figure 7D)

885    14. Toppgene analysis of those 19 overlapping genes (Figure 7D)

886 **SUPPLEMENTAL VIDEO LEGEND**

887 **Supplemental Videos**

888 Sup-video-1_hips-cm_beating-Related to Figure1

889 Sup-video-2_hips-cm_Ca-Related to Figure1

890 Sup-video-3_hips-cm_single_cell-Related to Figure1

891

892 **Sup-video-1_hips-cm_beating:** Videomicroscopy showing contraction of human iPSC-CM. The

893 iPSC-CM from control subject and Group I beat faster than iPSC-CM from Group II. Scale bar =

894 250 μm.

895

896 **Sup-video-2_hips-cm_Ca:** Calcium transients in the iPSC-CM are visualized using Rhod-2. Note

897 faster propagation of calcium transients in iPSC-CM from control subject and Group I patients as

898 compared to that of Group II. Scale bar = 250 μm.

899

900 **Sup-video-3_hips-cm_single_cell:** Videomicroscopy recording of individual beating iPSC-CM

901 from control subject, Group I and Group II patients. Robust contractions are seen in

902 cardiomyocytes from control and Group I, but only weak contractions are seen in Group II. Scale

903 bar = 10 μm.

904

## REFERENCE

Ahmad, A., Maitah, M.Y., Ginnebaugh, K.R., Li, Y., Bao, B., Gadgeel, S.M., and Sarkar, F.H. (2013). Inhibition of Hedgehog signaling sensitizes NSCLC cells to standard therapies through modulation of EMT-regulating miRNAs. J Hematol Oncol *6*, 77. 10.1186/1756-8722-6-77.

Alsoufi, B., Mahle, W.T., Manlhiot, C., Deshpande, S., Kogon, B., McCrindle, B.W., and Kanter, K. (2016). Outcomes of heart transplantation in children with hypoplastic left heart syndrome previously palliated with the Norwood procedure. J Thorac Cardiovasc Surg *151*, 167-174, 175 e161-162. 10.1016/j.jtcvs.2015.09.081.

Arakawa, K., Kudo, T., Ikawa, M., Morikawa, N., Kawai, Y., Sahashi, K., Lee, J.D., Kuriyama, M., Miyamori, I., Okazawa, H., and Yoneda, M. (2010). Abnormal myocardial energy-production state in mitochondrial cardiomyopathy and acute response to L-arginine infusion. C-11 acetate kinetics revealed by positron emission tomography. Circ J *74*, 2702-2711.

Artap, S., Manderfield, L.J., Smith, C.L., Poleshko, A., Aghajanian, H., See, K., Li, L., Jain, R., and Epstein, J.A. (2018). Endocardial Hippo signaling regulates myocardial growth and cardiogenesis. Dev Biol *440*, 22-30. 10.1016/j.ydbio.2018.04.026.

Ascah, A., Khairallah, M., Daussin, F., Bourcier-Lucas, C., Godin, R., Allen, B.G., Petrof, B.J., Des Rosiers, C., and Burelle, Y. (2011). Stress-induced opening of the permeability transition pore in the dystrophin-deficient heart is attenuated by acute treatment with sildenafil. Am J Physiol Heart Circ Physiol *300*, H144-153. 10.1152/ajpheart.00522.2010.

Bais, A.S., and Kostka, D. (2020). scds: computational annotation of doublets in single-cell RNA sequencing data. Bioinformatics *36*, 1150-1158. 10.1093/bioinformatics/btz698.

Beutner, G., Alanzalon, R.E., and Porter, G.A. (2017). Cyclophilin D regulates the dynamic assembly of mitochondrial ATP synthase into synthasomes. Sci Rep *7*. ARTN 14488 10.1038/s41598-017-14795-x.

Beutner, G., Eliseev, R.A., and Porter, G.A. (2014). Initiation of Electron Transport Chain Activity in the Embryonic Heart Coincides with the Activation of Mitochondrial Complex 1 and the Formation of Supercomplexes. PLoS One *9*. ARTN e113330 10.1371/journal.pone.0113330.

Biendarra-Tiegs, S.M., Li, X., Ye, D., Brandt, E.B., Ackerman, M.J., and Nelson, T.J. (2019). Single-Cell RNA-Sequencing and Optical Electrophysiology of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes Reveal Discordance Between Cardiac Subtype-Associated Gene Expression Patterns and Electrophysiological Phenotypes. Stem Cells Dev *28*, 659-673. 10.1089/scd.2019.0030.

Burridge, P.W., Matsa, E., Shukla, P., Lin, Z.C., Churko, J.M., Ebert, A.D., Lan, F., Diecke, S., Huber, B., Mordwinkin, N.M., et al. (2014). Chemically defined generation of human cardiomyocytes. Nat Methods *11*, 855-860. 10.1038/nmeth.2999.

Calvo, S.E., Compton, A.G., Hershman, S.G., Lim, S.C., Lieber, D.S., Tucker, E.J., Laskowski, A., Garone, C., Liu, S., Jaffe, D.B., et al. (2012). Molecular diagnosis of infantile mitochondrial disease with targeted next-generation sequencing. Sci Transl Med *4*, 118ra110. 10.1126/scitranslmed.3003310.

