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1 **Mitochondrial 4-HNE derived from MAO-A promotes mitoCa²⁺ overload**
2 **in chronic post-ischemic cardiac remodeling**
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30 **Abstract**

31 Chronic remodeling post-myocardial infarction consists in various maladaptive changes including
32 interstitial fibrosis, cardiomyocyte death and mitochondrial dysfunction that lead to Heart Failure (HF).
33 Reactive aldehydes such as 4-hydroxynonenal (4-HNE) are critical mediators of mitochondrial
34 dysfunction but the sources of mitochondrial 4-HNE in cardiac diseases together with its mechanisms of
35 action remain poorly understood. Here, we evaluated whether the mitochondrial enzyme monoamine
36 oxidase-A (MAO-A), which generates H₂O₂ as a by-product of catecholamine metabolism, is a source of
37 deleterious 4-HNE in HF.

38 We found that MAO-A activation increased mitochondrial ROS and promoted local 4-HNE production
39 inside the mitochondria through cardiolipin peroxidation in primary cardiomyocytes. Deleterious effects
40 of MAO-A/4-HNE on cardiac dysfunction were prevented by activation of mitochondrial aldehyde
41 dehydrogenase 2 (ALDH2), the main enzyme for 4-HNE metabolism. Mechanistically, MAO-A-derived
42 4-HNE bound to newly identified targets VDAC and MCU to promote ER-mitochondria contact sites and
43 MCU higher-order complex formation. The resulting mitochondrial Ca²⁺ accumulation participated in
44 mitochondrial respiratory dysfunction and loss of membrane potential, as shown with the protective
45 effects of the MCU inhibitor, RU360. Most interestingly, these findings were recapitulated in a chronic
46 model of ischemic remodeling where pharmacological or genetic inhibition of MAO-A protected the mice
47 from 4-HNE accumulation, MCU oligomer formation and Ca²⁺ overload, thus mitigating ventricular
48 dysfunction.

49 To our knowledge, these are the first evidences linking MAO-A activation to mitoCa²⁺ mishandling
50 through local 4-HNE production, contributing to energetic failure and post-ischemic remodeling.

51

52

53

54 **INTRODUCTION**

55 Post-myocardial infarction (MI) remodeling is a detrimental mechanism leading to Heart Failure (HF) and
56 poor patient outcome. Although it is known that chronic remodeling consists in various maladaptive
57 changes such as cardiomyocyte death, interstitial fibrosis, inflammation and contractile dysfunction, there
58 is still a strong need to better understand underlying pathological processes in order to develop novel
59 therapeutic strategies. Mitochondrial dysfunction has emerged as a key player in post-MI ventricular
60 failure and new advances put forward that mitochondria-targeted therapeutics may lead to improved
61 energy production in the heart and better contractile function (1). Mitochondria control key processes of
62 cardiac remodeling such as ATP production, lipid metabolism, cell survival/death and calcium (Ca^{2+})
63 buffering (2). Uptake of cytosolic Ca^{2+} into mitochondrial matrix regulates important enzymes of the
64 Krebs cycle and electron transport chain and is necessary for matching energy supply to the demand (3).
65 Hence, deregulation of mitochondrial Ca^{2+} (mito Ca^{2+}) levels contribute to energetic failure in rodent
66 models of HF, implying that its concentration needs to be fine-tuned thanks to the activity of the mito Ca^{2+}
67 uniporter (MCU) and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCLX)(4, 5). In the particular situation of chronic
68 ischemia, it is still unclear how mito Ca^{2+} is regulated and contributes to cardiac remodeling, especially
69 when it is accompanied by another stressor, oxidative stress (3).

70 Mitochondria are the main source of cellular reactive oxygen species (ROS) and, when exceeding
71 antioxidant capacities, ROS can lead to the oxidation of fatty acids in a process termed lipid peroxidation
72 (6). Mounting evidence has linked lipid peroxidation with various human pathological states, such as
73 neurodegenerative diseases, cancer, diabetes and HF. 4-hydroxy-trans-2-nonenal (4-HNE) is the most
74 abundant lipid peroxidation product and forms adducts with proteins, thereby affecting their biological
75 function to impair cellular homeostasis (7). Interestingly, 4-HNE levels are elevated in plasma of HF
76 patients and inversely correlate with cardiac function (8). Furthermore, enhancing the degradation of
77 mitochondrial 4-HNE is beneficial in rodent models of acute and chronic heart diseases (9, 10). At
78 present, there is a strong need to identify the source of mitochondrial 4-HNE in pathological states and to
79 understand the underlying mechanisms of its toxicity on mitochondrial function.

80 The monoamine oxidase-A (MAO-A) is an enzyme located at the outer mitochondrial membrane which
81 represents the main degradative pathway for catecholamines and serotonin. MAO-A generates H₂O₂ as a
82 by-product of substrate metabolism and has been recognized as a significant source of mitochondrial ROS
83 in the heart (11, 12). During post-MI remodeling, the sustained sympathetic drive and chronic elevation of
84 circulating catecholamines could fuel MAO-A activity and ROS generation leading to 4-HNE production.
85 To date, this putative role of MAO-A in post-MI remodeling has never been investigated.

86 In the present study, we found that MAO-A was responsible for an intra-mitochondrial generation of 4-
87 HNE that, in turn, bound to voltage-dependent anion channel (VDAC) and MCU proteins, leading to
88 mitoCa²⁺ overload and impaired mitochondrial function. In ischemic failing human heart samples, there
89 was a concomitant increase in MAO-A levels and 4-HNE-bound proteins and selective inactivation of
90 MAO-A in mice with chronic MI prevented cardiac remodeling, 4-HNE accumulation and mitoCa²⁺
91 overload.

92

93 **METHODS**

94 *Human heart tissues*

95 All studies are conformed to the Declaration of Helsinki and institutional ethical regulations. Informed
96 consent was obtained from all subjects. Human failing hearts samples were a kind gift from Dr J.L Samuel
97 (Lariboisière Hospital, France). Explanted failing hearts were obtained from patients undergoing cardiac
98 transplantation for end-stage cardiac HF secondary to ischemic cardiomyopathy. All patients had New
99 York Heart Association (NYHA) Class IV HF, with a mean pretransplant left ventricular EF of 22±4 %.
100 None had received chronic intravenous inotropic support over at least 7 days immediately prior to
101 transplantation. HF therapy consisted of angiotensin converting enzyme inhibitors and diuretics in all
102 patients. Non-failing hearts were obtained from prospective multiorgan donors who had died from head
103 trauma or intracranial bleeds; these hearts were unsuitable for transplantation for technical reasons. All
104 tissues were stored at -80 °C until further analyses.

105

106 ***Animal models***

107 All animal procedures were performed in accordance with International Guidelines on Animal
108 Experimentation and with a French Ministry of Agriculture license. Moreover, this investigation
109 conformed to the guide for Care and Use of Laboratory Animals published by the Directive 2010/63/EU
110 of the European Parliament. All mice were housed in temperature-controlled cages with a 12-h light-dark
111 cycle and given free access to water and food. *MAO-A cKO mice*: maoa gene on Chromosome X was
112 floxed with two LoxP sites around Ex12 in the laboratory of Jean C. Shih (University of Southern
113 California, CA, USA)⁽¹³⁾. The Myh6-Cre mice expressing Cre recombinase in cardiomyocytes were
114 purchased from Janvier labs. The males with cardiomyocyte-specific deletion of Maoa ($Maoa^{fl/Y}xCre^+$,
115 referred as MAO-A cKO) and their control littermates ($Maoa^{WT/Y}xCre^+$, referred as WT) on the C57Bl6J
116 background were obtained by crossing $Maoa^{WT/Y}xCre^+$ males with $Maoa^{WT/fl}$ females. *MAO-A Tg mice*:
117 MAO-A transgenic mice (MAO-A Tg) on the C57Bl6J background were maintained by breeding of
118 MAO-A Tg males (Myh6-maoa) with C57Bl6/J females (12). MAO-A Tg males and their WT littermates
119 were used for the experiments. *AAV9 model*: AAV9-cTnT-GFP and AAV9-cTnT-ALDH2 were purchased
120 from Penn Vector Core (Pennsylvania, USA). AAV9-cTnT-ALDH2 was made from the cDNA of mouse
121 ALDH2 cloned into pCMV6-Entry vector (OriGene, Rockville, USA). The AAV9 vectors were injected
122 by i.v. in the jugular vein of 3 week-old mice under anesthesia at a dose of 3×10^{11} vg/mouse. Mice were
123 kept for 4 months to assess cardiac remodeling.

124

125 ***Myocardial ischemia.***

126 Mice were anaesthetized by 2% isoflurane inhalation. After orotracheal intubation, mice were
127 mechanically ventilated (minivent type845, harvard apparatus) with 100% oxygen. Analgesia was
128 provided by buprenorphine injection (0.02 mg/kg s.c.) and local lidocaine injection (50 μ L of 10 mg/mL
129 solution). Electrocardiogram (ADInstrument) was used to monitor the mice during the procedure. A left
130 thoracotomy was performed at the 4th intercostal space to expose the heart. After opening of the

131 pericardium using an 8-0 pvsd suture, the left anterior descending (LAD) coronary artery was tied 1 mm
132 below the tip of the left auricle. Ischemia was verified by the sudden regional paleness of the myocardium
133 and ST elevation. The chest cavity was closed by bringing the ribs together with 6-0 suture while pressure
134 was applied at the level of the xyphoid process. Muscle and skin layers were closed and the anesthesia was
135 stopped. Mice were extubated at first sign of waking up and allowed to recover in a heating box. Only
136 LAD ligation was omitted in the SHAM procedure. *Moclobemide treatment*: moclobemide was given 15
137 min after coronary artery ligation by IP injection at a dose of 20 mg/kg. In the following day,
138 moclobemide was given in the drinking water at a concentration of 20/mg/kg/day and kept during the 4
139 remaining weeks. The number of animals required for the study has been evaluated from the first
140 validation experiment performed on a small cohort of mice (n=12). Power analysis was conducted to
141 estimate the appropriate sample size by setting the probability of a Type I error (α) at 0.05, power at 0.95,
142 and effect size at 1,5.

143 ***Echocardiography***

144 Mice were anesthetized with 2% isoflurane and examined with noninvasive echocardiography (Vivid 7
145 ultrasound, GE; vevo2100 Visual Sonics). Cardiac ventricular dimensions were measured in a blinded
146 fashion on M-MODE/2D images for the number of animals indicated.

147

148 ***Whole heart imaging***

149 Heart optical clearing was performed as follow. Formalin-fixed hearts were dehydrated with three
150 successive immersions in 100% methanol solutions. Samples were transferred into methanol/BABB
151 solution (one part benzyl alcohol, two parts benzyl benzoate and three parts of methanol) then in BABB
152 solution (one part benzyl alcohol and two parts benzyl benzoate), and incubated for 2 days. Cleared hearts
153 were imaged using Light-Sheet Fluorescence Microscopy (LSFM) as described in Abadie *et al.* (14).
154 Images were processed with the open-source image-processing Fiji. 3D volume rendering, segmentation

155 and measurements were performed with Amira ® software (FEI Visualization Sciences Group,
156 Hillsborough, OR, USA).

157
158 ***Primary cultures of cardiomyocytes***
159 *Neonatal rat ventricular myocytes (NRVM) isolation:* Neonatal rats of 1–2 days old were euthanized by
160 decapitation. The heart was excised and the atria were removed. Primary culture of NRVMs was
161 subsequently performed as previously described (15). NRVMs were transduced with an adenovirus
162 expressing rat MAO-A under the control of the CMV promoter to drive expression of MAO-A. 24h later,
163 the medium was replaced with Ham-F12 medium supplemented with 3% dialyzed FBS, and
164 pharmacological treatments were performed.

165 *Adult Mice Cardiomyocytes (AMCMs):* The heart was quickly excised, and the aorta was cannulated for
166 retrograde perfusion in a Langendorff apparatus at a constant flow rate of 3 mL/min at 37°C as previously
167 described (16). Freshly isolated cardiomyocytes were plated on laminin-coated culture dishes in M199
168 complete medium (M199 medium with added 100 IU/mL penicillin, 2 mmol/L L-carnitine, 5 mmol/L
169 creatine and 5 mmol/L taurine) at 37 °C in a humidified atmosphere with 5 % CO₂. The culture protocol
170 yielded an average of 80 % rod-shaped myocytes at a plating density of 50 cells/mm² that were viable at
171 pH 7.2 for 48 h.

172
173 ***Mitochondria isolation***
174 Hearts were excised, washed in phosphate-buffered saline (PBS), and crushed using a tissue grinder in
175 mitochondrial isolation buffer (0,3 M sucrose, 5 mM TES, 200 µM EGTA) supplemented with Proteinase
176 bacterial (Sigma-Aldrich). Mitochondrial isolation buffer supplemented with BSA was then added to the
177 homogenates before centrifugation at 500g for 10 min at 4°C. Supernatant was collected and centrifuged
178 at 3000g for 10 min at 4°C and pellet corresponding to mitochondria was either immediately used (for
179 mitoH₂O₂, mitoNAD⁺/NADH, mitoCa²⁺ measurements) or kept at -80°C (for western blots, LC-MS/MS
180 for cardiolipin and HODEs).

181
182 ***Immunofluorescence and histological studies***
183 Heart tissues were embedded in optimal cutting temperature compound (OCT) (Sigma-Aldrich) under ice-
184 cold 2-methylbutane. For immunofluorescence studies, frozen sections (5 µm) were fixed in 4%
185 paraformaldehyde, followed by permeabilization and blocking in PBS with 0.02% FBS, 1% bovine serum
186 albumine and 0.3% Triton X-100 at RT. Sections were immunostained overnight with the following
187 antibodies: anti-4-HNE (Abcam 46545), anti-Vinculin (Sigma-Aldrich V9131) followed by secondary
188 Alexafluor antibodies (Molecular Probes). Nuclei were visualized with DAPI. Images were acquired by
189 confocal Microscope Zeiss LSM 780 and ZEN image analysis software (Zeiss) or digitized with a
190 Hamamatsu NanoZoomer and fluorescence intensity was quantified by ImageJ software. Average
191 cardiomyocyte diameter and the number of cardiomyocytes per total myocardial area were measured
192 manually after vinculin staining (500-600 cells counted per heart) on 6 different regions of the peri-infarct
193 zone. For fibrosis, hearts were transversely sectioned at 10 µm thickness, fixed with 4% paraformaldehyde
194 and stained with Masson's Trichrome. Fibrosis was measured as positively stained area with Masson's
195 Trichrome and expressed as percent of total area, using ImageJ software (RSB). For NRVMs, cells stained
196 with MitoTracker™ Red (Thermo Fischer Scientific M22425) were fixed with 4% PFA and incubated in
197 TBS-0,2% triton O/N at 4°C with 4-HNE antibody. After secondary antibody (anti-rabbit IgG alexa-fluor
198 488, Invitrogen), slides were observed under confocal microscopy (LSM780, Carl Zeiss).

199
200 ***Western blot***

201 Total extracts of ventricle or NRVMs were lysed in RIPA buffer (50mM Tris pH 7.2, 500mM NaCl, 1%
202 Triton X-100, 1mM EDTA, 100mM sodium fluoride, 5mM sodium metavanadate, 10mM sodium
203 pyrophosphate) and equal protein amounts were subjected to SDS-PAGE. After electrophoresis, proteins
204 were transferred to PVDF membranes and immunoblotted with the following antibodies: anti-4-HNE
205 (Abcam 46545), anti-MAO-A (Abcam 126751), anti-ALDH2 (Abcam 108306), anti-GAPDH (CST5174),
206 anti-Vinculin (Sigma V9131), anti-COX-IV (CST4844), anti-CBARA1/MICU1 (CST12524), anti-

207 EFHA1/MICU2 (Abcam 101465), anti-MCU (CST14997), anti-VDAC1 (CST4866). Proteins were
208 detected by chemiluminescence with a Bio-Rad ChemiDoc. Relative densities were quantified using the
209 ImageLab 5.2.1 software (Bio-Rad). All data were normalized by internal controls.

210
211 ***Immunoprecipitation***
212 NRVMs, AMCMs or heart tissues were lysed in buffer containing 20 mmol/L Tris, pH 7.5, 150 mmol/L
213 NaCl, 1 mmol/L EDTA, 1% Triton X100, protease and phosphatase cocktail inhibitors (Roche). Proteins
214 were put in agitation O/N at 4°C with 5-10 µg of appropriated antibodies. Purification steps were
215 performed with protein A/G agarose according to the manufacturer's instructions (SantaCruz
216 Biotechnologies). For improved Western blotting detection to avoid heavy and light chains signals, blots
217 were incubated 2 h at RT with a Protein G-HRP (dilution 1:5000, Biorad), prepared in blocking buffer.
218 After washing with TBST, blots were developed with ECL Plus (Amersham Biosciences).

219
220 ***ALDH2 activity, GSH measurements and ATP content***
221 ALDH2 activity was determined in ventricular homogenates or NRVMs using ALDH2 assay kit (Abcam).
222 GSH content was evaluated in ventricle samples using "Total Glutathione detection kit" from Assay
223 Designs (Enzo life sciences). Intracellular ATP content was determined by measuring the conversion of
224 D-luciferin to oxyluciferin using ATP bioluminescence assay kit II according to the manufacturer's
225 instructions (Roche).

