

# **NADPH-oxidase 2 is required for molecular adaptations to high-intensity interval training in skeletal muscle.**

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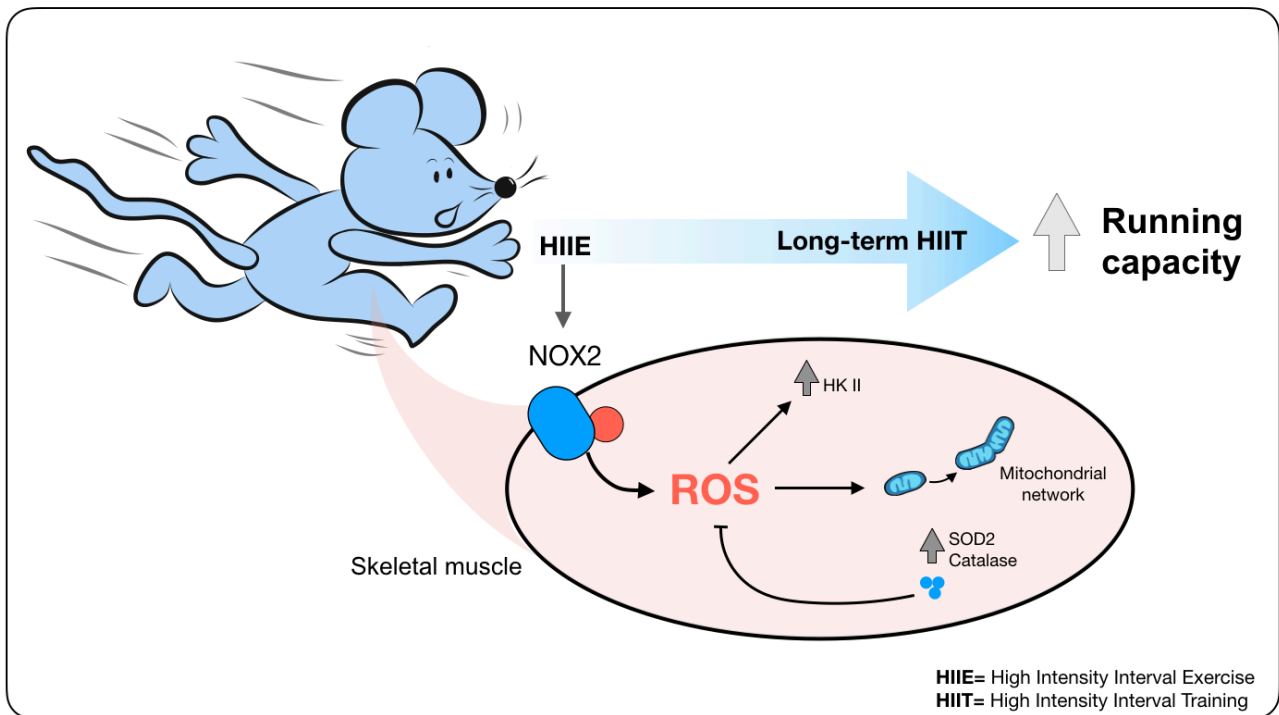
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24 **GRAPHICAL ABSTRACT**



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26

27 **Highlights:**

- 28 • Acute HIIE induces transient NOX2 complex activity *in vivo* in muscle
- 29 • Skeletal muscle adaptations to HIIT were impaired in *ncfl*-deficient mice
- 30 • Functional NOX2 is necessary for HIIT-induced increased expression of antioxidant enzymes
- 31 • *Ncfl*-deficient mice lack HIIT-induced mitochondrial adaptations

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39 **ABSTRACT**

40 **Objective:** Reactive oxygen species (ROS) have been proposed as signaling molecules mediating  
41 exercise training adaptation, but the ROS source has remained unclear. This study aimed to  
42 investigate the requirement for NADPH oxidase (NOX)2-dependent redox changes induced by acute  
43 and long-term high-intensity interval training (HIIT) in skeletal muscle in a mouse model lacking  
44 functional NOX2 complex due to deficient p47phox (*Ncf1*) subunit expression (*ncf1*\* mutation).

45 **Methods:** HIIT was investigated after an acute bout of exercise and after a chronic intervention (3x  
46 week for 6 weeks) in wildtype (WT) vs. NOX2 activity-deficient (*ncf1*\*) mice. NOX2 activation  
47 during HIIT was measured using a genetically-encoded biosensor. Immunoblotting and single-fiber  
48 staining were performed to measure classical exercise-training responsive endpoints in skeletal  
49 muscle.