Chen, J., Bardes, E.E., Aronow, B.J., and Jegga, A.G. (2009). ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. Nucleic Acids Res *37*, W305-311. 10.1093/nar/gkp427.

Cleves, M.A., Ghaffar, S., Zhao, W., Mosley, B.S., and Hobbs, C.A. (2003). First-year survival of infants born with congenital heart defects in Arkansas (1993-1998): a survival analysis using

950  registry data. Birth Defects Res A Clin Mol Teratol *67*, 662-668. 10.1002/bdra.10119.

951  Cui, Y., Zheng, Y., Liu, X., Yan, L., Fan, X., Yong, J., Hu, Y., Dong, J., Li, Q., Wu, X., et al. (2019).

952  Single-Cell Transcriptome Analysis Maps the Developmental Track of the Human Heart. Cell Rep

953  *26*, 1934-1950 e1935. 10.1016/j.celrep.2019.01.079.

954  Cyganek, L., Tiburcy, M., Sekeres, K., Gerstenberg, K., Bohnenberger, H., Lenz, C., Henze, S.,

955  Stauske, M., Salinas, G., Zimmermann, W.H., et al. (2018). Deep phenotyping of human induced

956  pluripotent stem cell-derived atrial and ventricular cardiomyocytes. JCI Insight *3*.

957  10.1172/jci.insight.99941.

958  Driscoll, D.J., Offord, K.P., Feldt, R.H., Schaff, H.V., Puga, F.J., and Danielson, G.K. (1992). Five-

959  to fifteen-year follow-up after Fontan operation. Circulation *85*, 469-496. 10.1161/01.cir.85.2.469.

960  Dubois, N.C., Craft, A.M., Sharma, P., Elliott, D.A., Stanley, E.G., Elefanty, A.G., Gramolini, A.,

961  and Keller, G. (2011). SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived

962  from human pluripotent stem cells. Nat Biotechnol *29*, 1011-U1082. 10.1038/nbt.2005.

963  Elia, A.E., Lalli, S., Monsurro, M.R., Sagnelli, A., Taiello, A.C., Reggiori, B., La Bella, V.,

964  Tedeschi, G., and Albanese, A. (2016). Tauroursodeoxycholic acid in the treatment of patients with

965  amyotrophic lateral sclerosis. Eur J Neurol *23*, 45-52. 10.1111/ene.12664.

966  Feinstein, J.A., Benson, D.W., Dubin, A.M., Cohen, M.S., Maxey, D.M., Mahle, W.T., Pahl, E.,

967  Villafane, J., Bhatt, A.B., Peng, L.F., et al. (2012). Hypoplastic left heart syndrome: current

968  considerations and expectations. J Am Coll Cardiol *59*, S1-42. 10.1016/j.jacc.2011.09.022.

969  Fortini, P., Ferretti, C., Iorio, E., Cagnin, M., Garribba, L., Pietraforte, D., Falchi, M., Pascucci, B.,

970  Baccarini, S., Morani, F., et al. (2016a). The fine tuning of metabolism, autophagy and

971  differentiation during in vitro myogenesis. Cell Death Dis *7*, e2168. 10.1038/cddis.2016.50.

972  Fortini, P., Iorio, E., Dogliotti, E., and Isidoro, C. (2016b). Coordinated Metabolic Changes and

973  Modulation of Autophagy during Myogenesis. Front Physiol *7*, 237. 10.3389/fphys.2016.00237.

974  funakoshi, s., , M.N., , C.O., , K.M., , T.K., , S.Y., , A.W., and , Y.Y. (2018). Abstract 16184: Single

975  Cell RNA Sequencing Reveals Dynamic and Heterogeneous Changes of Transcriptome During

976  Cardiac Differentiation in vitro. Circulation *2014;130:A16184*.

977  Fusaki, N., Ban, H., Nishiyama, A., Saeki, K., and Hasegawa, M. (2009). Efficient induction of

978  transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus

979  that does not integrate into the host genome. P Jpn Acad B-Phys *85*, 348-362. 10.2183/pjab.85.348.

980  Gaber, N., Gagliardi, M., Patel, P., Kinnear, C., Zhang, C., Chitayat, D., Shannon, P., Jaeggi, E.,

981  Tabori, U., Keller, G., and Mital, S. (2013). Fetal reprogramming and senescence in hypoplastic

982  left heart syndrome and in human pluripotent stem cells during cardiac differentiation. Am J Pathol

983  *183*, 720-734. 10.1016/j.ajpath.2013.05.022.

984  Galie, N., Ghofrani, H.A., Torbicki, A., Barst, R.J., Rubin, L.J., Badesch, D., Fleming, T., Parpia,

985  T., Burgess, G., Branzi, A., et al. (2005). Sildenafil citrate therapy for pulmonary arterial

986  hypertension. N Engl J Med *353*, 2148-2157. 10.1056/NEJMoa050010.

987  Galmiche, L., Serre, V., Beinat, M., Assouline, Z., Lebre, A.S., Chretien, D., Nietschke, P., Benes,

988  V., Boddaert, N., Sidi, D., et al. (2011). Exome sequencing identifies MRPL3 mutation in

989  mitochondrial cardiomyopathy. Hum Mutat *32*, 1225-1231. 10.1002/humu.21562.