226
227 ***Mitochondrial H₂O₂***
228 Cells were incubated with mitoPY1 probe (Mitochondria Peroxy Yellow-1, Sigma) at a final
229 concentration of 10 µM at 37°C for 60 min. Mitochondrial specificity was confirmed by co-staining with
230 MITO-ID Red (Enzo life sciences). Representative pictures were taken with a confocal microscope
231 (LSM780, Carl Zeiss). Mitochondrial H₂O₂ production in mouse hearts was measured using fluorescent

232 probe Amplex Red (10 $\mu\text{mol/L}$) in the presence of 0.6 U/ml horseradish peroxidase (excitation and
233 emission wavelengths set to 530 and 590 nm, respectively) on isolated mitochondria.

234
235 ***Mitochondrial membrane potential ($\Delta\Psi\text{m}$)***
236 TMRE (Tetramethylrhodamine, ethyl ester) probe was added to each well at a final concentration of 500
237 nM at 37°C for 30 min. Then, medium was replaced by HBSS and the fluorescence was recorded in the
238 presence or not of FCCP at Ex/Em = 549/575 nm, using a fluorimeter TECAN infinite pro F200.

239
240 ***Mitochondrial respiration***
241 Oxygen consumption rate (OCR) was measured in AMCMs with a Seahorse XFe24 Analyzer (Agilent).
242 Cells were plated in laminin-coated Seahorse 24-well assay plates (2500 cells per well) in M199 complete
243 medium. After 2 h of cell attachment, cell medium was replaced with XF base medium supplemented with
244 10 mM glucose, 4 mM L-glutamine and 1 mM sodium pyruvate (pH 7.4) and maintained at 37°C for 1h.
245 The wells of a hydrated sensor cartridge were then loaded with 1 μM oligomycin (port A), 1 μM FCCP
246 (port B) and 1 μM antimycin A + 1 μM rotenone (port C). Data were analyzed with Seahorse Wave
247 software. Maximal respiratory capacity was calculated by subtracting the non-mitochondrial OCR
248 (minimum rate measurement after antimycin A/rotenone injection) from the OCR after FCCP injection.
249 Spare respiratory capacity was calculated by subtracting the baseline OCR from the OCR after FCCP
250 injection.

251
252 ***Measurement of mitochondrial Ca^{2+} uptake***
253 Intact cardiomyocytes or freshly isolated mitochondria were suspended in Tyrode buffer and incubated
254 with 10 $\mu\text{mol/L}$ Rhod-2-acetoxymethyl ester (Rhod-2-AM; Life Technologies, France) for 30 min at 4°C,
255 and then for 30 min at 37°C(16). With this cold loading/warm incubation procedure, Rhod-2-AM
256 localized to mitochondria. Typically, successive 1ml aliquots of cells/mitochondria suspension were
257 loaded and experimented on for the same amount of time. Fluorescence (λ excitation: 540 nm, λ emission:

258 605nm) was acquired from cells/mitochondria treated or not with Rhod-2-AM on a Varioskan Flash
259 Multimode Microplate Reader for 270 sec. In each experiment, 20 $\mu\text{mol/L}$ CaCl_2 was added. Experiments
260 were performed at least with 5-6 mice/group.

261

262 ***Duolink Proximity Ligation In Situ Assay***

263 Briefly adult mouse cardiomyocytes were fixed with 4 % paraformaldehyde and permeabilized with 0.1 %
264 Triton X-100. Subsequent blocking, antibody hybridizations, proximity ligations, and detections were
265 performed according to recommendations from manufacturers (OLINK Bioscience, Sigma–Aldrich,
266 France). The cells were incubated with the primary antibodies overnight at 4°C and then washed 3 times
267 with TBS–0.05 % Tween 20. Briefly, after incubation with primary antibodies, we applied combinations
268 of corresponding *in situ* proximity ligation assay (PLA) probes for 1 h at 37°C. The cells were washed
269 with TBS–0.05 % Tween 20, incubated for 30 min with ligase, and finally washed with TBS–0.05%
270 Tween 20. Then cells were incubated with polymerase for 100 min. Fluorescence was analyzed with a
271 laser confocal Zeiss LSM780 microscope. Quantification of signals was performed with BlobFinder
272 software (Center for Image Analysis, Uppsala University) and expressed as interactions per cell relative to
273 the non-treated group. Experiments were performed at least 4 times, with a minimum of 6-8 fields taken
274 per condition.

275

276 ***Size-exclusion Chromatography***

277 Cells were washed in PBS and lysed in IP buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1 mmol/L
278 EDTA, 1 % Triton X100, protease and phosphatase inhibitors cocktail (Roche). Using a AKTA purifier 10
279 (GE Life Science), lysates were injected (200 μL per injection) onto a Superose 6 10/300GL column (GE
280 Life Sciences) equilibrated with IP Buffer (pH 7.5). The flow rate was 0.2 mL.min⁻¹ and 40x400 μL
281 fractions were collected using a low protein binding 96-deep-well plate Eppendorf (Hamburg, Germany)
282 and tested on western blots. The system was previously calibrated with High molecular weight markers
283 (GE Life Science).

284
285 **Statistical analysis**
286 Statistical analysis was carried out using Student's t-test or 2-way ANOVA with the Tukey post hoc test,
287 when appropriate. Lung edema was analyzed statistically using chi square test. The results are shown as
288 the mean \pm SEM. Values of $p < 0.05$ were considered to be significant.

289
290 **RESULTS**

291 ***MAO-A activation promotes intra-mitochondrial 4-HNE formation through cardiolipin peroxidation***

292 We first sought to determine whether monoamine oxidase-A (MAO-A) influenced mitochondrial 4-HNE.
293 Application of tyramine (Tyr), a MAO substrate, led to 4-HNE mitochondrial accumulation in adult mice
294 cardiomyocytes (AMCMs), as revealed by co-staining of 4-HNE with the mitochondrial marker Mito-ID
295 (Fig. 1A, Supp Fig. 1A). In transgenic mice with cardiac overexpression of MAO-A, a model of oxidative
296 stress and HF (MAO-A Tg) (12), isolated mitochondria showed accumulation of 4-HNE-bound proteins
297 compared to WT mice (Fig. 1B). 4-HNE can be formed upon ROS-dependent peroxidation of lipids, in
298 particular cardiolipin (CL) the signature phospholipids of mitochondrial inner membrane, via the synthesis
299 of hydroxyoctadecadienoic acids (HODEs) (17). We thus investigated whether MAO-A favored intra-
300 mitochondrial 4-HNE accumulation through ROS-dependent CL peroxidation. In AMCMs, application of
301 Tyr for 30 min increased mitochondrial H_2O_2 , as shown by overlapped staining of the H_2O_2 -sensitive
302 probe MitoPY1 with Mito-ID red (Fig. 1C, Supp Fig. 1B). Consistently, mitochondria of MAO-A Tg
303 hearts also displayed elevated levels of H_2O_2 compared to WT (Fig. 1D). In neonatal rat ventricular
304 myocytes (NRVMs) transduced with MAO-A adenovirus, Tyr-induced mito H_2O_2 accumulation preceded
305 the accumulation of 4-HNE (at 1 h and later) (Supp Fig. 1C-E). We next performed LC-MS/MS to analyze
306 mitochondrial content in cardiolipins and HODEs. The major species tetralinoleoylcardiolipin (L₄CL or
307 72:8) was significantly decreased in MAO-A Tg mitochondria compared to WT (Fig. 1E, Supp Fig. 1F).
308 This is particularly interesting since L₄CL is the most abundant CL in mammalian tissues, and is the only
309 form made of four linoleic moieties, the main precursor of 4-HNE. Consequently, we observed an increase

310 in 12(S), 13(S) and 9(S) species of HODEs, which are different oxidation products of linoleic acid (Fig.
311 1F). Our results indicate that MAO-A-dependent ROS generation promotes intra-mitochondrial formation
312 of 4-HNE through L₄CL peroxidation and HODEs intermediate formation (Fig. 1G).

313
314 ***MitoALDH2 mitigates MAO-A-induced cardiac dysfunction***

315 In order to evaluate if 4-HNE could mediate the deleterious effects of MAO-A, we overexpressed
316 mitochondrial ALDH2, the main enzyme for 4-HNE detoxification, in hearts of MAO-A Tg mice using a
317 cardiotropic AAV9-ALDH2 (Fig. 2A). Major 4-HNE detoxification systems were inhibited in Tg hearts,
318 as shown by a decrease in cardiac glutathione content (GSH) and mitochondrial ALDH2 activity (Fig. 2B-
319 C). As expected, AAV9-ALDH2 restored basal level of ALDH2 activity and reduced 4-HNE
320 accumulation in MAO-A Tg hearts (Fig. 2D-F). Importantly, this effect of ALDH2 was associated with a
321 reduction in reactive hypertrophy and interstitial fibrosis (Fig. 2E-H). An improved cardiac function was
322 also observed in AAV9-ALDH2 Tg mice compared to AAV9-GFP Tg mice, as demonstrated by the
323 echocardiographic data (Fig. 2I-J, Table 1). These results show that mitochondrial ALDH2 protects from
324 MAO-A-induced 4-HNE accumulation and cardiac dysfunction in MAO-A Tg.

325
326 ***MAO-A-induced 4-HNE accumulation alters mitochondrial function***

327 As we demonstrated that mitochondrial 4-HNE mediated the deleterious effects of MAO-A, we next
328 sought to investigate by which downstream mechanisms this occurred. Quantitative mass spectrometry-
329 based (nanoLC-MS/MS) proteomic study after immunoprecipitation with an anti-4-HNE antibody
330 identified 53 proteins enriched at least 2-fold in MAO-A Tg vs WT cardiomyocytes, with a cutoff p-value
331 <0.05 (Table 2). The majority of proteins were mitochondrial, confirming the existence of an organelle-
332 specific regulation of 4-HNE downstream MAO-A. Analysis of over-represented networks of 4-HNE-
333 bound proteins with Reactome (<https://reactome.org/>) identified five major pathways that belong to
334 mitochondrial metabolism (respiratory electron transport, citric acid cycle), mitoCa²⁺ transport, glucose
335 metabolism and muscle contraction (Supp Fig. 2). We thus assessed the functional relevance of these

336 pathways in response to MAO-A/4-HNE axis in AMCMs of WT mice. Activation of MAO-A with Tyr
337 altered mitochondrial respiration, as shown by the impairment in oxygen consumption rate (OCR), an
338 effect prevented with the selective MAO-A inhibitor moclobemide and with Alda-1, an allosteric activator
339 of ALDH2 (Fig. 3A-B, Supp Fig. 3A-D). Interestingly, direct application of 4-HNE on AMCMs was
340 sufficient to impair mitochondrial respiration (Supp Fig. 4). A decrease in ATP concentration was
341 observed after Tyr stimulation, and this effect was prevented by Moclobemide and Alda-1 (Fig. 3C).
342 MAO-A activation also decreased NAD⁺/NADH ratio and altered mitochondrial membrane potential but
343 not in the presence of Moclobemide and Alda-1 (Fig. 3D-E). Altogether, our data show that mitochondrial
344 4-HNE mediates the deleterious effects of MAO-A on mitochondrial function.

345

346 ***MAO-A/4-HNE axis induces mitochondrial Ca²⁺ accumulation***

347 Because mitoCa²⁺ handling proteins were identified as potential 4-HNE targets (Supp Fig. 2), we
348 measured the effects of MAO-A/4-HNE on mitoCa²⁺. Cardiomyocytes or mitochondria of MAO-A Tg
349 mice showed elevated levels of mitoCa²⁺ compared to WT mice, as shown by the colocalization of the
350 fluorescent probe Rhod-2-AM with MitoTracker (Fig. 4A, 4B, 4C). Interestingly, activation of MAO-A
351 with Tyr in WT AMCMs or mitochondria induced mitoCa²⁺ accumulation, an effect prevented with
352 moclobemide or Alda-1 (Fig. 4D, 4E, 4F).

353 MitoCa²⁺ uptake is regulated at different levels. It is driven by close interactions between the endoplasmic
354 reticulum (ER) and the mitochondria called MAMs, resulting in the transfer of Ca²⁺ through the outer
355 membrane by VDACs, followed by the accumulation Ca²⁺ into the matrix via the MCU on the inner
356 membrane (18). As observed in the proteomic study, an increased association between VDAC1 and 4-
357 HNE was shown by co-immunoprecipitation experiments in AMCMs stimulated with Tyr (Fig. 5A). We
358 evaluated the interactions between ER and mitochondria, which involve associations of VDAC1, IP3R1
359 and GRP75. Proximity Ligation Assay showed that AMCMs of MAO-A Tg mice displayed increased
360 interactions between VDAC1/IP3R1, VDAC1/GRP75 and IP3R1/GRP75 compared to WT mice,
361 potentially favoring Ca²⁺ transfer (Fig. 5B-E). Interestingly, in WT AMCMs, histamine, a known

362 stimulator of IP3R-mediated Ca^{2+} transfer from ER to mitochondria, enhanced Tyr-induced mito Ca^{2+}
363 accumulation (Fig. 5F, 5G). On the other hand, Xestospongin C, a specific inhibitor of IP3R, reduced Tyr-
364 mediated mito Ca^{2+} transfer (Fig. 5F, 5G). Thus, MAO-A/4-HNE axis may potentiate Ca^{2+} transfer through
365 increased interactions between ER and mitochondria and enhanced Ca^{2+} outer membrane transfer through
366 VDAC1.

367

368 ***4-HNE binding to MCU induces Mito Ca^{2+} overload and mitochondrial dysfunction***

369 Ca^{2+} transport through the inner membrane occurs via the MCU, which is regulated by its two main
370 partners MICU1 and MICU2 (19). We investigated whether MCU also participated in mito Ca^{2+}
371 accumulation in response to MAO-A/4-HNE. Tyr-mediated mito Ca^{2+} accumulation was significantly
372 reduced by the MCU inhibitor Ru360, in both isolated WT AMCMs and purified mitochondria (Fig. 4E,
373 4F). In line with these findings, direct application of 4-HNE on AMCMs promoted mito Ca^{2+}
374 accumulation, an effect inhibited by RU360 (Fig. 6A). This finding prompted us to determine whether
375 MCU, MICU1 or MICU2 were potential targets of 4-HNE covalent binding. In AMCMs of MAO-A Tg
376 mice, immunoprecipitation assays showed that 4-HNE levels increased on MCU, but not on MICU1 and
377 MICU2 (Fig. 6B, Supp Fig. 5A). In NRVMs treated with Tyr, we also found increased association of
378 MCU and 4-HNE, but not MICU1 or MICU2 (Fig. 6C, Supp Fig. 5B). In order to better understand how
379 4-HNE adducts could modify MCU-mediated Ca^{2+} uptake, we evaluated MCU complex formation, the
380 active form of the channel. Indeed, the MCU per se is not able to form the channel but should organize in
381 oligomers allowing efficient mito Ca^{2+} uptake (20, 21). Size exclusion chromatography followed by
382 western blot on NRVMs showed that MCU was eluted and detected in a high molecular weight fraction
383 ($F1 \approx 400$ kDa) only in the Tyr-stimulated condition, which corresponded to higher-order oligomers (Fig.
384 6D). Furthermore, in non-reducing western blots conditions, MAO-A Tg cardiomyocytes displayed larger
385 MCU complexes compared to WT (Fig. 6E). Similar findings were observed in NRVMs treated with Tyr
386 or 4-HNE (Fig. 6F, Supp. Fig 5C-D). Importantly, Alda-1 prevented MCU oligomerization, confirming
387 the role of 4-HNE in MAO-A-induced MCU conformational modifications (Fig 6F). In order to assess if

388 MCU functionally regulated mitochondrial function in response to MAO-A activation, we incubated WT
389 AMCMs with Tyr in the presence of the MCU inhibitor RU360. Seahorse measurements indicated that
390 respiration impairment induced by MAO-A could be prevented with RU360 (Fig. 6G, 6H and Supp Fig.
391 6). Loss of ATP content and mitochondrial membrane depolarization induced by MAO-A were also
392 partially reduced in the presence of RU360 in WT AMCMs (Fig. 6I, 6J). Altogether, these results identify
393 for the first time MAO-A as a regulator of MCU channel through 4-HNE-mediated conformational
394 changes and show that MCU activation participates in mitochondrial dysfunction.