50 **Results:** A single bout of HIIT increased NOX2 activity measured using electroporated p47roGFP  
51 oxidation immediately after exercise but not 1h after exercise. After a 6-week of HIIT regime,  
52 improvements in maximal running capacity and some muscle training-markers responded less to HIIT  
53 in the *ncf1*\* mice compared to WT, including superoxide dismutase (SOD)2, catalase, hexokinase II  
54 (HK II), pyruvate dehydrogenase (PDH) and protein markers of mitochondrial oxidative  
55 phosphorylation complexes. Strikingly, HIIT-training increased mitochondrial network area and  
56 decreased fragmentation in WT mice only.

57 **Conclusion:** This study provided evidence that HIIT exercise activates NOX2 complex in skeletal  
58 muscle and that the presence of functional NOX2 is required for specific skeletal muscle adaptations  
59 to HIIT relating to antioxidant defense, glucose metabolism, and mitochondria.

60

61 **KEYWORDS: Redox, Reactive oxygen species, Exercise, high-intensity interval training.**

62 **Non-standard abbreviations:** HIIE, high-intensity interval exercise; HIIT, high-intensity interval training; HK II,  
63 Hexokinase II; SOD2, superoxide dismutase 2; NOX2, NADPH oxidase 2; *ncf1*\*, B10.Q. p47phox mutated PDH,  
64 pyruvate dehydrogenase; RER, respiratory exchange ratio; ROS, reactive oxygen species.

## 65 1. INTRODUCTION

66 Physical inactivity is regarded as cause of morbidity and premature mortality worldwide [1].  
67 Inactivity and sedentary behavior are estimated to be responsible for between 6% -10% of the burden  
68 of disease from non-communicable diseases [2]. Exercise intensity is a significant variable explaining  
69 the health benefits induced by physical activity [3, 4]. Indeed, structured high-intensity interval  
70 training (HIIT) has been demonstrated to improve both whole-body and skeletal muscle metabolic  
71 health in different populations [5-7]. Despite the proven efficacy of HIIT to promote metabolic health,  
72 the underlying mechanisms improving adaptation in the HIIT-exercised musculature are not yet fully  
73 understood. Gaining a deeper understanding of the mechanistic basis of the signaling mechanisms  
74 governing the acute and chronic responses to HIIT in skeletal muscle would support the development  
75 of more effective exercise training regimes and the identification of potential drug targets.

76 Reactive oxygen species (ROS) act as intracellular compartmentalized second  
77 messengers mediating skeletal muscle adaptations in both health and disease [8, 9]. Sprint interval  
78 bicycle exercise has been shown to elicit greater post-exercise plasma hydrogen peroxide compared  
79 to moderate exercise in humans [10], suggesting that exercise-induced ROS production in skeletal  
80 muscle may be intensity dependent. Specific ROS may be required for adaptation to chronic exercise,  
81 since ROS scavengers have been shown to disrupt some of the acute and long-term responses to  
82 exercise in skeletal muscle (reviewed in [11]). Furthermore, elevated levels of systemic oxidative  
83 stress markers were associated with greater adaptations after 6-weeks of exercise training in humans  
84 [12]. Taken together, this suggests that ROS may contribute to the intensity-dependent myocellular  
85 exercise training adaptation in skeletal muscle.

86 Although many studies have suggested the importance of ROS molecules to exercise  
87 training adaptation, the exact myocellular source of ROS has remained unclear [13]. For many years,  
88 mitochondria were believed to be as the primary source of ROS during exercise in skeletal muscle.  
89 More recently, non-mitochondrial sources have emerged as potential ROS sources during contractile

90 activity in skeletal muscle [14, 15]. Based on studies using electrically evoked contractions in isolated  
91 rodent muscle, the professional superoxide-producing enzyme complex NADPH oxidase 2 (NOX2)  
92 was strongly suggested to mediate contraction-induced ROS production [15]. Moreover,  
93 pharmacological inhibition of NOX2 has been shown to disrupt acute signaling and gene expression  
94 elicited by moderate-intensity endurance exercise in mice [14]. Thus, whether NOX2 is a significant  
95 source of ROS during high-intensity exercise and its role in the specific context of HIIT requires  
96 clarification.