990  Ganapathiraju, M.K., Thahir, M., Handen, A., Sarkar, S.N., Sweet, R.A., Nimgaonkar, V.L.,

991  Loscher, C.E., Bauer, E.M., and Chaparala, S. (2016). Schizophrenia interactome with 504 novel

992  protein-protein interactions. Npj Schizophr *2*. UNSP 16012

993  10.1038/npjschz.2016.12.

994  Garcia, A.M., Beatty, J.T., and Nakano, S.J. (2020). Heart failure in single right ventricle

995  congenital heart disease: physiological and molecular considerations. Am J Physiol Heart Circ

996    Physiol *318*, H947-H965. 10.1152/ajpheart.00518.2019.

997    Gentles, T.L., Gauvreau, K., Mayer, J.E., Jr., Fishberger, S.B., Burnett, J., Colan, S.D., Newburger,
998    J.W., and Wernovsky, G. (1997). Functional outcome after the Fontan operation: factors
999    influencing late morbidity. J Thorac Cardiovasc Surg *114*, 392-403; discussion 404-395.
1000   10.1016/s0022-5223(97)70184-3.

1001   Guglin, M., Rajagopalan, N., Anaya, P., and Charnigo, R. (2016). Sildenafil in heart failure with
1002   reactive pulmonary hypertension (Sildenafil HF) clinical trial (rationale and design). Pulm Circ *6*,
1003   161-167. 10.1086/685548.

1004   Guimaraes-Camboa, N., Stowe, J., Aneas, I., Sakabe, N., Cattaneo, P., Henderson, L., Kilberg,
1005   M.S., Johnson, R.S., Chen, J., McCulloch, A.D., et al. (2015). HIF1alpha Represses Cell Stress
1006   Pathways to Allow Proliferation of Hypoxic Fetal Cardiomyocytes. Dev Cell *33*, 507-521.
1007   10.1016/j.devcel.2015.04.021.

1008   Heallen, T., Morikawa, Y., Leach, J., Tao, G., Willerson, J.T., Johnson, R.L., and Martin, J.F. (2013).
1009   Hippo signaling impedes adult heart regeneration. Development *140*, 4683-4690.
1010   10.1242/dev.102798.

1011   Hom, J.R., Quintanilla, R.A., Hoffman, D.L., de Mesy Bentley, K.L., Molkentin, J.D., Sheu, S.S.,
1012   and Porter, G.A., Jr. (2011). The permeability transition pore controls cardiac mitochondrial
1013   maturation and myocyte differentiation. Dev Cell *21*, 469-478. 10.1016/j.devcel.2011.08.008.

1014   Hrstka, S.C.L., Li, X., Nelson, T.J., and Pipeline, W.P.G. (2017). NOTCH1-Dependent Nitric
1015   Oxide Signaling Deficiency in Hypoplastic Left Heart Syndrome Revealed Through Patient-
1016   Specific Phenotypes Detected in Bioengineered Cardiogenesis. Stem Cells *35*, 1106-1119.
1017   10.1002/stem.2582.

1018   Hsu, D.T., Zak, V., Mahony, L., Sleeper, L.A., Atz, A.M., Levine, J.C., Barker, P.C., Ravishankar,
1019   C., McCrindle, B.W., Williams, R.V., et al. (2010). Enalapril in infants with single ventricle: results
1020   of a multicenter randomized trial. Circulation *122*, 333-340.
1021   10.1161/CIRCULATIONAHA.109.927988.

1022   Huang, S., Wang, X., Wu, X., Yu, J., Li, J., Huang, X., Zhu, C., and Ge, H. (2018). Yap regulates
1023   mitochondrial structural remodeling during myoblast differentiation. Am J Physiol Cell Physiol
1024   *315*, C474-C484. 10.1152/ajpcell.00112.2018.

1025   Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D., and Yamamoto, M. (1999).
1026   Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to
1027   the amino-terminal Neh2 domain. Genes Dev *13*, 76-86. 10.1101/gad.13.1.76.

1028   Jiang, Y., Habibollah, S., Tilgner, K., Collin, J., Barta, T., Al-Aama, J.Y., Tesarov, L., Hussain, R.,
1029   Trafford, A.W., Kirkwood, G., et al. (2014a). An Induced Pluripotent Stem Cell Model of
1030   Hypoplastic Left Heart Syndrome (HLHS) Reveals Multiple Expression and Functional
1031   Differences in HLHS-Derived Cardiac Myocytes. Stem Cells Transl Med *3*, 416-423.
1032   10.5966/sctm.2013-0105.

1033   Jiang, Y., Habibollah, S., Tilgner, K., Collin, J., Barta, T., Al-Aama, J.Y., Tesarov, L., Hussain, R.,
1034   Trafford, A.W., Kirkwood, G., et al. (2014b). An induced pluripotent stem cell model of
1035   hypoplastic left heart syndrome (HLHS) reveals multiple expression and functional differences in
1036   HLHS-derived cardiac myocytes. Stem Cells Transl Med *3*, 416-423. 10.5966/sctm.2013-0105.

1037   Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., Katayama, T., Kawashima,
1038   S., Okuda, S., Tokimatsu, T., and Yamanishi, Y. (2008). KEGG for linking genomes to life and the
1039   environment. Nucleic Acids Res *36*, D480-D484. 10.1093/nar/gkm882.