395
396 ***MAO-A inactivation protects from 4-HNE accumulation and cardiac remodeling during chronic***
397 ***ischemia***

398 In order to evaluate the pathophysiological relevance of our findings, we developed a model of myocardial
399 infarction (MI) induced by permanent coronary artery ligation in mice. Four weeks post-MI, while
400 ALDH2 expression remained unchanged, both MAO-A levels and 4-HNE-bound proteins were elevated
401 compared to sham-operated mice (Fig. 7A). The same observation was made in ventricular samples of
402 human ischemic cardiomyopathy (Fig. 7B). In order to test if MAO-A/4-HNE axis could be associated
403 with chronic cardiac remodeling, mice were treated with the selective and reversible MAO-A inhibitor,
404 moclobemide (20 mg/kg/day), after the surgery and the treatment was sustained during 4 weeks (Fig. 7C).
405 Moclobemide efficiently inhibited MAO-A activity and reduced 4-HNE accumulation post-MI (Supp Fig.
406 7A; Fig. 7D). This was accompanied by reduced cardiac remodeling, as shown by improved ejection
407 fraction, reduced ventricular dilatation, decreased heart-to-body weight ratios and lung edema (Fig. 7E,
408 Table 3, Supp Fig. 7D-E). In order to test if cardiomyocytes were the primary cells responsible for these
409 effects, we used mice with cardio-selective deletion of MAO-A (*Maoa^{fl}* crossed with *α MHC-Cre⁺* mice)
410 (Supp Fig. 7B-C). Cardiomyocyte-specific deletion of MAO-A reduced 4-HNE accumulation and
411 mitigated contractility impairment and dilatation at 4 weeks post-MI (Fig. 7D-E, Supp Fig. 7G-H, Table
412 3). Heart-to-body weight ratios and lung congestions were also reduced in MAO-A cKO mice compared
413 to WT mice (Supp Fig. 7D-E). To evaluate the volume of the scar zone, we performed 3D-whole heart

414 imaging using high-resolution LSM. As shown by 3D reconstructions in Fig. 7F, the scar zone (blue)
415 represented 14 % of total heart in WT mice while it was reduced to 5% in MAO-A cKO mice, suggesting
416 that MAO-A deletion limited expansion of the infarct (Supp Fig. 7F). These results were confirmed with
417 Masson's Trichrome showing a marked decrease in the scar area of MAO-A cKO hearts compared to WT
418 (Fig. 7F, Supp Fig. 7F). Histologically, MAO-A cKO hearts had lessened reactive hypertrophy in the
419 remote zone of the infarct compared to WT mice (Fig. 7G) while interstitial fibrosis was slightly
420 decreased (Supp Fig. 7G-I). Next, we evaluated if the newly identified mechanism of action of 4-HNE on
421 MCU/mitoCa²⁺ was dysregulated in ischemic remodeling. Most interestingly, we observed a co-
422 immunoprecipitation of 4-HNE with MCU in MI hearts, associated with MCU oligomer formation, and
423 this was inhibited by moclobemide (Fig. 7H-I). Moreover, cardiac mitochondria of ischemic hearts
424 displayed higher mitoCa²⁺ levels compared to SHAM mice, and this effect was attenuated in MAO-A
425 cKO mice (Fig. 7J). Altogether, these results strongly support a role for cardiac MAO-A in the generation
426 of deleterious 4-HNE and MCU-mediated Ca²⁺ overload during chronic remodeling.

427

428 **Discussion**

429 In this study, we show for the first time that overactivation of MAO-A leads to intra-mitochondrial
430 generation of 4-HNE with functional consequences on mitoCa²⁺ transport, mitochondrial dysfunction and
431 chronic ventricular remodeling post-MI (Fig. 8). To our knowledge, this constitutes the first evidence of a
432 link between MAO-A activation during cardiac injury and the previously recognized deleterious effects of
433 4-HNE. Recently, it has been shown that human endstage ischemic hearts displayed an increase in MAO-
434 A and MAO-B activities, but the causative role of these enzymes in the pathogenesis of chronic ischemic
435 remodeling and the associated mechanisms were not investigated (22). Here, we show that
436 pharmacological or genetic inhibition of MAO-A reduces deleterious remodeling in mice with coronary
437 artery ligation. The role of MAO-A has been previously demonstrated in acute ischemia/reperfusion injury
438 (23) and in pressure overload-induced cardiac remodeling (24). Its mechanisms of action have been
439 delineated in a mouse model of MAO-A overexpression that, despite high MAO-A expression levels, is

440 useful in deciphering specific pathways downstream MAO-A (25). However, previous studies mainly
441 focused on the mechanisms of action of H₂O₂. Recently, Kaludercic et al. suggested a role for aldehydes in
442 MAO-B-induced deleterious effects, showing a worsening effect of *in vitro* ALDH2 inhibition after
443 dopamine treatment (26). Here, we unravel for the first time that lipid-derived aldehydes, in particular the
444 most abundant 4-HNE, act downstream MAO-A to mediate mitochondria dysfunction. Mounting evidence
445 has linked lipid peroxidation aldehydes such as 4-HNE with various human pathological states (8) and
446 with mice models of HF (10). However, the mechanisms of 4-HNE mitochondrial accumulation as well as
447 the identification of its mitochondrial targets remained poorly understood (27). Here, we show that MAO-
448 A is one of the factors driving 4-HNE mitochondrial accumulation through L₄CL peroxidation. The loss of
449 L₄CL in mitochondrial membrane constitutes a hallmark of cardiomyopathy, with consequences on the
450 enzymatic activity of cytochrome oxidase and respiratory capacity (28). However, this alteration of L₄CL
451 is not sufficient to explain the mitochondrial dysfunction observed with MAO-A, as ALDH2 activation
452 conferred significant protection. This indicates that 4-HNE mitochondrial accumulation plays a major role
453 in MAO-dependent mitochondrial damage.

454 Our results also put forward that 4-HNE acts at its site of formation since: i) 4-HNE accumulates within
455 the mitochondria after MAO activation, ii) overexpression of ALDH2 activity, a mitochondrial enzyme,
456 rescues MAO-A-mediated HF ; iii) Mass spectrometry of 4-HNE-bound proteins shows over-
457 representation of mitochondrial proteins. The main pathways identified by proteomic study were
458 “mitochondrial metabolism, glucose metabolism, mitoCa²⁺ transport and muscle contraction”.
459 Interestingly, two targets of 4-HNE (NDUFS2, SDHA) were also found in large-scale analysis of 4-HNE-
460 bound proteins in doxorubicin-induced cardiomyopathy, which suggests the existence of common targets
461 in stressed cardiomyocytes (29).

462 Due to its key role in bioenergetics, redox homeostasis and cell death, mitochondrial dysfunction is a
463 critical factor in the development and progression of HF (1). Of particular interest, mitoCa²⁺ overload
464 seems to be an important determinant of ischemic ventricular remodeling. A recent study showed that
465 blockade of mitoCa²⁺ extrusion by NCX channel promoted infarct expansion and death of border

466 cardiomyocytes (4). Santelli et al. also demonstrated that leaky Ryr2 caused mitoCa²⁺ overload and
467 dysfunction in HF (5). Interestingly, mitoCa²⁺ amounts were lessened in MAO-A cKO mice compared to
468 WT after ischemia, which was associated with a better cardiac function, identifying MAO-A as an
469 activator of mitoCa²⁺ entry. In addition, direct 4-HNE treatment led to mitoCa²⁺ overload and
470 mitochondrial dysfunction in both isolated cardiomyocytes and mitochondria. The mechanism by which
471 Ca²⁺ overload induces mitochondrial dysfunction remains incompletely understood. One hypothesis could
472 be that mitoCa²⁺ exacerbates ROS generation, further impairing mitochondrial function through the
473 generation of superoxide due to mild uncoupling of mitochondria (4).

474 Of particular importance, we identified VDAC and MCU as relevant targets of 4-HNE adduct formation
475 following MAO-A activation. The entry of Ca²⁺ inside the mitochondria is highly regulated by the
476 communication between the ER and the mitochondria called MAMs (18) and the association of the
477 VDAC1/GRP75/IP3R complex (30). MAO-A Tg mice exhibited higher levels of MAMs compared to WT
478 mice, which could favor mitoCa²⁺ entry from the ER. As we and others (31) demonstrated that 4-HNE
479 covalently bound to VDAC1, it is possible that the carbonylated form of VDAC1 has better interactions
480 with its partners GRP75 and IP3R1, strengthening mitoCa²⁺ transfer during MAO-A activation. We also
481 observed that MAO-A-dependant 4-HNE formation led to the formation of higher-order MCU oligomers.
482 MCU oligomer formation is sensitive to post-translational modifications, among which oxidation of Cys-
483 97, resulting in persistent MCU channel activity and higher mitoCa²⁺ uptake (32). As 4-HNE exhibits the
484 strongest reactivity for Cys residues, we can hypothesize that increased 4-HNE binding could modify
485 MCU activity through similar conformational changes. These results put forward MAO-A as a regulator
486 of MCU through 4-HNE-mediated post-translational modification and subsequent conformational
487 changes. Finally, we provide evidences that MCU-mediated mitoCa²⁺ overload following MAO-A/4-HNE
488 activation is deleterious, as mitochondrial dysfunction is prevented with the MCU inhibitor RU360. While
489 it has been clearly demonstrated that increasing the levels of mitoCa²⁺ was deleterious in pathological
490 cardiac remodeling (4, 5), the selective inhibition of MCU has given conflicting results. Global MCU-/-
491 had normal response to isoproterenol or pressure overload stress (33, 34), suggesting the existence of

492 compensatory mechanisms. On the other hand, cardiac-specific ablation of MCU in adult mice protected
493 mice from I/R damage (35) and pharmacological inhibition of MCU was beneficial in pressure-overloaded
494 hearts (36). In the present study, our results highlight for the first time a deleterious role for MCU-
495 dependent Ca^{2+} overload in chronic ischemic remodeling. In conclusion, we propose a model whereby
496 excessive activation of MAO-A in cardiac stress conditions promotes mitochondrial 4-HNE accumulation
497 and binding on VDAC1 and MCU, both acting in a synergistic way to promote excessive mitoCa^{2+} entry
498 and mitochondrial dysfunction (Fig. 8).

499 Finally, our identification of MAO-A as a major source of mitochondrial 4-HNE during post-MI
500 remodelling paves the way toward targeted therapies aimed at decreasing aldehyde load, mitoCa^{2+}
501 dysregulation and energetic depletion in HF. As the MAO-A inhibitor moclobemide is approved in Europe
502 and used as an antidepressant, it would be interesting to consider the possibility of repurposing this drug
503 for HF therapy.

504

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514

515 **Conflict of interest.** none

516

517

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618

619 **Figure legends**

620 **Fig 1. MAO-A activation leads to intra-mitochondrial 4-HNE production through H₂O₂-mediated**
 621 **L₄CL peroxidation.** (A) Representative confocal images of 4-HNE staining (green) on AMCMs isolated
 622 from WT mice loaded with Mito-ID Red and counterstained with DAPI (blue). Cells were treated with 50
 623 μM Tyr for 1 h. Scale Bar = 10 μm. n=5. (B) Immunoblots of MAO-A expression and 4-HNE protein
 624 adducts and quantifications in isolated mitochondria of WT and MAO-A Tg hearts at 12-weeks (n=6 per
 625 group). (C) Representative confocal images of MitoPY1 (green), Mito-ID (red) and Hoechst 33258
 626 staining on AMCMs isolated from WT mice treated with 50 μM Tyr for 30 min. Scale Bar = 10 μm. n=5.
 627 (D) Amplex red measurements in isolated mitochondria of WT and MAO-A Tg hearts (n=5) at 12-weeks.
 628 (E-F) Quantifications by LCMS/MS in isolated mitochondria of WT (n=6) and MAO-A Tg (n=4) hearts
 629 at 12 weeks showing (E) L₄CL content as percent of total cardiolipin and (F) each type of HODEs species
 630 as ng/μg prot. (G) Proposed mechanism for intra-mitochondrial 4-HNE production through H₂O₂-
 631 mediated L₄CL peroxidation downstream MAO-A. Data are expressed as means ± sem (**p*<0.05,
 632 ***p*<0.01 vs WT mice).

634 **Fig 2. 4-HNE-mediated adverse remodeling in MAO-A Tg hearts is prevented by ALDH2 gene**
 635 **transduction.** (A) Model of AAV9-GFP or AAV9-ALDH2 (3x10¹¹ vg/mouse) transduction in WT or
 636 MAO-A Tg mice. (B) Cardiac GSH content and (C) mitochondrial ALDH2 activity in WT and MAO-A
 637 Tg hearts at 12 weeks (n=4). (D) Mitochondrial ALDH2 activity on cardiac homogenates of MAO-A Tg
 638 mice after AAV9 transduction (n=7). (E) 4-HNE (Scale Bar = 100μm) and Vinculin (Scale Bar = 50 μm)
 639 immunofluorescence, and interstitial fibrosis with Sirius Red (Scale Bar = 1 mm) on cardiac cryosections
 640 of mice after AAV9 transduction. (F) Quantification of 4-HNE fluorescence as % of total area (n=6-7).
 641 (G) Quantification of cardiomyocytes area on at least 100 cells/mouse in 3 distinct regions of the left
 642 ventricle (n=6-7). (H) Quantification of fibrosis as % of total area (n= 4-6). (I, J) Echocardiographic
 643 measurements with (I) Fractional Shortening (%) and (J) Systolic LV Internal Dimension on mice after
 644 AAV9 transduction (n=6-7). Data are expressed as means ± sem (**p*<0.05, ***p*<0.01, ****p*<0.001 vs WT
 645 GFP mice; #*p*<0.05, ##*p*<0.01 vs MAO-A Tg GFP mice).

647 **Fig 3. Mitochondrial MAO-A/4-HNE axis impairs organelle function.** (A) Oxygen consumption rate
 648 (OCR) measurements in AMCMs of WT mice treated with Tyr (50 μM, 3 h) in the presence of Moclo (15
 649 μM) or Alda-1 (100 μM) at baseline and after addition of Oligomycin, FCCP and Antimycin A +
 650 Rotenone (n=6). (B) Maximal respiratory capacity measured from oxygen consumption rate (OCR) (n=6).
 651 (C) ATP content in AMCMs isolated from WT mice stimulated with Tyr (50 μM, 3h) in the presence of
 652 Moclo (15 μM) or Alda-1 (100 μM) (n=6). (D) NAD⁺/NADH ratio in mitochondria isolated from WT
 653 mice and treated with Tyr (50 μM, 3 h) in the presence of Moclo (15 μM) or Alda-1 (100 μM) (n=5). (E)
 654 TMRE fluorescence normalized to fluorescence in the presence of FCCP (F/F_{FCCP}), in AMCMs isolated
 655 from WT mice stimulated with Tyr (50 μM, 3 h) in the presence of Moclo (15 μM) or Alda-1 (100 μM)
 656 (n=6). Data are expressed as means ± sem (***p*<0.01, ****p*<0.001 vs CTL; #*p*<0.05, ##*p*<0.01,
 657 ###*p*<0.001 vs Tyr).

659 **Fig 4. MAO-A activation induces 4-HNE-dependent increase in mitoCa²⁺ levels.** (A) Representative
 660 images of mitochondrial Ca²⁺ (mitoCa²⁺) accumulation in WT or MAO-A Tg AMCMs. The Ca²⁺
 661 fluorescent probe Rhod-2-AM overlapped with the mitochondrial marker Mitotracker green.
 662 Quantification of mitoCa²⁺ accumulation in AMCMs (B) and isolated mitochondria (C) of MAO-A Tg
 663 mice (n=5-6). (D, E) Representative images and quantification of mitoCa²⁺ accumulation in AMCMs of
 664 WT mice treated with Tyr (50 μM, 3 h) in the presence of Moclo (15 μM), Alda-1 (100 μM) or RU360
 665 (10 μM) (n=5-6). (F) Quantification of Ca²⁺ accumulation in isolated mitochondria of WT mice treated
 666 with Tyr (50 μM, 30 min) (n=5-6). Data are expressed as means ± sem (**p*<0.05, ****p*<0.001 vs Veh or
 667 WT; ##*p*<0.01, ###*p*<0.001 vs MI WT or Tyr).

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669 **Fig 5. Implication of MAMs in MAO-A-induced mitoCa²⁺ increase.** (A) Representative immunoblots
 670 showing the interaction of 4-HNE with VDAC1 in NRVMs stimulated with Tyr. Immunoprecipitation
 671 (IP) experiments were performed with 4-HNE or VDAC1 antibodies. IgG was used as a negative control
 672 for IP. Input is a control of cell lysates. n=4. (B) Representative images of *in situ* interactions (red
 673 fluorescent dots) between IP3R1 and GRP75 or IP3R1 and VDAC1 or GRP75 and VDAC1 in WT or
 674 MAO-A Tg cardiomyocytes. Nuclei are stained with DAPI. Scale bar, 20 μ m. (C-E) Quantifications of
 675 the proximity ligation assay (n=3). (F) Representative images of mitoCa²⁺ accumulation in AMCMs of
 676 WT mice treated with Tyr and challenged with histamine (100 μ M) or Xestospongine C (2 μ M). (G)
 677 Quantifications of mitoCa²⁺ accumulation (n=5). Data are expressed as means \pm sem (*p<0.05, **p<0.01,
 678 ***p<0.001 vs WT or Veh, ###p<0.001 vs Tyr).

680 **Fig 6. MAO-A/4-HNE axis regulates mitoCa²⁺-induced mitochondrial dysfunction through MCU-**
 681 **binding and higher order complex formation.** (A) Quantifications of mitoCa²⁺ accumulation in
 682 AMCMs isolated from WT mice treated with 4-HNE (5 μ M, 15 min) in the presence of RU360 (10 μ M).
 683 n=4. (B, C) Representative immunoblots showing the interaction of 4-HNE with MCU (B) in AMCMs of
 684 MAO-A Tg mice, (C) in NRVMs stimulated with Tyr (500 μ M, 1h). Immunoprecipitation (IP)
 685 experiments were performed with 4-HNE or MCU antibodies. IgG was used as a negative control for IP.
 686 Input is a control of cell lysates. n=4. (D) Representative elution profile and immunoblots showing MCU
 687 expression in different protein complexes obtained after size exclusion chromatography (SEC) of non-
 688 denatured NRVMs lysates using HPLC (AKTA purifier 10, GE Life Science) coupled to a Superose 6
 689 10/300GL column, after stimulation with Tyr (500 μ M, 4h). The elution profile of molecular complexes
 690 are shown in four different fractions (F1-F4) with estimated sizes: F1 at \approx 400 kDa, F2 at \approx 100 kDa ; F3
 691 at \approx 75 kDa and F4 at \approx 35 kDa. The annotated molecular weights were estimated from the elution profiles
 692 of standard markers injected onto the SEC column. (E, F) Representative non-reducing immunoblots
 693 showing MCU higher order complex formation (E) in AMCMs of MAO-A Tg mice and (F) in NRVMs
 694 stimulated with Tyr in the presence of Alda-1 (100 μ M). (G) Oxygen consumption rate (OCR)
 695 measurements in AMCMs of WT mice treated with Tyr (50 μ M, 3 h) in the presence of RU360 (10 μ M)
 696 at baseline and after addition of Oligomycin, FCCP and Antimycin A + Rotenone (n=5-6). (H) Oxygen
 697 consumption rate (OCR) associated with maximal respiratory capacity (n=5-6). (I) TMRE fluorescence
 698 normalized to fluorescence in the presence of FCCP (F/F_{FCCP}) in AMCMs of WT mice stimulated with
 699 Tyr (50 μ M, 3 h) in the presence of RU360 (10 μ M) (n=6). (J) ATP content in AMCMs of WT mice
 700 stimulated with Tyr (50 μ M, 3 h) in the presence of RU360 (10 μ M) (n=6). Data are expressed as means
 701 \pm sem (*p<0.05, **p<0.01, ***p<0.001 vs CTL; #p<0.05, vs 4-HNE or Tyr).

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 703 **Fig 7. Effect of Moclobemide or MAO-A deficiency on myocardial infarction (MI)-induced 4-HNE**
 704 **accumulation and cardiac remodeling.** (A-B) Immunoblots and quantifications of ALDH2, MAO-A and
 705 4-HNE protein adducts in (A) cardiac homogenates of SHAM or MI mice (n=4) and (B) left ventricular
 706 myocardium of CTL (n=4) or human ischemic cardiomyopathy patients (hICM) (n=5). (C) Model of MI
 707 experiments with moclobemide treatment (20 mg/kg/day) or in mice with deletion of MAO-A in
 708 cardiomyocytes (MAO-A cKO). (D) Quantifications of 4-HNE protein adducts in mouse hearts after MI.
 709 (MAO cKO: n=4 sham, n=8 MI; Moclobemide: n=5 sham, n=5 MI). (E) Echocardiographic parameters of
 710 Ejection Fraction (EF, %). (F) Representative pictures of 3D-reconstructed hearts by LSFM with scar
 711 zone in blue in upper panel (Scale Bar = 2 mm) or Masson's Trichrome staining in lower panel (Scale Bar
 712 = 1 mm). (G) Vinculin (Scale Bar = 50 μ m) immunofluorescence staining with quantifications of mean
 713 cardiomyocyte area. (MAO-A cKO: n=4 sham, n=8 MI). (H) Immunoprecipitation experiments with
 714 MCU or 4-HNE in heart homogenates of mice subjected to 4 weeks ischemia (MI) in the presence of
 715 moclobemide (20 mg/kg/day). (n=3 per group). (I) Representative non-reducing immunoblots showing
 716 MCU higher order complex formation in heart homogenates of mice subjected to 4 weeks ischemia (MI)
 717 in the presence of moclobemide (20 mg/kg/day). (n=3 per group). (J) Quantification of Ca²⁺ accumulation
 718 in isolated mitochondria of WT and MAO-A cKO mice subjected to 1 week ischemia (n=5-7).

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720 **Fig 8.** Schema illustrating the mechanisms associated with MAO-A/4-HNE signaling during chronic
721 ischemia (right panel) compared to normal conditions (left panel).
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771 TABLE 1. Echocardiographic parameters of WT and MAO-A Tg mice transduced with AAV9-cTnT-GFP
 772 or AAV9-cTnT-ALDH2 (M-MODE)

AAV9-cTnT	WT		MAO-A Tg	
	GFP	ALDH2	GFP	ALDH2
No of mice	6	6	7	7
IVSs (cm)	0,18 ± 0,01	0,17 ± 0,004	0,16 ± 0,01***	0,15 ± 0,003**
LVIDs (cm)	0,19 ± 0,02	0,17 ± 0,01	0,27 ± 0,01***	0,23 ± 0,01 [#]
LVIDd (cm)	0,34 ± 0,02	0,31 ± 0,01	0,38 ± 0,01	0,36 ± 0,01
LVPWs (cm)	0,16 ± 0,01	0,19 ± 0,01	0,15 ± 0,01	0,17 ± 0,01
EDV (ml)	0,10 ± 0,01	0,08 ± 0,01	0,14 ± 0,01	0,12 ± 0,01
ESV (ml)	0,020 ± 0,004	0,013 ± 0,002	0,053 ± 0,006***	0,036 ± 0,003 [#]
EF (%)	81,8 ± 1,7	82,5 ± 1,5	62,1 ± 2,2***	72,0 ± 1,4** ^{##}
FS (%)	44,6 ± 1,7	45,2 ± 1,5	28,7 ± 1,4***	35,7 ± 1,1** ^{##}
HR (bpm)	596 ± 12	571 ± 8	621 ± 9	620 ± 10

773 IVSs, systolic LV interventricular septal thickness ; LVIDs, systolic LV internal dimension ;
 774 LVIDd, diastolic LV internal dimension; LVPWs, systolic LV posterior wall thickness ; LVPWs,
 775 systolic LV posterior wall thickness ; EDV, end-diastolic volume ; ESV, end-systolic volume ; EF,
 776 ejection fraction ; FS, fractional shortening ; HR, heart rate. **p<0.01, ***p<0.001 vs.
 777 corresponding WT mice; #p<0.05, ##p<0.01 vs. AAV9-GFP for MAO-A Tg mice
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806 TABLE 2. List of 53 4-HNE-bound proteins in cardiomyocytes of MAO-A Tg mice compared to WT
 807 mice (cut-off ratio>2 and p-value<0.05)

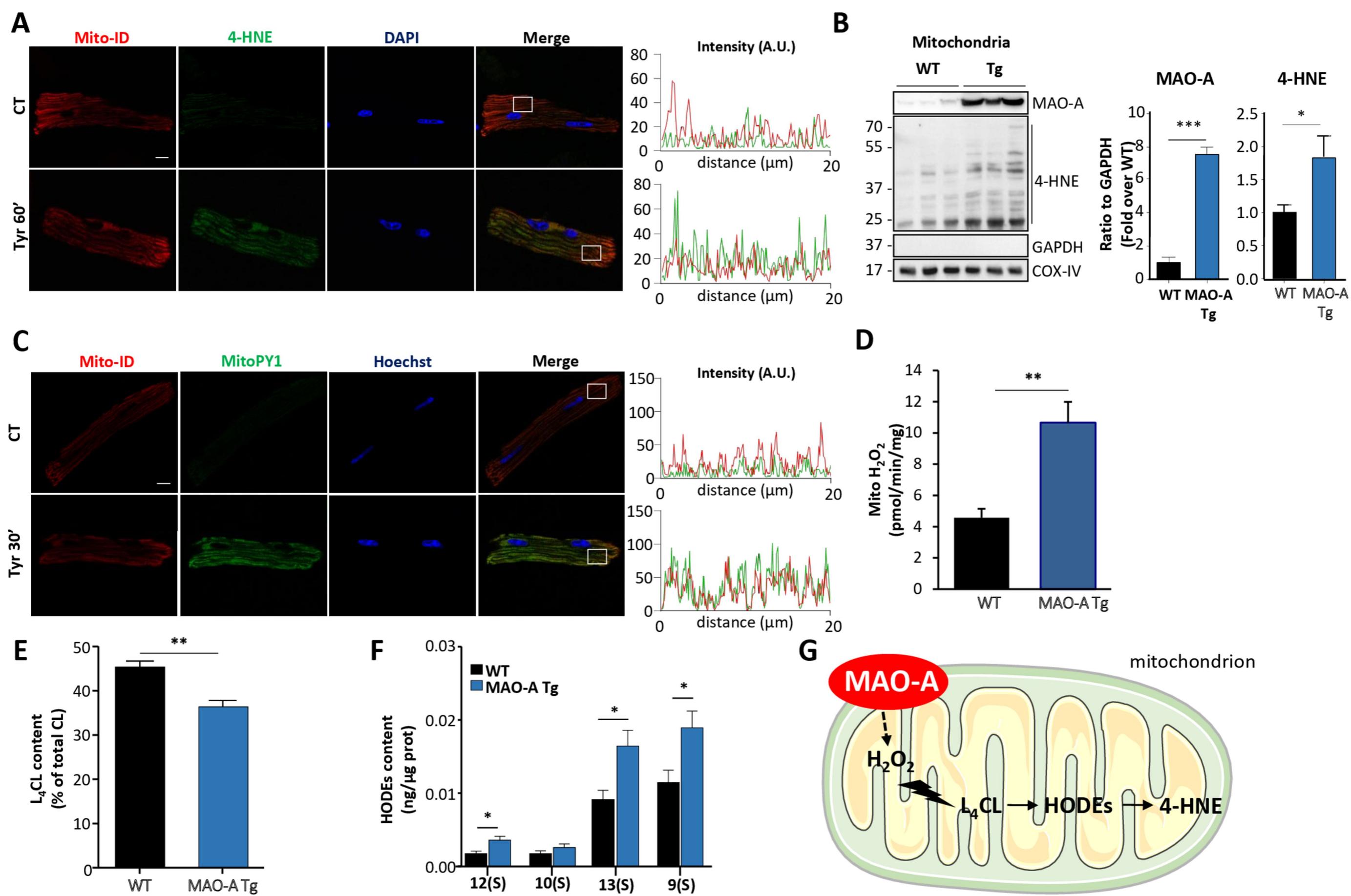
Proteins	MW (kDa)	Fold change	t-test
Astn2	99,9	15,6	4,96E-05
COX2	26,0	4,9	4,20E-05
Ces1d	61,8	4,6	1,10E-03
Myh11	223,4	4,5	1,05E-02
Bcam	21,1	4,4	2,59E-03
Col6a4	250,8	4,2	1,04E-02
Tmx1	14,3	3,6	3,52E-02
Hk1	108,3	3,2	9,82E-04
Macrold1	35,3	3,1	6,89E-05
COX3	29,9	3,1	7,27E-04
Sdha	72,6	3,0	8,69E-08
Cox4i1	19,5	2,9	3,56E-05
Actn2	103,9	2,9	9,13E-05
Uqcrc1	52,8	2,8	4,60E-05
Bsg	24,1	2,8	6,66E-04
Mybpc3	141,3	2,7	7,28E-04
Vdac2	30,4	2,7	6,58E-04
Uqcrc2	48,2	2,7	2,90E-05
Hibadh	35,5	2,6	6,87E-04
Cox6c	8,5	2,6	6,92E-03
Glud1	61,3	2,5	1,43E-02
Trim21	53,2	2,5	3,13E-02
Atp1b1	35,2	2,5	2,72E-03
Sdhb	27,2	2,5	2,45E-05
Ndufs2	52,6	2,4	4,56E-04
Uqcrcfs1	29,4	2,4	9,50E-04
Ndufs1	79,7	2,4	7,22E-05
Akr1b10	32,7	2,4	2,24E-02
Ndufa10	40,6	2,3	8,03E-04
Mdh2	35,6	2,3	2,64E-06
Ndufs7	24,7	2,3	1,79E-02
Coq9	27,9	2,2	9,89E-04
Ugp2	55,5	2,2	9,17E-05
Vdac1	30,7	2,2	1,37E-04
Ndufa8	20,0	2,2	4,44E-02
Bdh1	38,3	2,1	4,43E-05
Try4	26,3	2,1	4,42E-04
Ldha	34,5	2,1	7,77E-05
Etfdh	61,2	2,1	3,99E-04
Hibch	43,0	2,1	4,99E-04
Kdm2b	144,9	2,1	1,60E-02
Atp1a1	113,0	2,1	2,36E-03
Sirt5	34,1	2,1	2,98E-03
Slc16a1	38,2	2,1	6,76E-05
Ndufa12	17,4	2,1	1,98E-04
Akr7a2	40,6	2,1	3,21E-03
Mdh1	36,5	2,0	1,02E-04
Reep5	21,1	2,0	3,34E-04
Atp2a2	109,8	2,0	4,65E-02
Got2	47,4	2,0	8,99E-07
Aldoa	39,3	2,0	1,29E-05
Aldh4a1	61,8	2,0	2,96E-04
Aco2	85,5	2,0	4,66E-06