1040   Karczewski, K.J., Francioli, L.C., Tiao, G., Cummings, B.B., Alfoldi, J., Wang, Q., Collins, R.L.,
1041   Laricchia, K.M., Ganna, A., Birnbaum, D.P., et al. (2020). The mutational constraint spectrum

1042    quantified from variation in 141,456 humans. Nature *581*, 434-443. 10.1038/s41586-020-2308-7.

1043    Kobayashi, J., Yoshida, M., Tarui, S., Hirata, M., Nagai, Y., Kasahara, S., Naruse, K., Ito, H., Sano,
1044    S., and Oh, H. (2014). Directed differentiation of patient-specific induced pluripotent stem cells
1045    identifies the transcriptional repression and epigenetic modification of NKX2-5, HAND1, and
1046    NOTCH1 in hypoplastic left heart syndrome. PLoS One *9*, e102796.
1047    10.1371/journal.pone.0102796.

1048    Liu, X., Wu, C., Li, C., and Boerwinkle, E. (2016). dbNSFP v3.0: A One-Stop Database of
1049    Functional Predictions and Annotations for Human Nonsynonymous and Splice-Site SNVs. Hum
1050    Mutat *37*, 235-241. 10.1002/humu.22932.

1051    Liu, X., Yagi, H., Saeed, S., Bais, A.S., Gabriel, G.C., Chen, Z., Peterson, K.A., Li, Y., Schwartz,
1052    M.C., Reynolds, W.T., et al. (2017). The complex genetics of hypoplastic left heart syndrome. Nat
1053    Genet *49*, 1152-1159. 10.1038/ng.3870.

1054    Marin-Garcia, J., and Akhmedov, A.T. (2016). Mitochondrial dynamics and cell death in heart
1055    failure. Heart Fail Rev *21*, 123-136. 10.1007/s10741-016-9530-2.

1056    Martel, C., Huynh le, H., Garnier, A., Ventura-Clapier, R., and Brenner, C. (2012). Inhibition of
1057    the Mitochondrial Permeability Transition for Cytoprotection: Direct versus Indirect Mechanisms.
1058    Biochem Res Int *2012*, 213403. 10.1155/2012/213403.

1059    McCarthy, D.J., Campbell, K.R., Lun, A.T.L., and Wills, Q.F. (2017). Scater: pre-processing,
1060    quality control, normalization and visualization of single-cell RNA-seq data in R. Bioinformatics
1061    *33*, 1179-1186. 10.1093/bioinformatics/btw777.

1062    McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K.,
1063    Altshuler, D., Gabriel, S., Daly, M., and DePristo, M.A. (2010). The Genome Analysis Toolkit: a
1064    MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res *20*,
1065    1297-1303. 10.1101/gr.107524.110.

1066    Meng, Z., Moroishi, T., and Guan, K.L. (2016). Mechanisms of Hippo pathway regulation. Genes
1067    Dev *30*, 1-17. 10.1101/gad.274027.115.

1068    Miao, Y., Tian, L., Martin, M., Paige, S.L., Galdos, F.X., Li, J., Klein, A., Zhang, H., Ma, N., Wei,
1069    Y., et al. (2020). Intrinsic Endocardial Defects Contribute to Hypoplastic Left Heart Syndrome.
1070    Cell Stem Cell *27*, 574-589 e578. 10.1016/j.stem.2020.07.015.

1071    Mills, R.J., Titmarsh, D.M., Koenig, X., Parker, B.L., Ryall, J.G., Quaife-Ryan, G.A., Voges, H.K.,
1072    Hodson, M.P., Ferguson, C., Drowley, L., et al. (2017). Functional screening in human cardiac
1073    organoids reveals a metabolic mechanism for cardiomyocyte cell cycle arrest. Proc Natl Acad Sci
1074    U S A *114*, E8372-E8381. 10.1073/pnas.1707316114.

1075    Myung, S.K., Ju, W., Cho, B., Oh, S.W., Park, S.M., Koo, B.K., Park, B.J., and Korean Meta-
1076    Analysis Study, G. (2013). Efficacy of vitamin and antioxidant supplements in prevention of
1077    cardiovascular disease: systematic review and meta-analysis of randomised controlled trials. BMJ
1078    *346*, f10. 10.1136/bmj.f10.

1079    Nakano, H., Minami, I., Braas, D., Pappoe, H., Wu, X., Sagadevan, A., Vergnes, L., Fu, K.,
1080    Morselli, M., Dunham, C., et al. (2017). Glucose inhibits cardiac muscle maturation through
1081    nucleotide biosynthesis. Elife *6*. 10.7554/eLife.29330.

1082    Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., Hong, H., Nakagawa,
1083    M., Tanabe, K., Tezuka, K., et al. (2011). A more efficient method to generate integration-free
1084    human iPS cells. Nat Methods *8*, 409-U452. 10.1038/Nmeth.1591.

1085    Oster, M.E., Lee, K.A., Honein, M.A., Riehle-Colarusso, T., Shin, M., and Correa, A. (2013).
1086    Temporal trends in survival among infants with critical congenital heart defects. Pediatrics *131*,
1087    e1502-1508. 10.1542/peds.2012-3435.