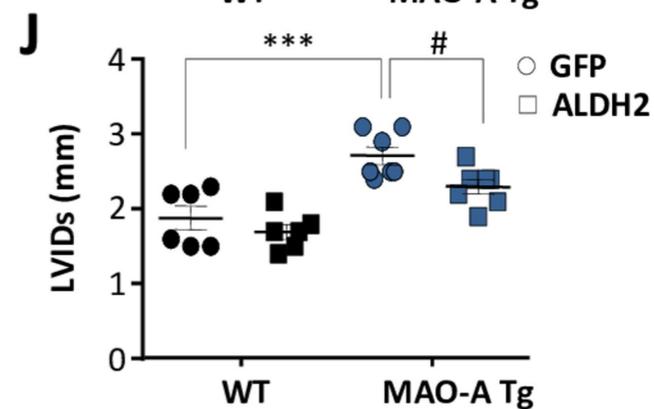
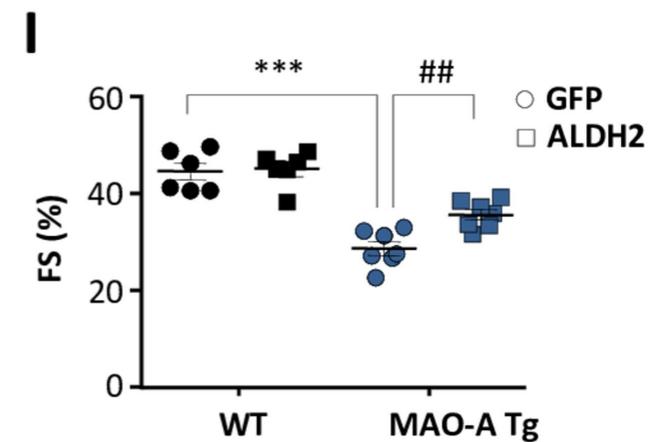
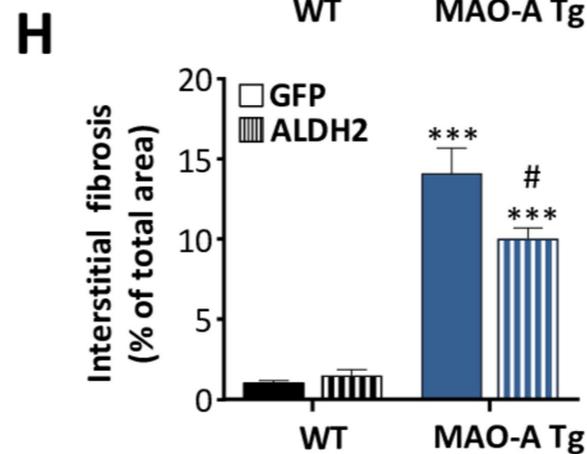
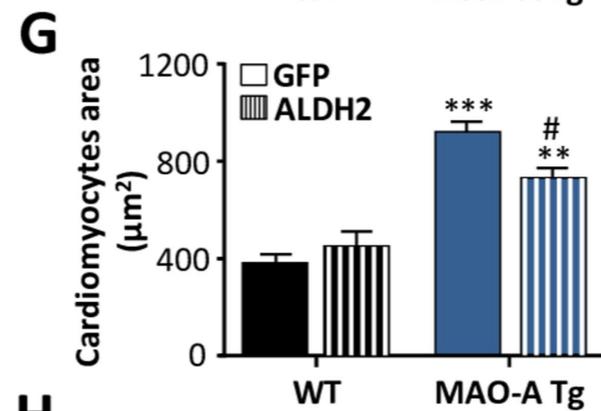
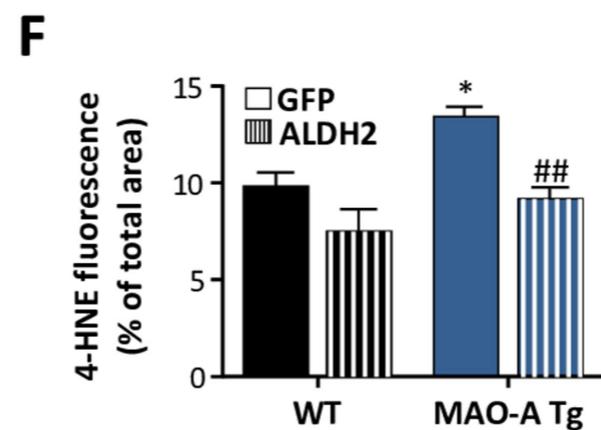
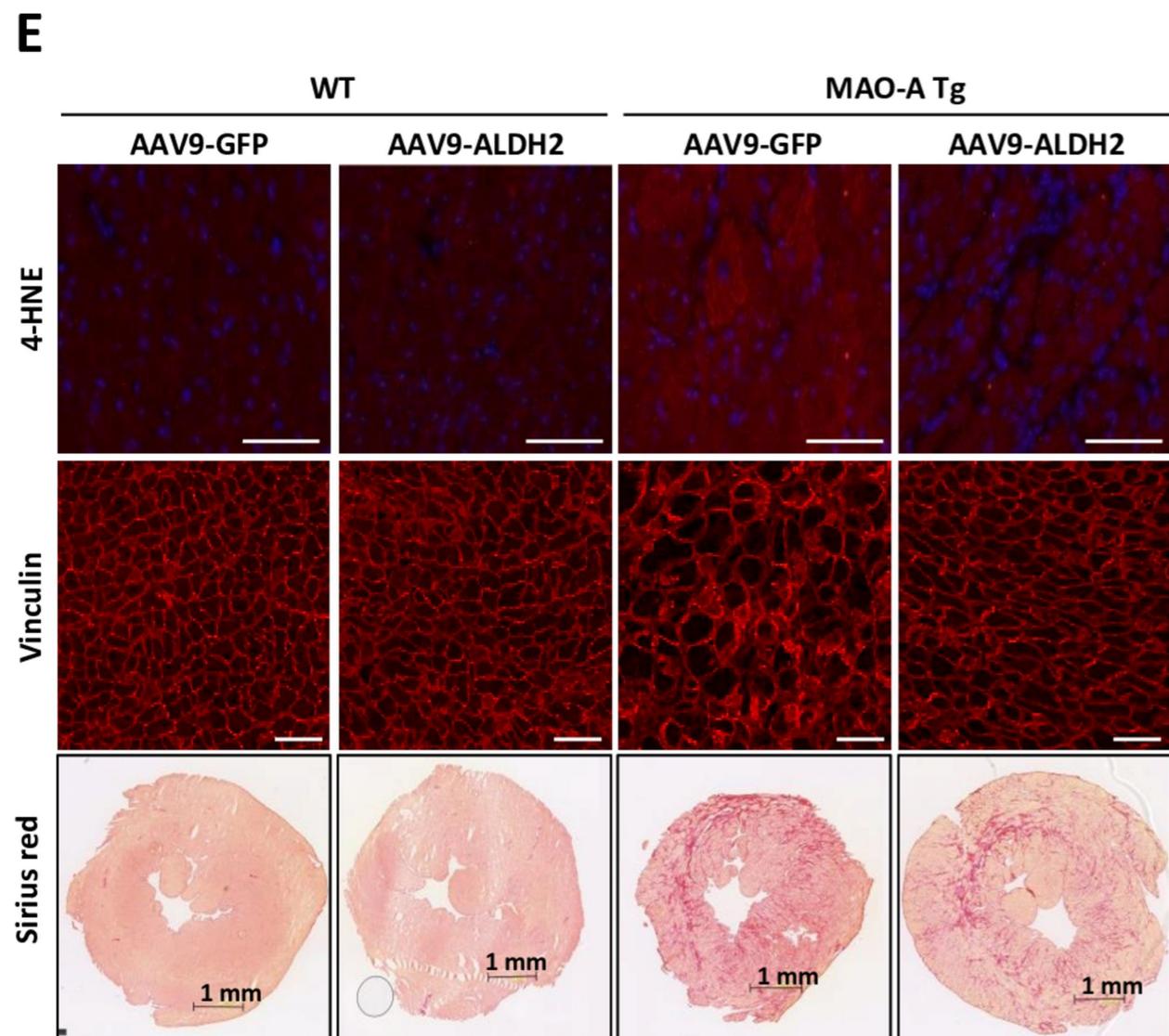
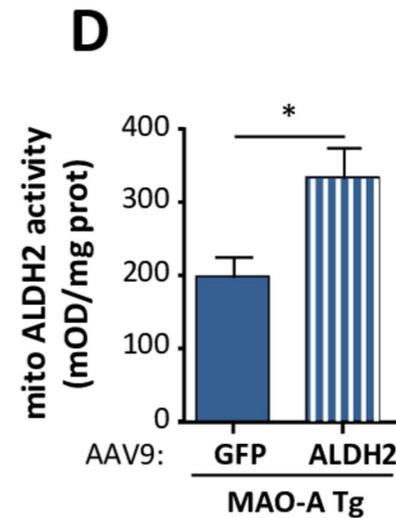
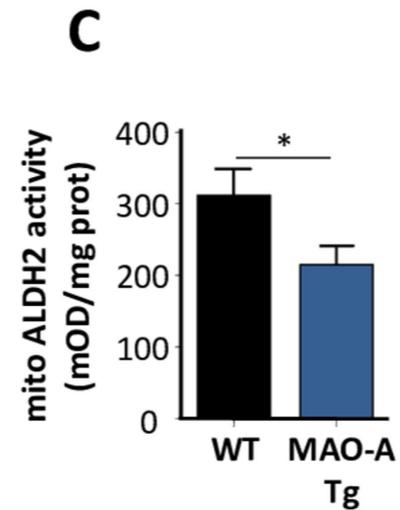
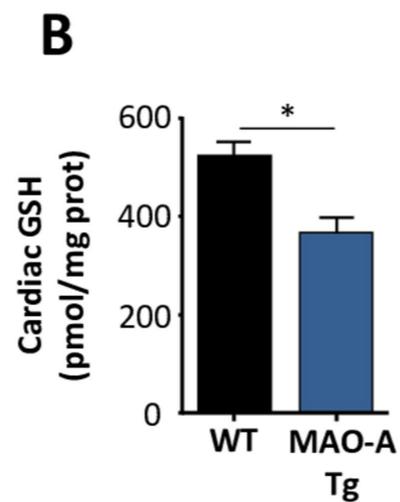
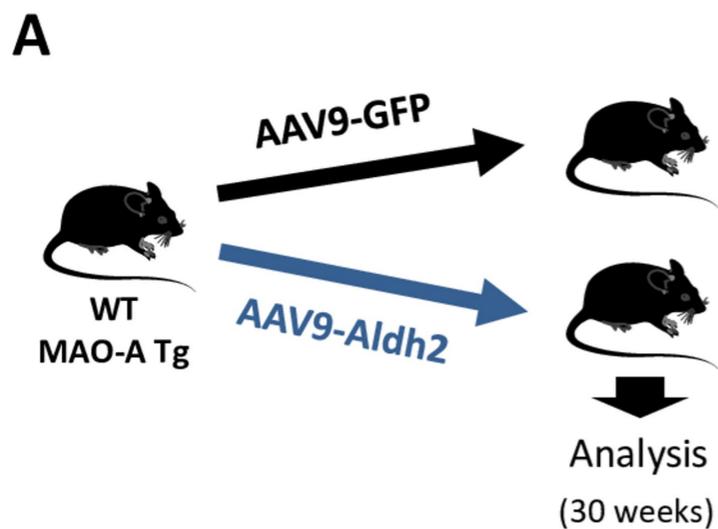
808 TABLE 3. Echocardiographic parameters of mice treated with Moclobemide (20 mg/kg/d) or MAO-A
 809 cKO mice 4 weeks after myocardial ischemia (2D imaging)

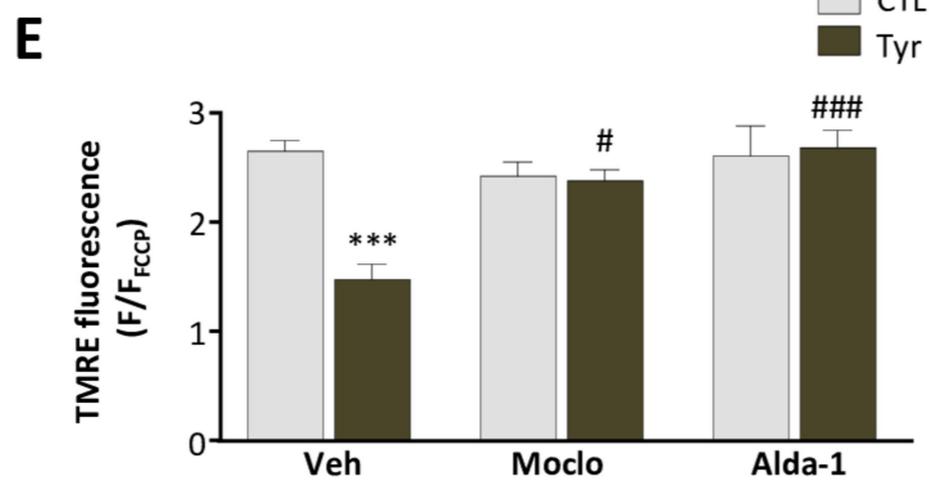
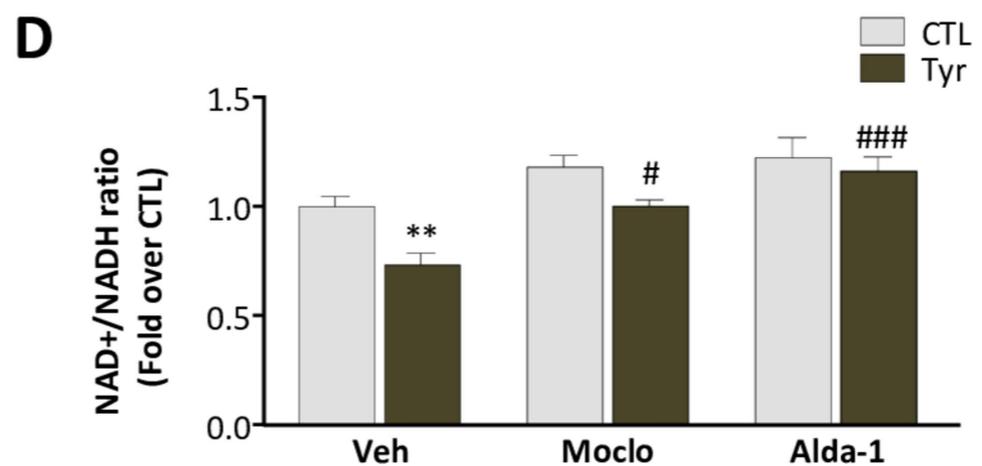
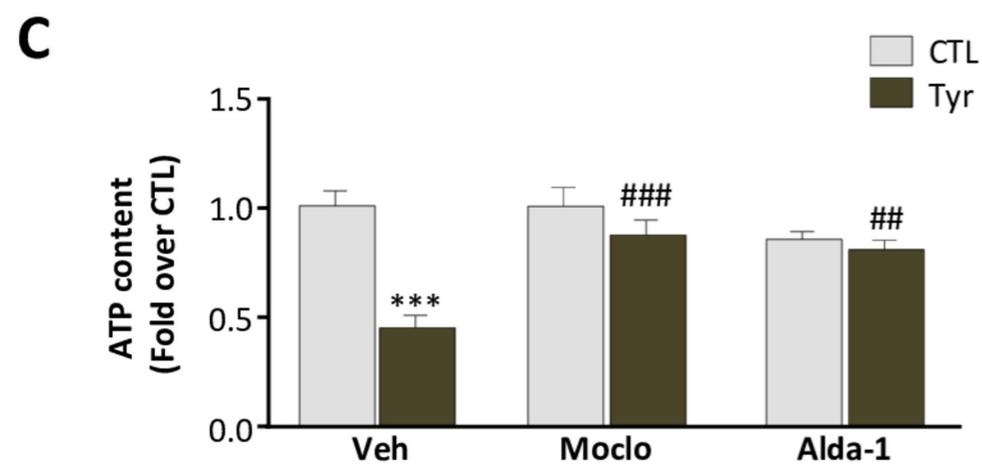
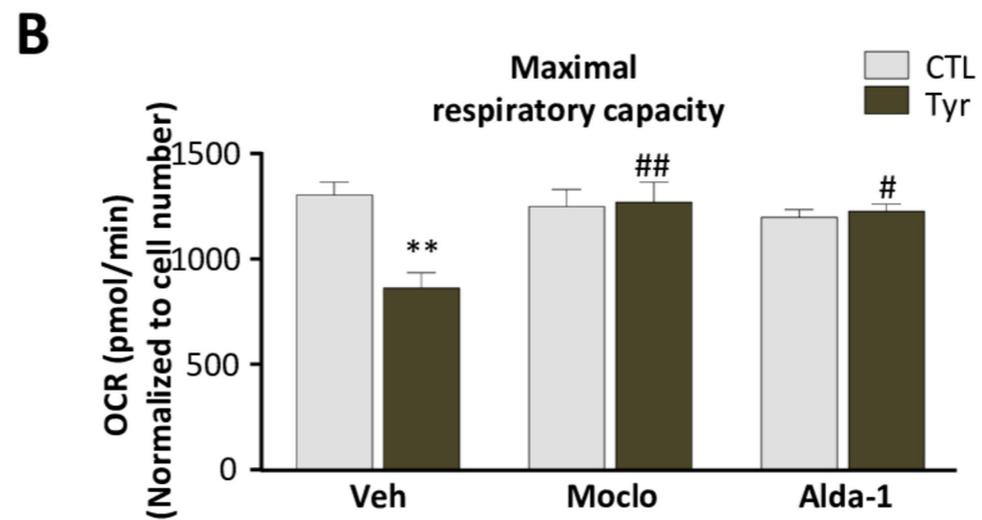
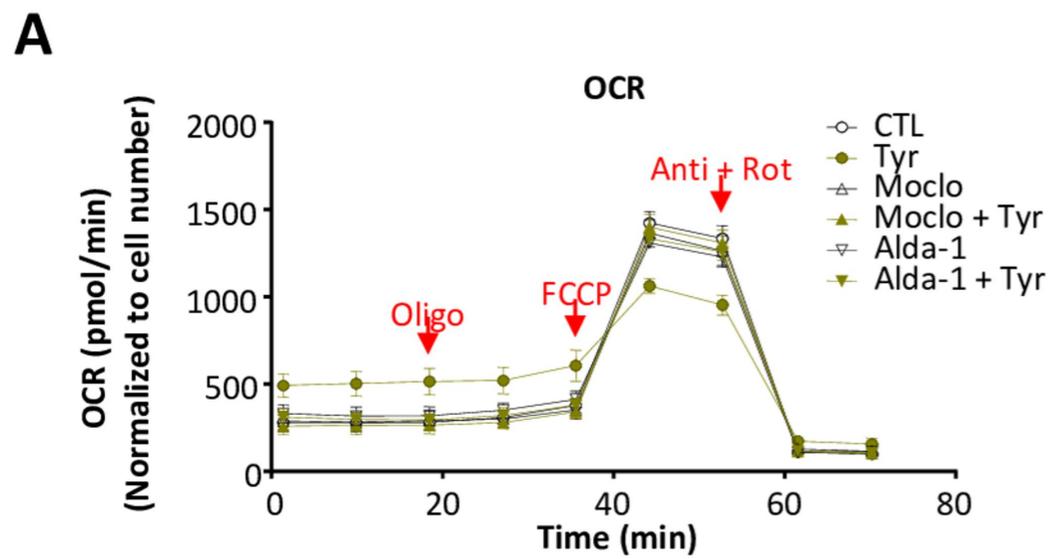
	SHAM		MI	
	Vehicle	Moclobemide	Vehicle	Moclobemide
N	6	5	13	11
Ad, mm²	24,3 ± 1,2	26,6 ± 0,6	43,7 ± 1,6***	38,1 ± 2,3**,#
EDV, ml	0,068 ± 0,005	0,077 ± 0,002	0,199 ± 0,093***	0,15 ± 0,01***,##
As, mm²	15,7 ± 0,7	19,0 ± 0,6	40,4 ± 1,7***	32,95 ± 2,25***,##
ESV, ml	0,033 ± 0,002	0,045 ± 0,002	0,170 ± 0,012***	0,125 ± 0,014***,##
EF (%)	50,7 ± 0,7	43,9 ± 1,6	11,7 ± 1,7***	20,0 ± 2,5***,##
HR, bpm	479,38 ± 12,61	398,49 ± 6,88*	503,79 ± 18,65	470,66 ± 15,91

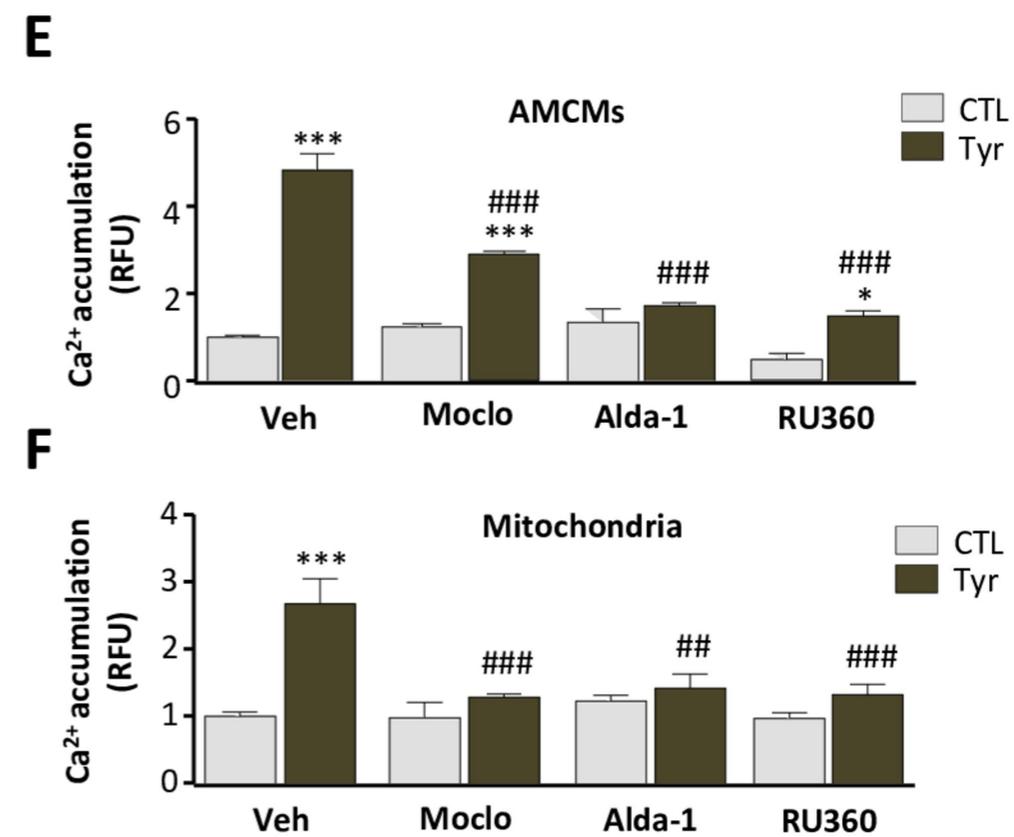
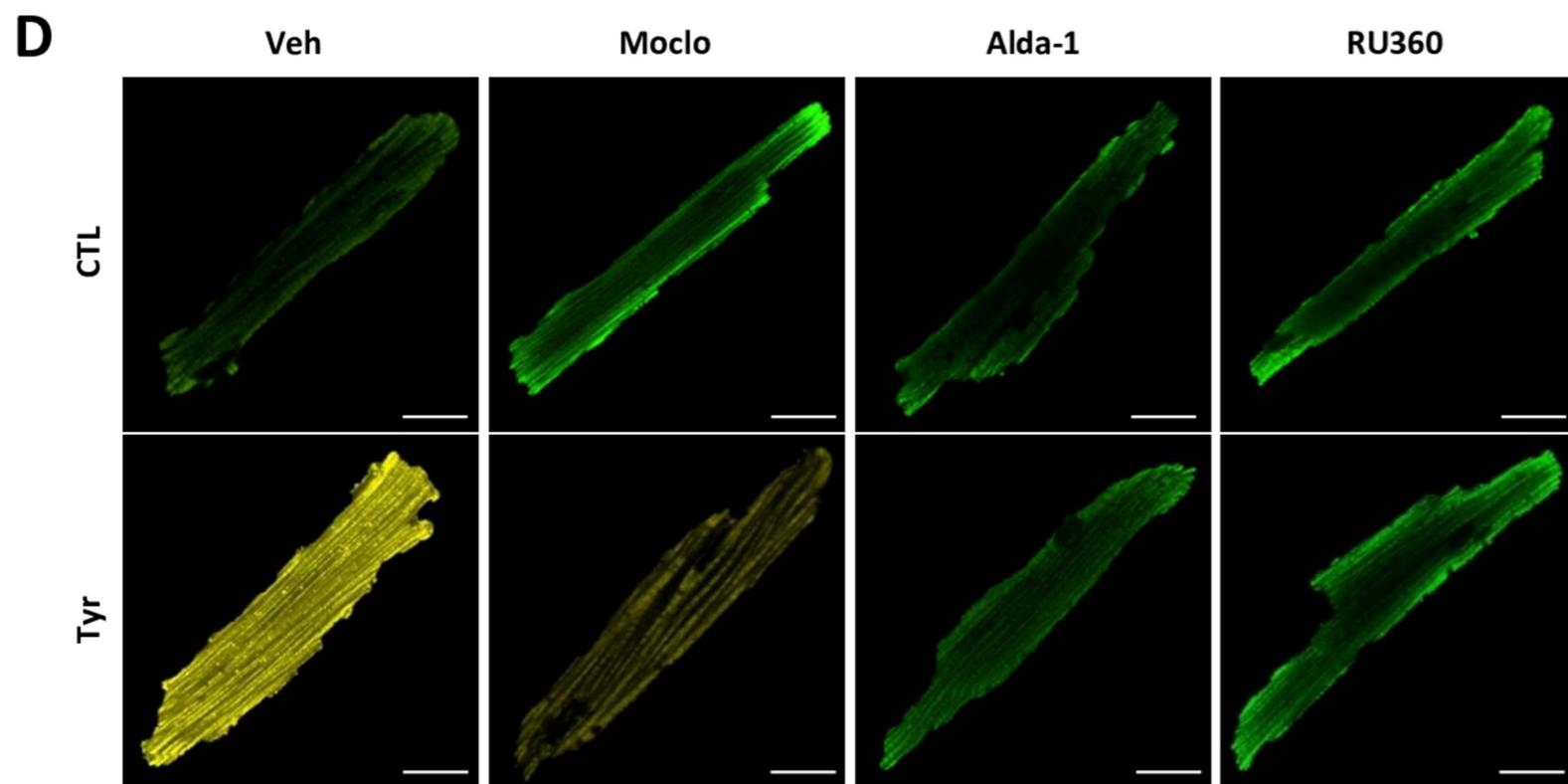
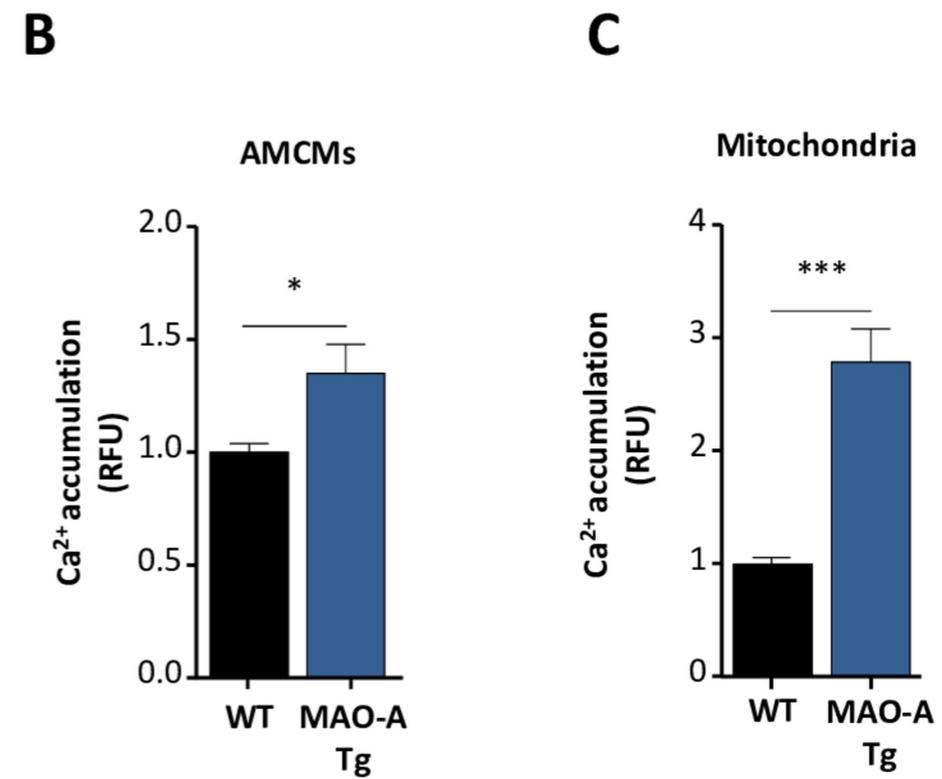
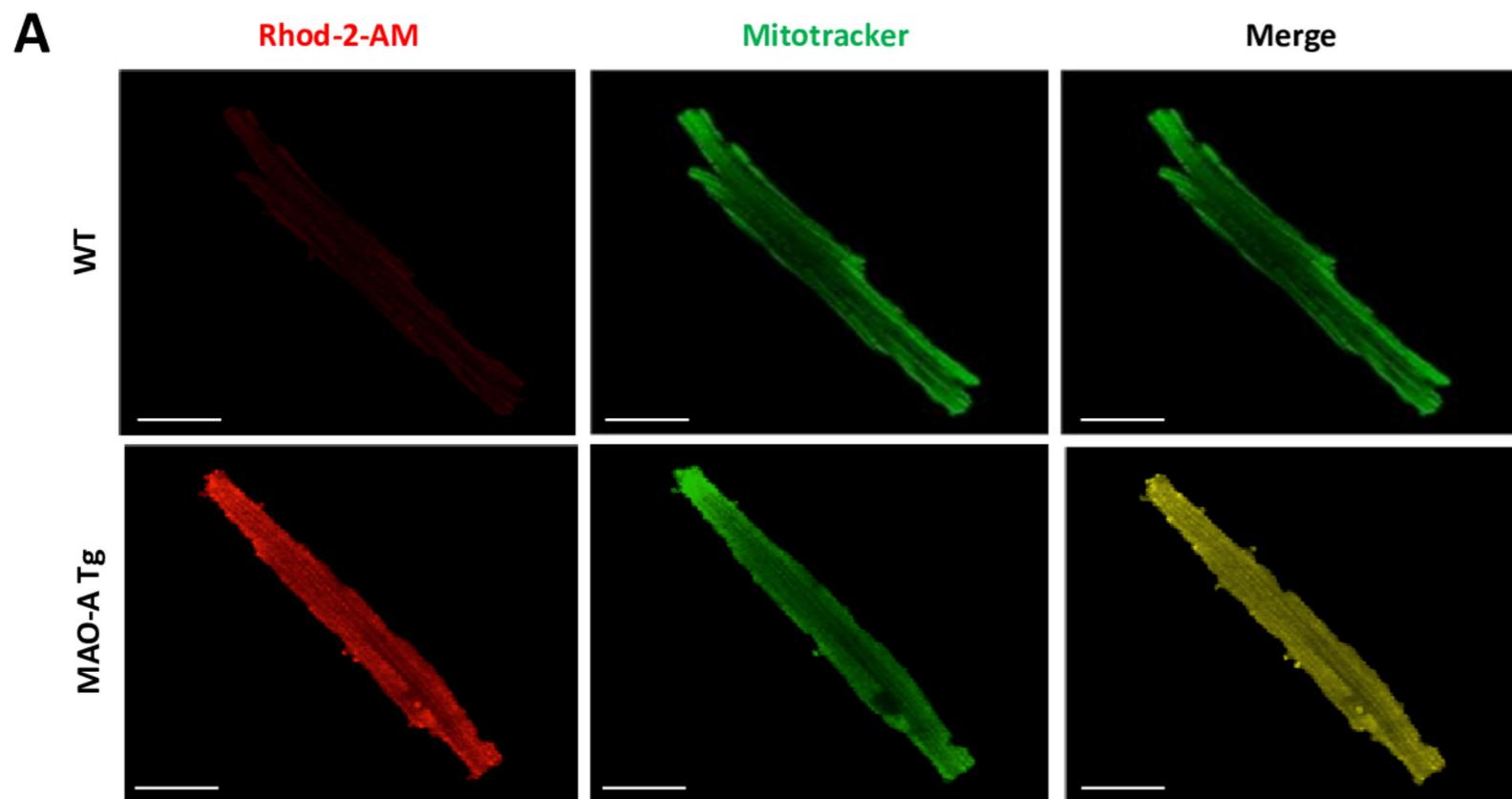
	SHAM		MI	
	WT	MAO-A cKO	WT	MAO-A cKO
N	4	4	8	8
Ad, mm²	26,10 ± 0,97	29,45 ± 0,98	49,94 ± 3,15***	37,82 ± 1,69*,##
EDV, ml	0,078 ± 0,005	0,073 ± 0,018	0,234 ± 0,025***	0,151 ± 0,012*,#
As, mm²	12,5 ± 2,5	17,5 ± 2,5	42,9 ± 3,6***	32,5 ± 1,6***,##
ESV, ml	0,030 ± 0,004	0,035 ± 0,006	0,188 ± 0,023***	0,106 ± 0,010*,##
EF (%)	47,5 ± 1,2	48,3 ± 0,6	21,2 ± 2,2***	29,5 ± 1,8***,##
HR, bpm	500,25 ± 22,93	460,75 ± 14,59	540,13 ± 21,17	466,78 ± 21,52