1088  Pagliarini, D.J., Calvo, S.E., Chang, B., Sheth, S.A., Vafai, S.B., Ong, S.E., Walford, G.A., Sugiana,
1089  C., Boneh, A., Chen, W.K., et al. (2008). A mitochondrial protein compendium elucidates complex
1090  I disease biology. Cell *134*, 112-123. 10.1016/j.cell.2008.06.016.
1091  Paige, S.L., Galdos, F.X., Lee, S., Chin, E.T., Ranjbarvaziri, S., Feyen, D.A.M., Darsha, A.K., Xu,
1092  S., Ryan, J.A., Beck, A.L., et al. (2020). Patient-Specific Induced Pluripotent Stem Cells Implicate
1093  Intrinsic Impaired Contractility in Hypoplastic Left Heart Syndrome. Circulation *142*, 1605-1608.
1094  10.1161/CIRCULATIONAHA.119.045317.
1095  Perrelli, M.G., Pagliaro, P., and Penna, C. (2011). Ischemia/reperfusion injury and cardioprotective
1096  mechanisms: Role of mitochondria and reactive oxygen species. World J Cardiol *3*, 186-200.
1097  10.4330/wjc.v3.i6.186.
1098  Pile, L.A., Spellman, P.T., Katzenberger, R.J., and Wassarman, D.A. (2003). The SIN3 deacetylase
1099  complex represses genes encoding mitochondrial proteins: implications for the regulation of
1100  energy metabolism. J Biol Chem *278*, 37840-37848. 10.1074/jbc.M305996200.
1101  Prabhu, L.V., Rai, R., Ranade, A.V., Kini, H., Krishnamurthy, A., and Bernhardt, K.L. (2013).
1102  Effect of sildenafil-induced nitric oxide on the histomorphology of cardiomyocytes in male rats.
1103  Int J Appl Basic Med Res *3*, 84-87. 10.4103/2229-516X.117056.
1104  Prasad, T.S.K., Goel, R., Kandasamy, K., Keerthikumar, S., Kumar, S., Mathivanan, S.,
1105  Telikicherla, D., Raju, R., Shafreen, B., Venugopal, A., et al. (2009). Human Protein Reference
1106  Database-2009 update. Nucleic Acids Res *37*, D767-D772. 10.1093/nar/gkn892.
1107  Rooney, J.P., Ryde, I.T., Sanders, L.H., Howlett, E.H., Colton, M.D., Germ, K.E., Mayer, G.D.,
1108  Greenamyre, J.T., and Meyer, J.N. (2015). PCR based determination of mitochondrial DNA copy
1109  number in multiple species. Methods Mol Biol *1241*, 23-38. 10.1007/978-1-4939-1875-1_3.
1110  Schiattarella, G.G., Altamirano, F., Tong, D., French, K.M., Villalobos, E., Kim, S.Y., Luo, X.,
1111  Jiang, N., May, H.I., Wang, Z.V., et al. (2019). Nitrosative stress drives heart failure with preserved
1112  ejection fraction. Nature *568*, 351-356. 10.1038/s41586-019-1100-z.
1113  Schuster, J., Halvardson, J., Pilar Lorenzo, L., Ameur, A., Sobol, M., Raykova, D., Anneren, G.,
1114  Feuk, L., and Dahl, N. (2015). Transcriptome Profiling Reveals Degree of Variability in Induced
1115  Pluripotent Stem Cell Lines: Impact for Human Disease Modeling. Cell Reprogram *17*, 327-337.
1116  10.1089/cell.2015.0009.
1117  Shaddy, R.E., Boucek, M.M., Hsu, D.T., Boucek, R.J., Canter, C.E., Mahony, L., Ross, R.D., Pahl,
1118  E., Blume, E.D., Dodd, D.A., et al. (2007). Carvedilol for children and adolescents with heart
1119  failure: a randomized controlled trial. JAMA *298*, 1171-1179. 10.1001/jama.298.10.1171.
1120  Stark, C., Breitkreutz, B.J., Chatr-Aryamontri, A., Boucher, L., Oughtred, R., Livstone, M.S.,
1121  Nixon, J., Van Auken, K., Wang, X., Shi, X., et al. (2011). The BioGRID Interaction Database:
1122  2011 update. Nucleic Acids Res *39*, D698-704. 10.1093/nar/gkq1116.
1123  Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W.M., 3rd, Hao, Y.,
1124  Stoeckius, M., Smibert, P., and Satija, R. (2019). Comprehensive Integration of Single-Cell Data.
1125  Cell *177*, 1888-1902 e1821. 10.1016/j.cell.2019.05.031.
1126  Tan, Y., and Cahan, P. (2019). SingleCellNet: A Computational Tool to Classify Single Cell RNA-
1127  Seq Data Across Platforms and Across Species. Cell Syst *9*, 207-213 e202.
1128  10.1016/j.cels.2019.06.004.
1129  Tao, G., Kahr, P.C., Morikawa, Y., Zhang, M., Rahmani, M., Heallen, T.R., Li, L., Sun, Z., Olson,
1130  E.N., Amendt, B.A., and Martin, J.F. (2016). Pitx2 promotes heart repair by activating the
1131  antioxidant response after cardiac injury. Nature *534*, 119-123. 10.1038/nature17959.
1132  Tsutsui, H., Kinugawa, S., and Matsushima, S. (2011). Oxidative stress and heart failure. Am J
1133  Physiol Heart Circ Physiol *301*, H2181-2190. 10.1152/ajpheart.00554.2011.