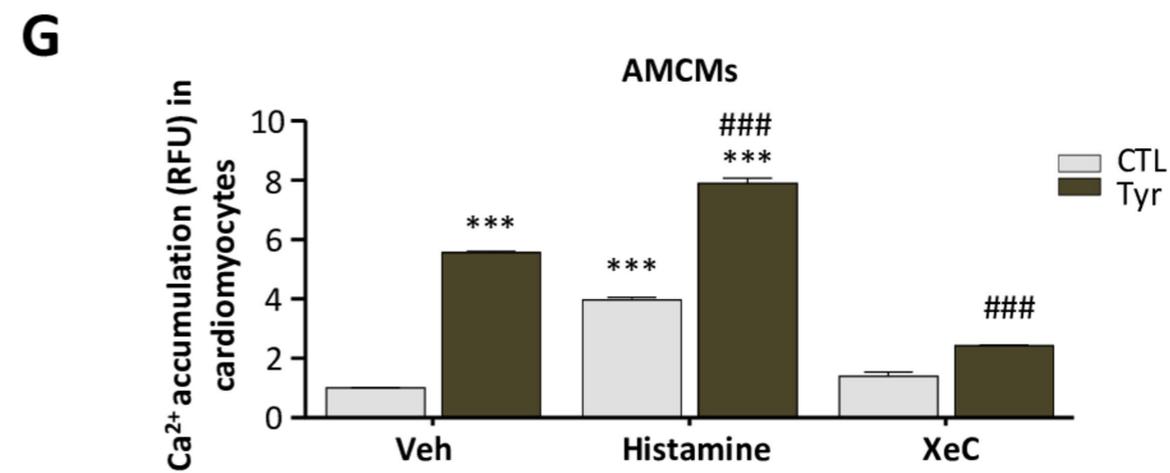
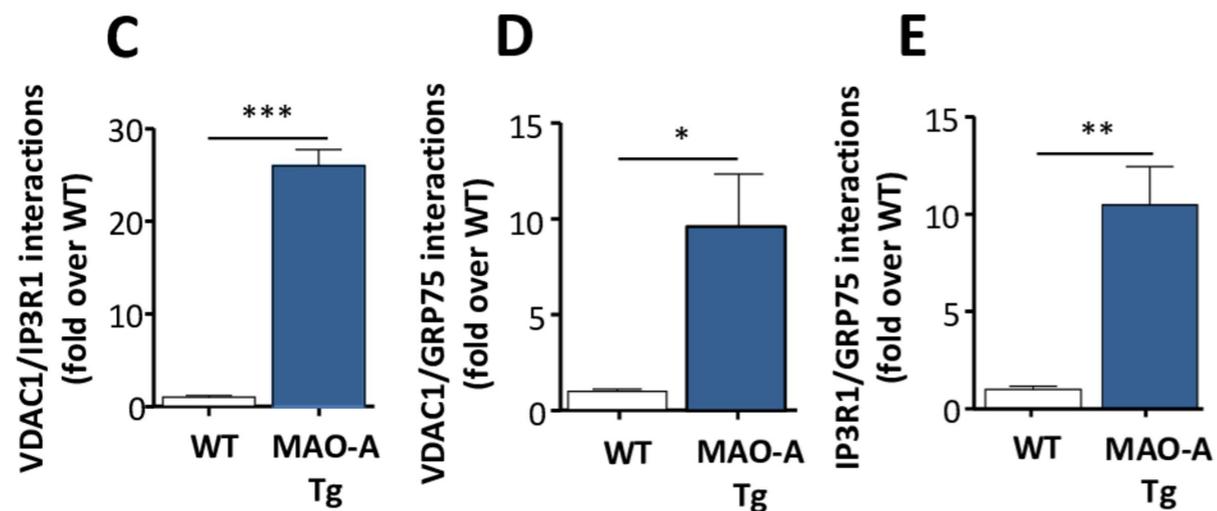
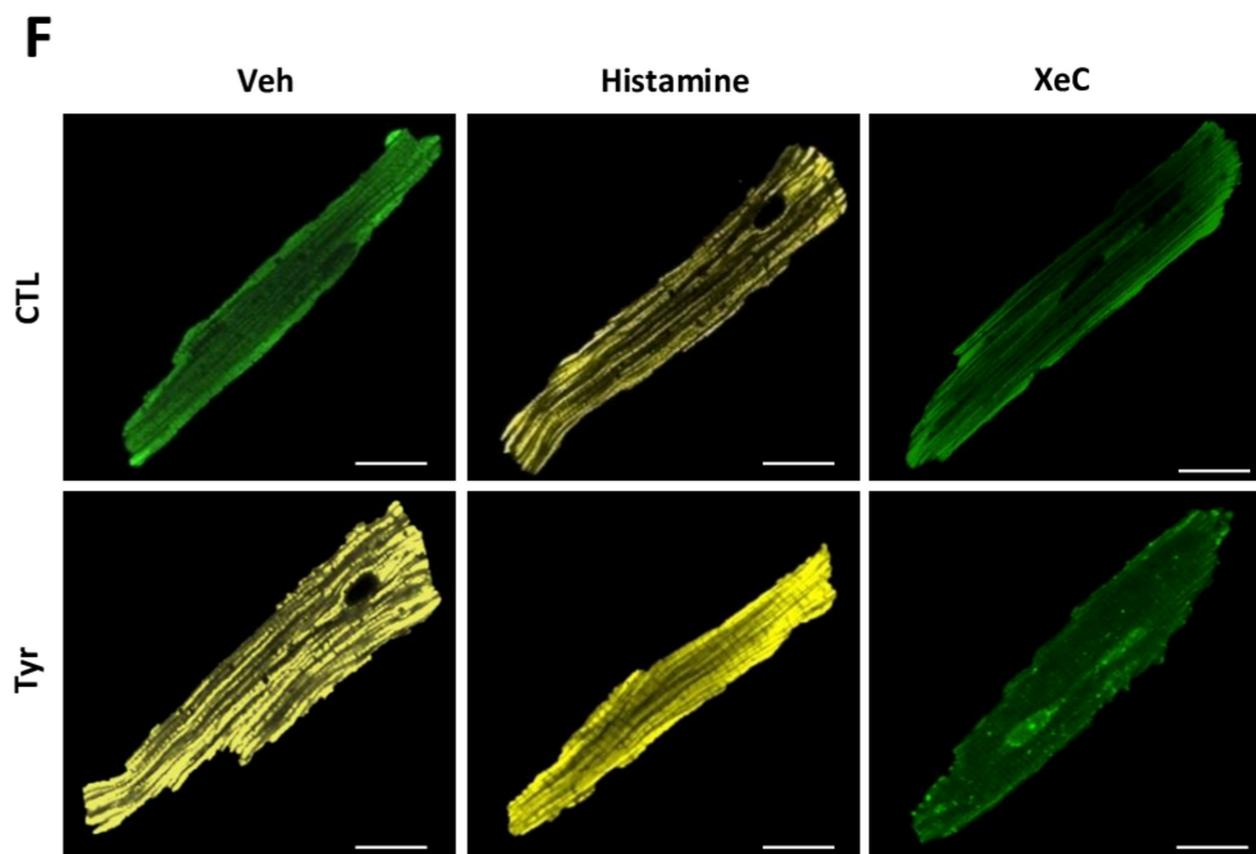
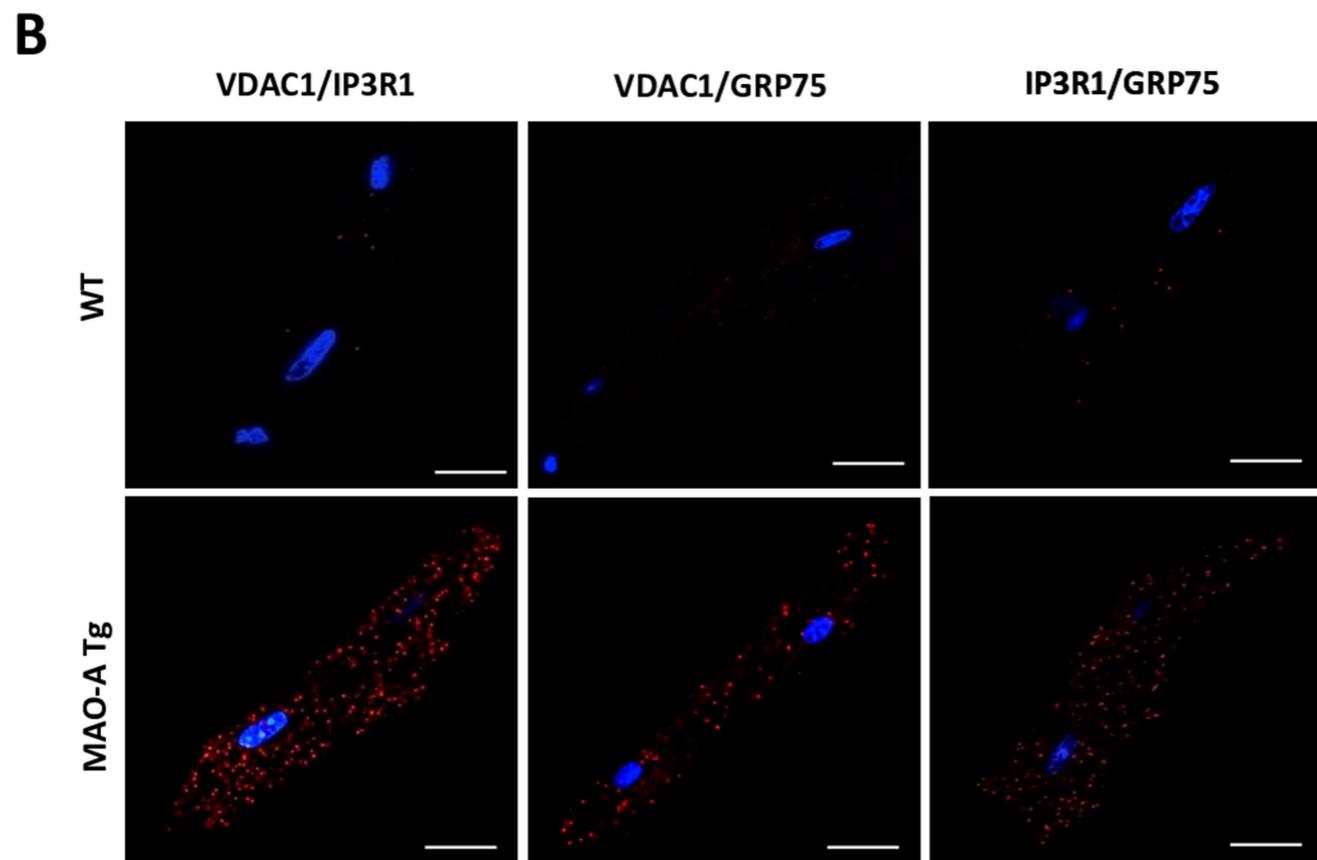
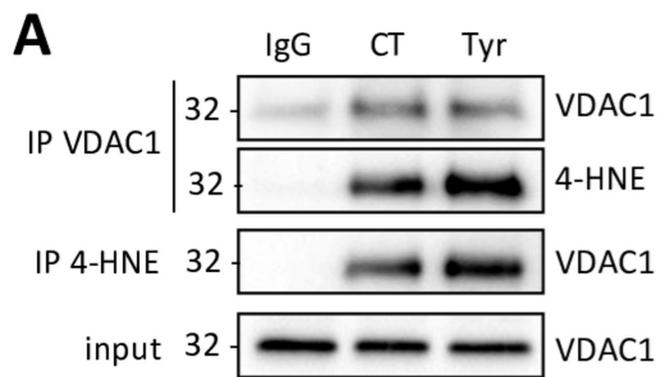
810 Ad, end-diastolic area ; EDV, end-diastolic volume ; As, end-systolic area ; ESV, end-systolic volume ; EF,
 811 ejection fraction. **p<0.01, ***p<0.001 vs. corresponding SHAM mice; #p<0.05, ##p<0.01 vs. mice treated
 812 with Vehicle or WT mice for MI condition.

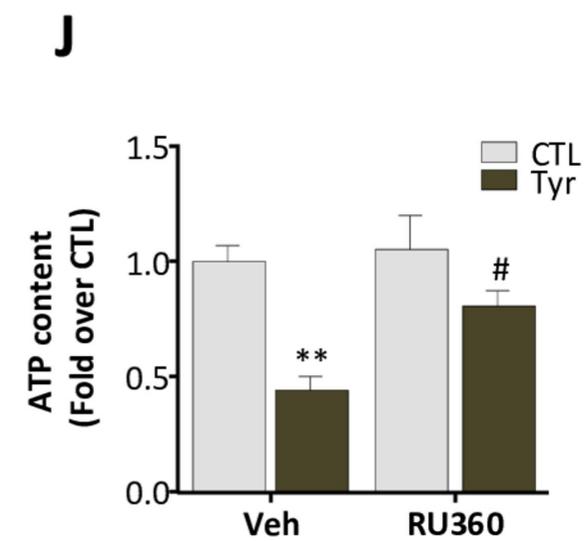
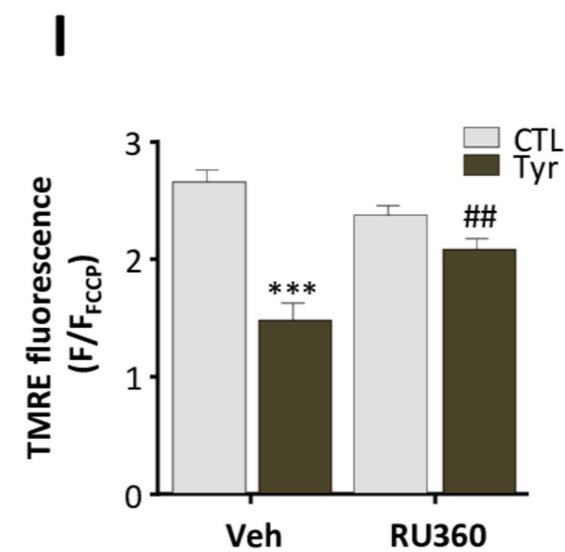
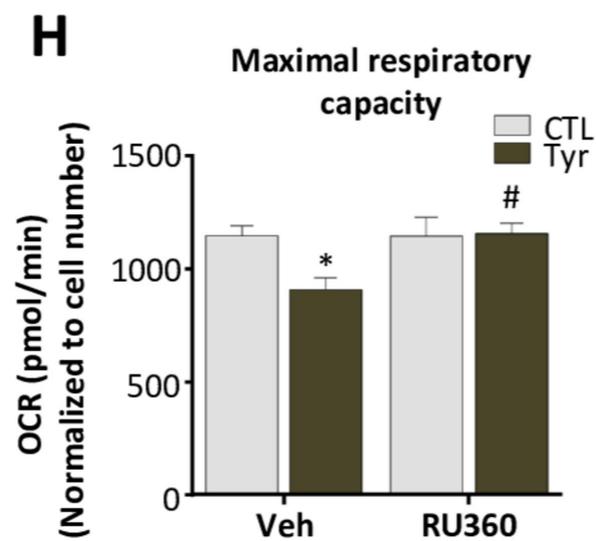
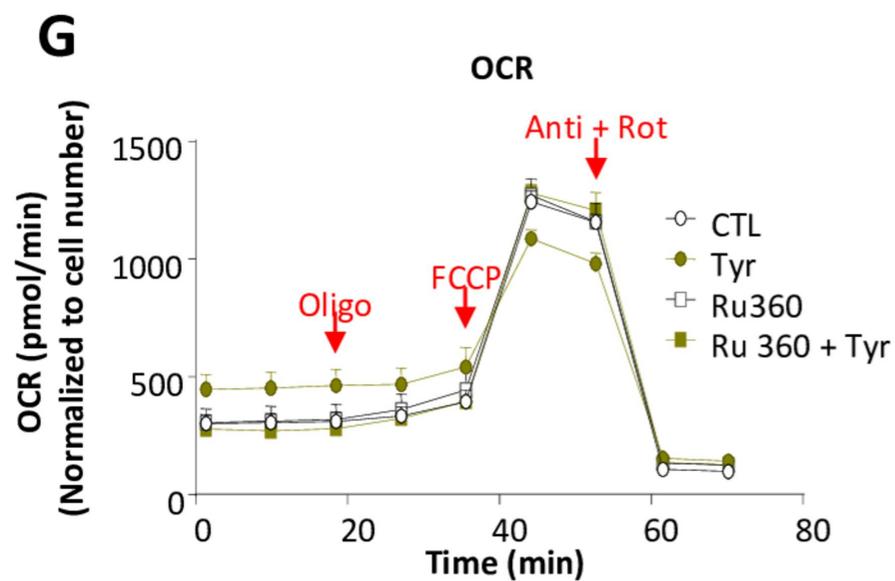
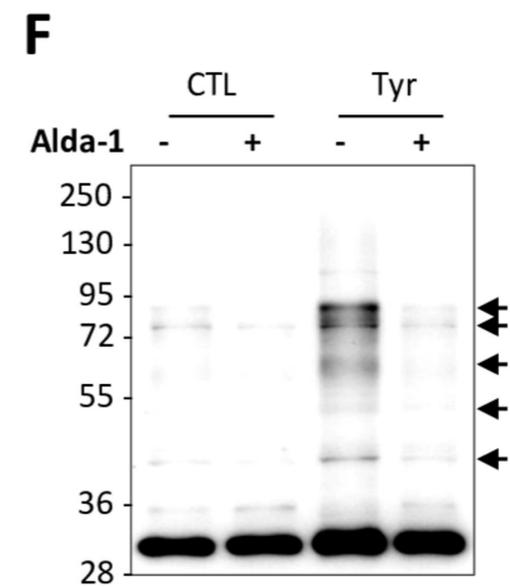
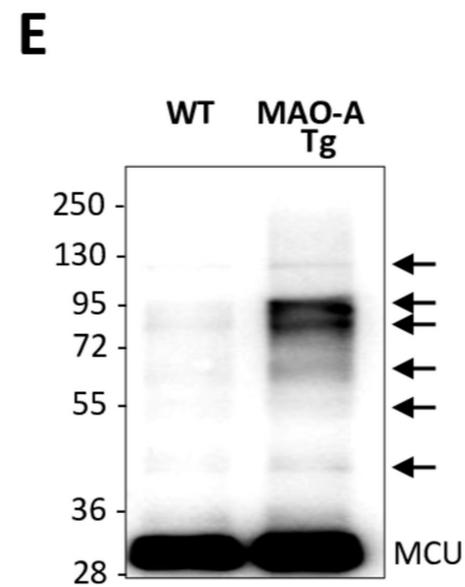
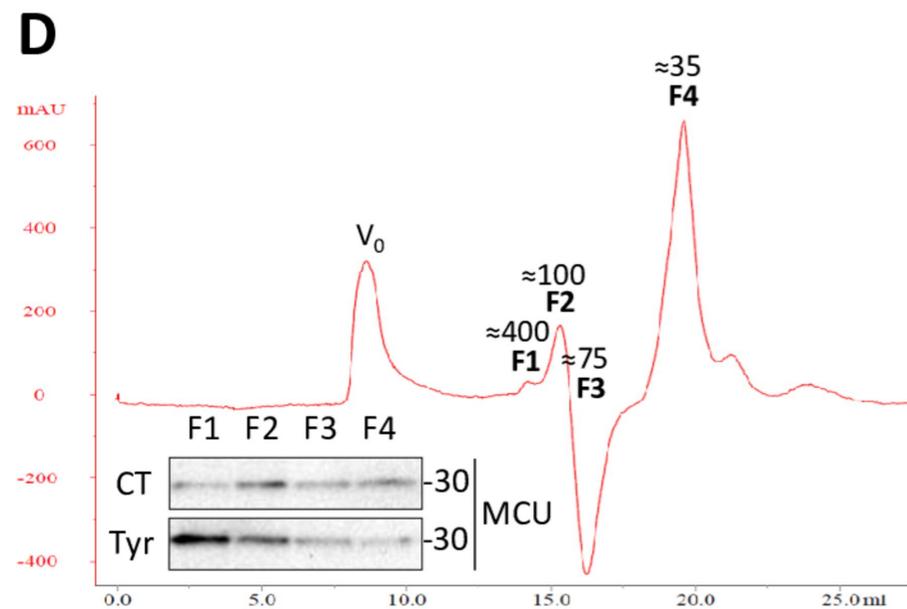
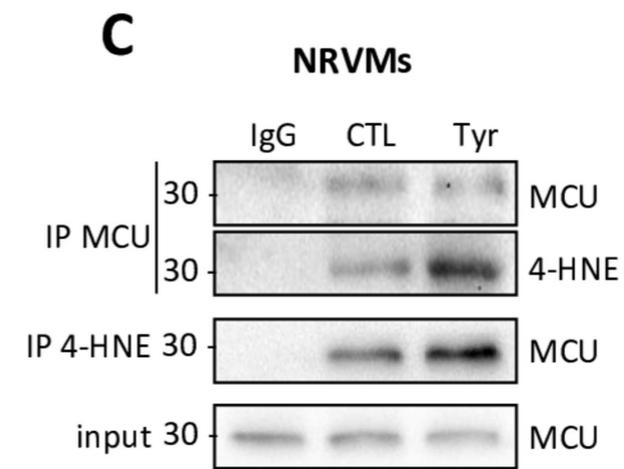
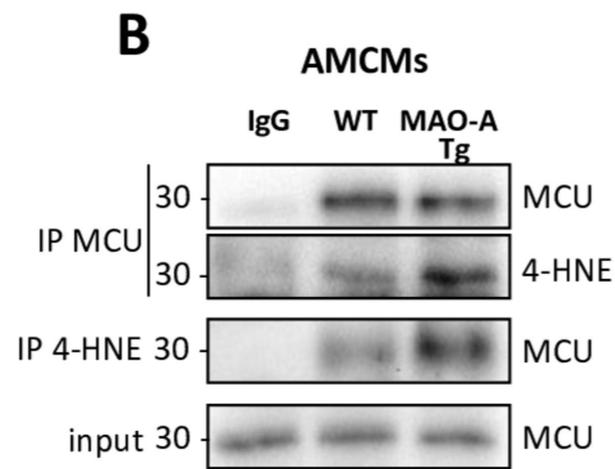
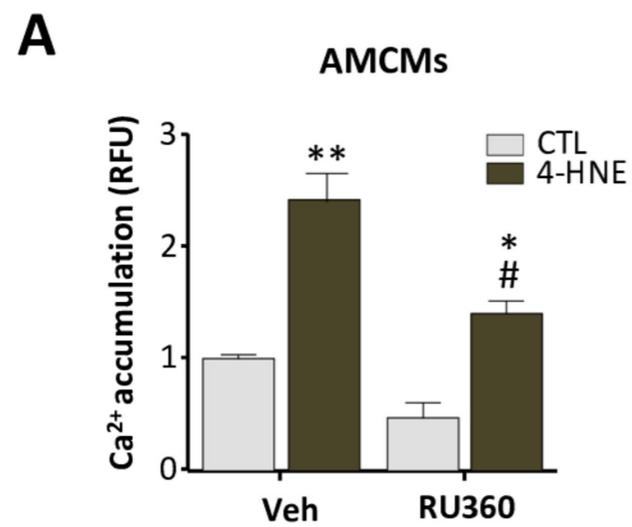