1134    Tweddell, J.S., Hoffman, G.M., Mussatto, K.A., Fedderly, R.T., Berger, S., Jaquiss, R.D.,
1135    Ghanayem, N.S., Frisbee, S.J., and Litwin, S.B. (2002). Improved survival of patients undergoing
1136    palliation of hypoplastic left heart syndrome: lessons learned from 115 consecutive patients.
1137    Circulation *106*, I82-89.
1138    Ventura-Clapier, R., Garnier, A., Veksler, V., and Joubert, F. (2011). Bioenergetics of the failing
1139    heart. Bba-Mol Cell Res *1813*, 1360-1372. 10.1016/j.bbamcr.2010.09.006.
1140    von Gise, A., Lin, Z., Schlegelmilch, K., Honor, L.B., Pan, G.M., Buck, J.N., Ma, Q., Ishiwata, T.,
1141    Zhou, B., Camargo, F.D., and Pu, W.T. (2012). YAP1, the nuclear target of Hippo signaling,
1142    stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. Proc Natl Acad
1143    Sci U S A *109*, 2394-2399. 10.1073/pnas.1116136109.
1144    Wang, K., Li, M., and Hakonarson, H. (2010). ANNOVAR: functional annotation of genetic
1145    variants from high-throughput sequencing data. Nucleic Acids Res *38*, e164. 10.1093/nar/gkq603.
1146    Wolf, F.A., Angerer, P., and Theis, F.J. (2018). SCANPY: large-scale single-cell gene expression
1147    data analysis. Genome Biol *19*, 15. 10.1186/s13059-017-1382-0.
1148    Xinxiu Xu, T.T., Jiuann-Huey Ivy Lin, Phillips Adams, Xiaoqin Liu, Feinstein Timothy N , Omar
1149    Khalifa, George A Porter Jr, Sruti S. Shiva, Cecilia W. Lo (2018). Intrinsic Cardiomyocyte
1150    Mitochondrial Defects Underlie Cardiac Dysfunction and Heart Failure Risk Associated with
1151    Hypoplastic Left Heart Syndrome. Circulation. *2018;138:A15746*.