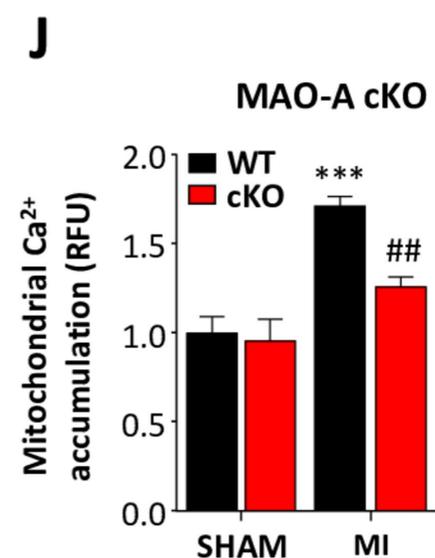
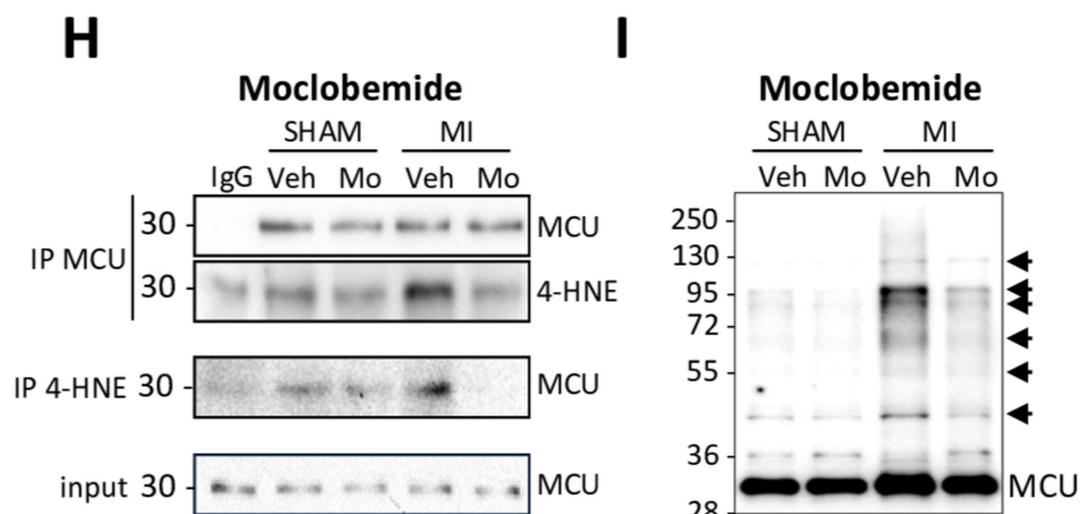
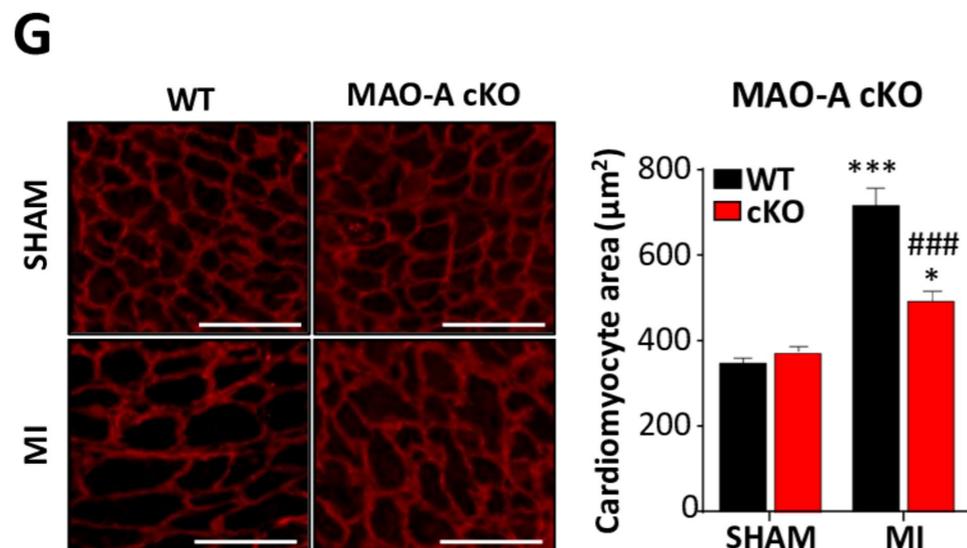
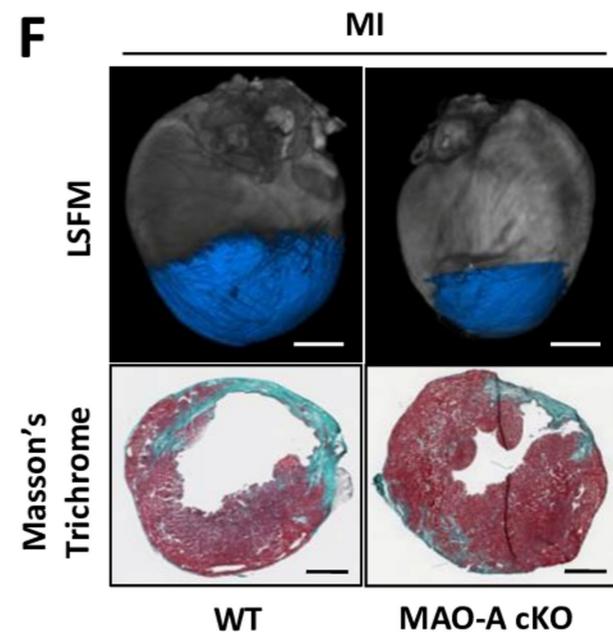
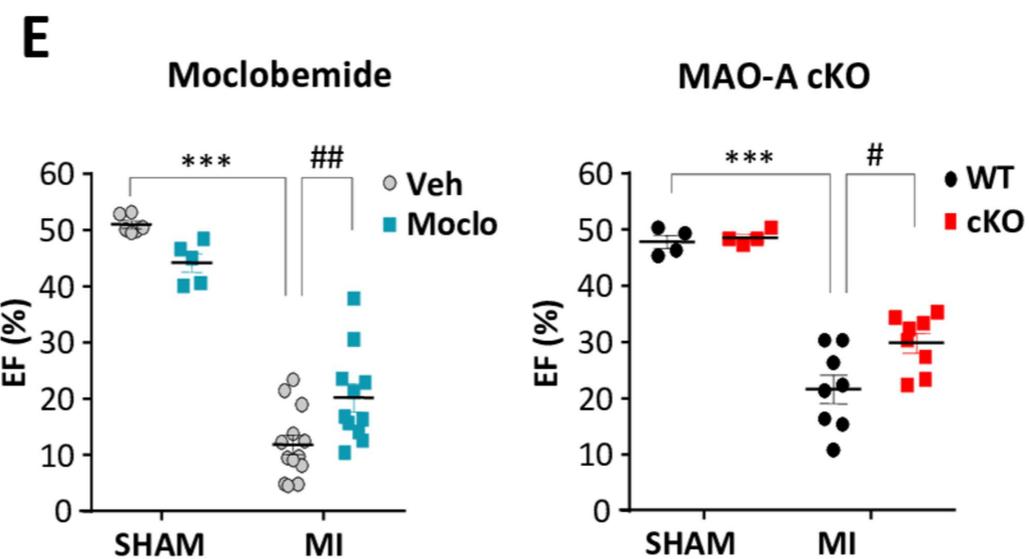
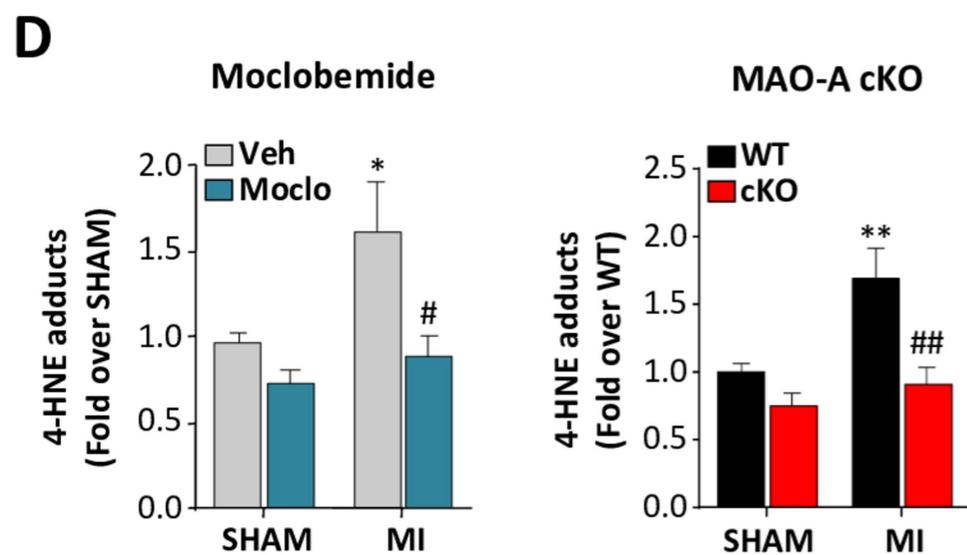
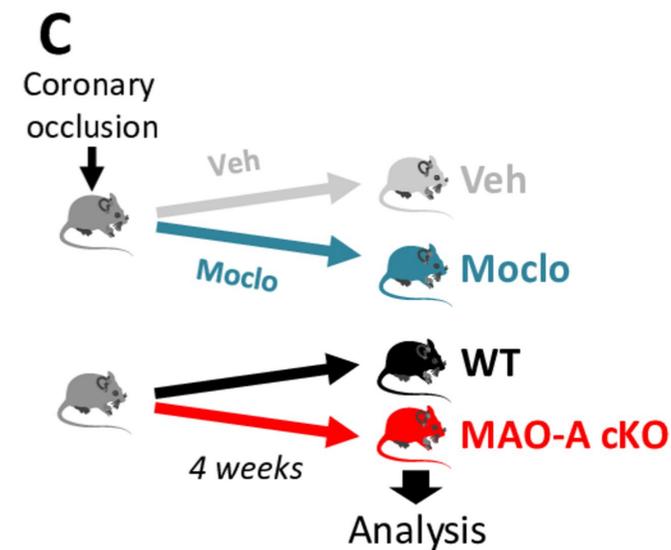
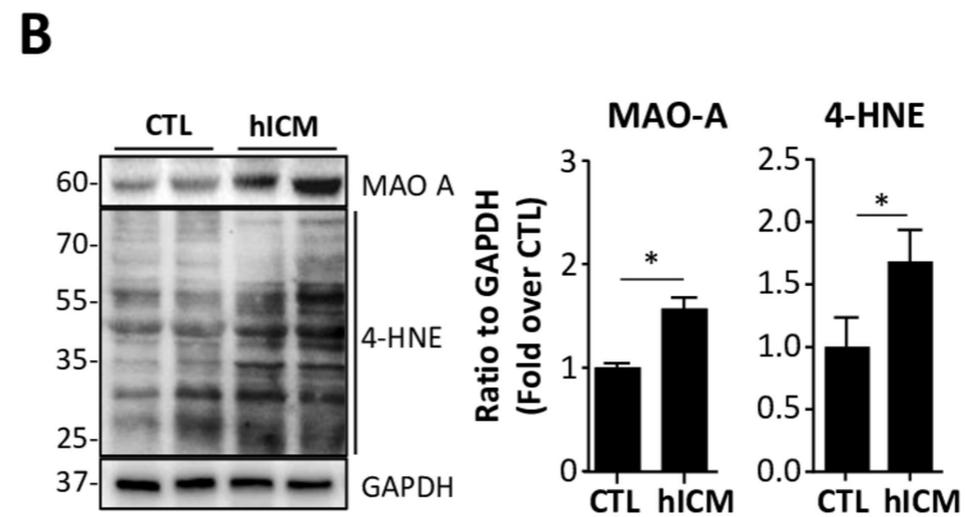
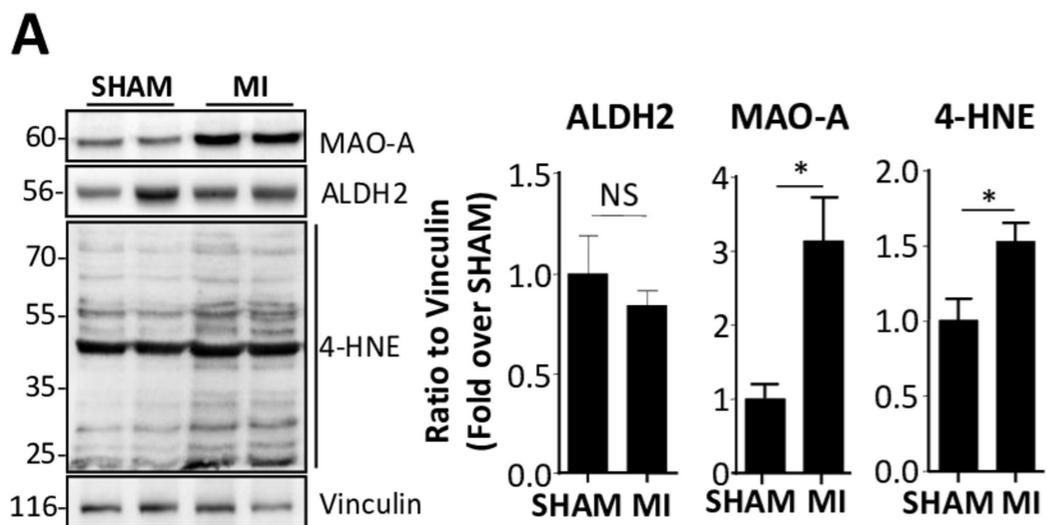




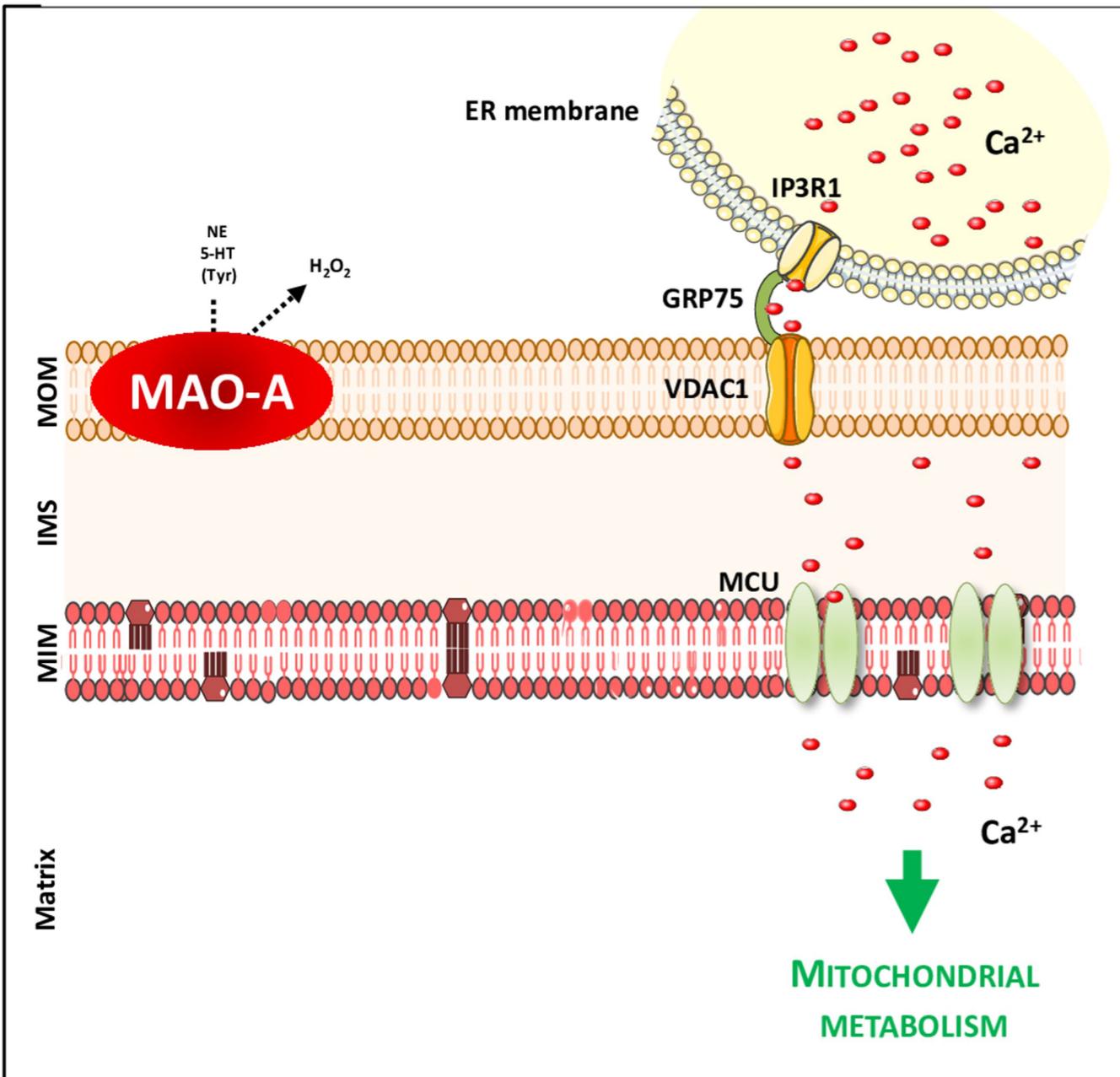








NORMAL HEART



MYOCARDIAL INFARCTION