1152    Xu, X., Wang, Q., Long, Y., Zhang, R., Wei, X., Xing, M., Gu, H., and Xie, X. (2013). Stress-
1153    mediated p38 activation promotes somatic cell reprogramming. Cell Res *23*, 131-141.
1154    10.1038/cr.2012.143.
1155    Ye, Y., Li, J., and Yuan, Z. (2013). Effect of antioxidant vitamin supplementation on cardiovascular
1156    outcomes: a meta-analysis of randomized controlled trials. PLoS One *8*, e56803.
1157    10.1371/journal.pone.0056803.
1158    Zhou, Q., Li, L., Zhao, B., and Guan, K.L. (2015). The hippo pathway in heart development,
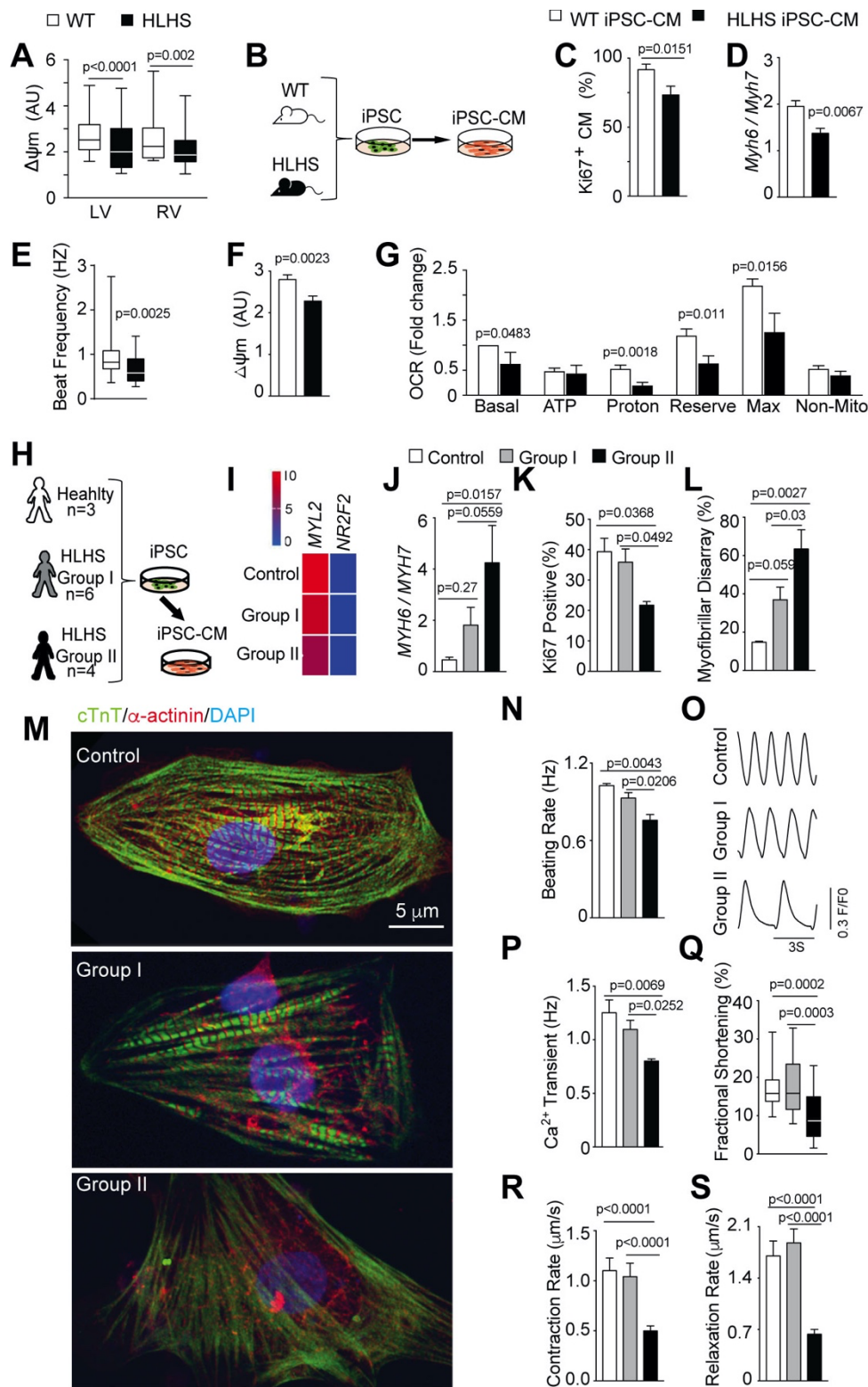1159    regeneration, and diseases. Circ Res *116*, 1431-1447. 10.1161/CIRCRESAHA.116.303311.
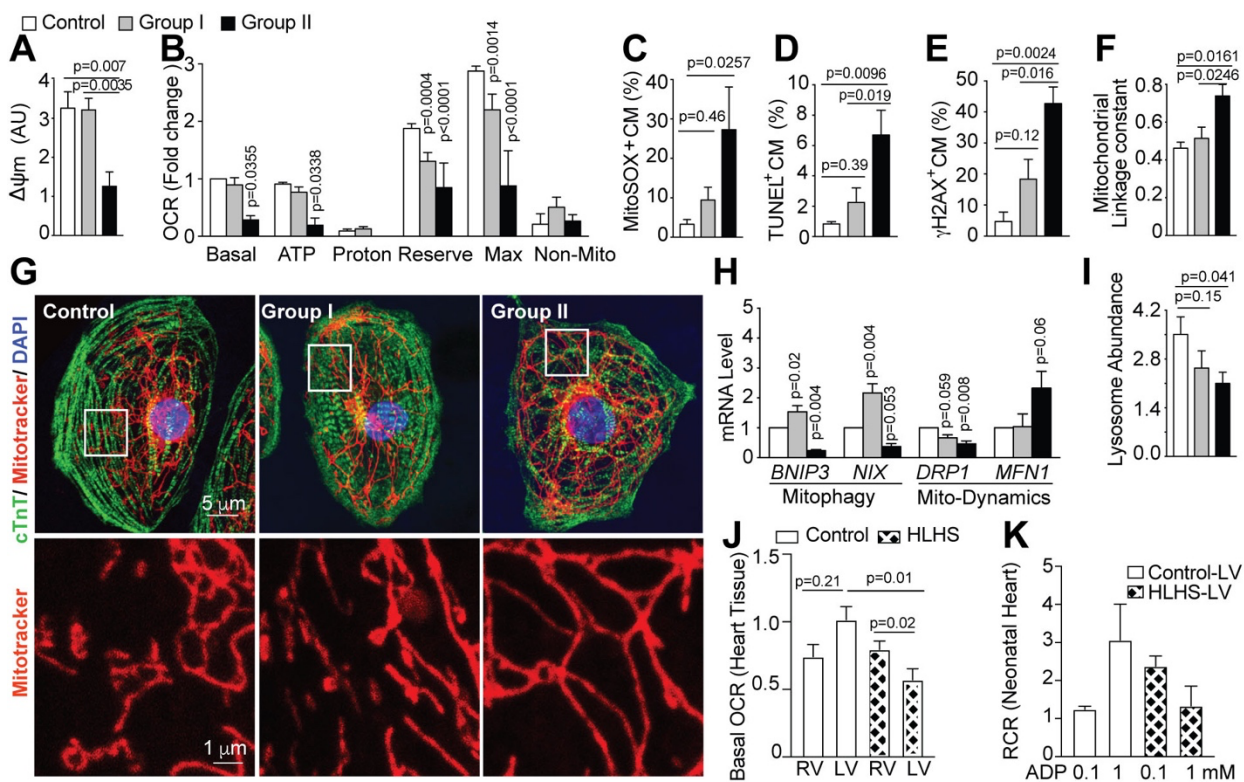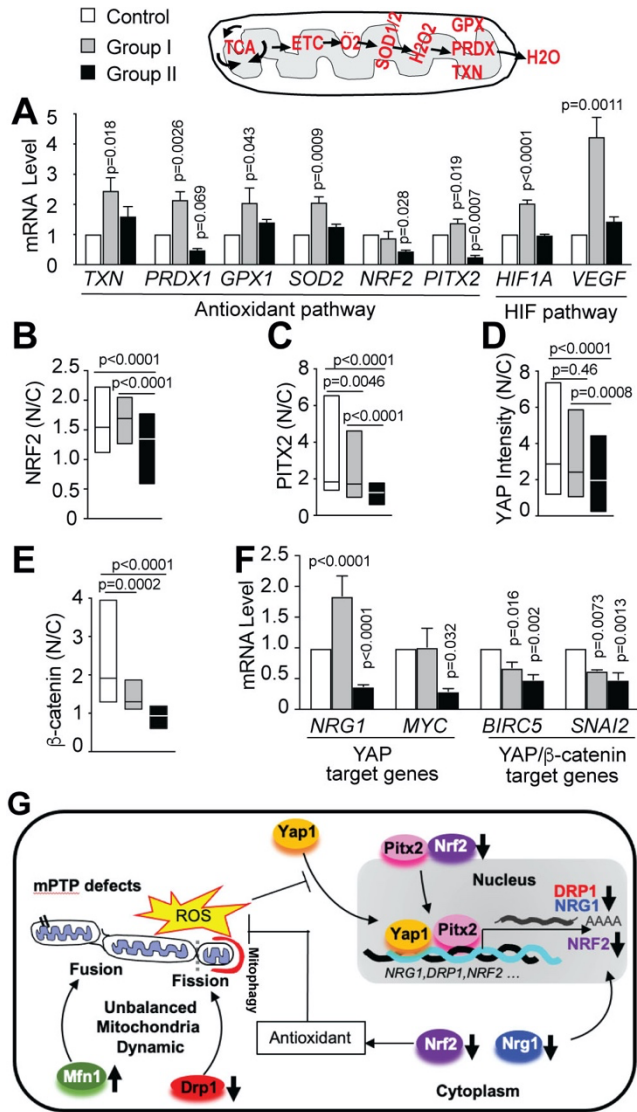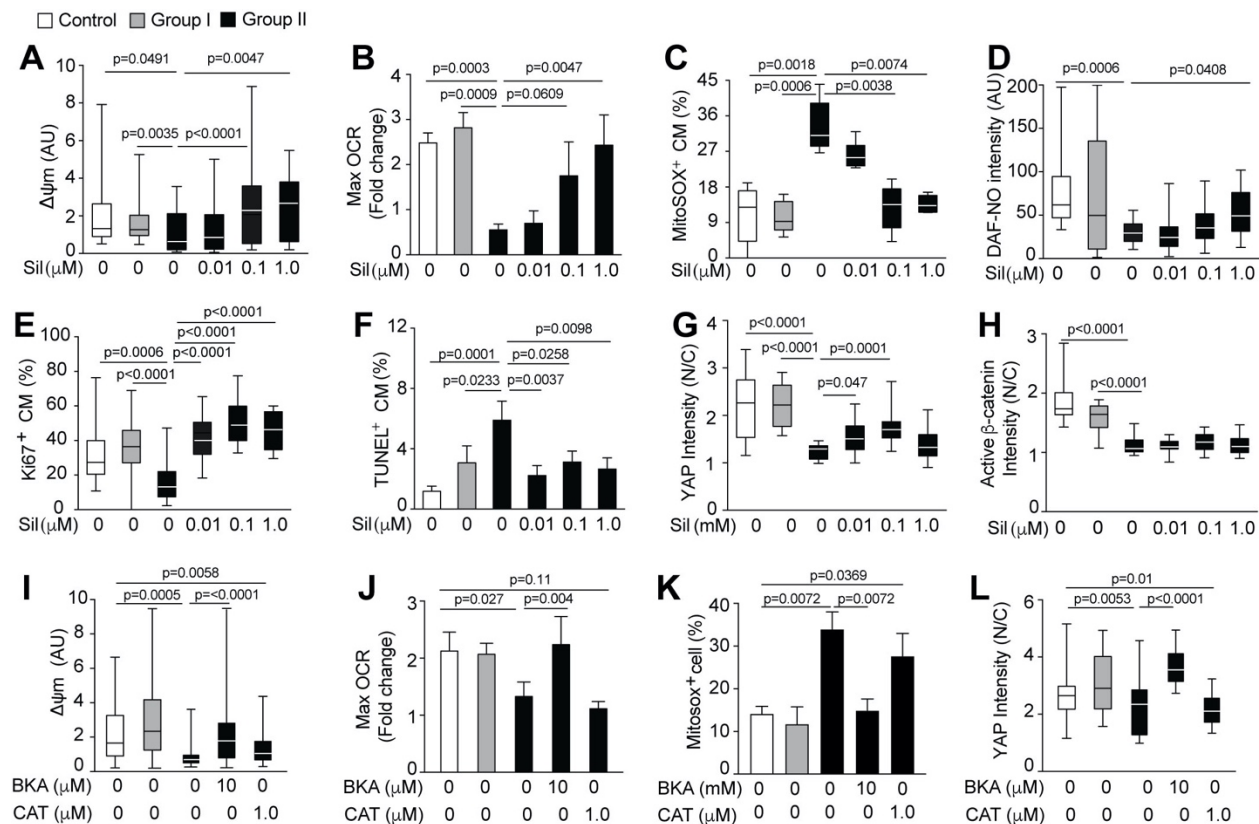1160

Fig1

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