97 In the present study, we investigated whether NOX2 is activated in mouse skeletal  
98 muscle by physiological acute high-intensity exercise (HIIE). Furthermore, we investigated if NOX2  
99 activity was required for the long-term skeletal muscle adaptations to HIIT using a mouse model  
100 lacking functional NOX2 due to a mutation in its regulatory subunit p47phox. We hypothesized that  
101 NOX2 was a major ROS source in skeletal muscle during this high-intensity exercise modality and  
102 required for long-term HIIT adaptations.

103

## 104 2. RESULTS

### 105 2.1 Acute HIIE increased NOX2-dependent redox changes in skeletal muscle.

106 To assess NOX2-specific ROS production, we used a genetically- encoded probe expressing human  
107 p47<sup>phox</sup> fused to the N-terminus of redox-sensitive green fluorescent protein 2 (p47roGFP) [16].  
108 Oxidation of roGFP in this probe causes a ratio metric change in fluorescence, measureable as a  
109 reduction in the 470 nm and an increase/maintenance in the 405 nm emission. The p47roGFP reporter  
110 was expressed in tibialis anterior muscle (TA) via *in vivo* electroporation 1 week before exercise.  
111 Acute HIIE elicited an increase in p47roGFP oxidation immediately after exercise (time 0) which  
112 returned to baseline at 1h after HIIE (Figure 1). This demonstrated that NOX2-dependent ROS  
113 production is increased transiently by HIIE.

114

115 Phosphorylation of p38 MAPK and ERK1/2 were increased by acute HIIE (Figure 2). Moreover,  
116 phosphorylation of AMPK and its substrate ACC2 increased immediately after exercise in Quad and  
117 SOL muscles (Figure 2).

118 To evaluate whether an acute HIIE increased the autophagy-associated LC3 I lipidation in skeletal  
119 muscle, we measured LC3 I and II expression in muscle lysates. Neither Quad nor SOL muscle  
120 changed their LC3 II/I ratio up to 4h post-HIIT (Figure 3), suggesting that HIIE does not increase  
121 autophagy during exercise or in the immediate post-exercise recovery period.

122

## 123 **2.2 Lack of NOX2 complex activity reduced responsiveness in running capacity**

124 Having confirmed that an acute bout of HIIE activated NOX2, we next tested whether NOX2 is  
125 required for long-term HIIT adaptation. We used *Ncf1*\* mice, a described whole-body loss-of-  
126 function model for NOX2 activity [17] with undetectable ROS production [18]. *Ncf1*\* mice were  
127 indistinguishable from WT and no cases of the previously reported spontaneous post-partum  
128 arthritis[19] were observed during >1y of breeding. Parallel results from our laboratory demonstrated  
129 the absence of in vivo treadmill exercise-stimulated ROS production in skeletal muscle of *Ncf1*\* mice  
130 [20], in agreement with earlier in vitro studies using electrical stimulation [16].

131 Presently, WT and *nclf*\* mice performed a three-times per week HIIT regimen for 6-weeks on a  
132 motorized treadmill [21]. The HIIT intervention increased maximal running capacity in WT but not  
133 *nclf*\* mice, compared to untrained mice (Figure 4A: +23% vs. 10% in WT and *nclf*\* respectively).  
134 Similar body weight and composition were observed in the untrained state, but trained *nclf*\* mice  
135 displayed lower body weight compared to trained WT mice (Figure 4B). Reduced body fat and  
136 increased lean mass were observed in the *nclf*\* HIIE group compared with the trained WT group  
137 (Figure 4C-D). Neither training nor genotype affected energy intake (Figure 4 E-F). Overall, *nclf*\*  
138 mice showed a tendency towards lower RER than WT mice (Figure 4G), but a significant genotype  
139 main effect was only observed in the trained group (Figure 4H). Similar oxygen consumption was

140 observed between genotypes in both the untrained and trained state (Figure 4I-J). Thus, NOX2-  
141 deficient mice display impaired HIIT-induced improvements in maximal running capacity and lower  
142 body fat content after HIIT training.

143

### 144 **2.3 NOX2 deficiency impaired the HIIT-induced increase in specific antioxidant enzymes**

145 ROS scavengers decrease the moderate-intensity training-stimulated improvements in antioxidant  
146 capacity in skeletal muscle [22, 23]. Furthermore, acute pharmacological blockade of NOX2 reduces  
147 mRNA levels of key antioxidant enzymes in mouse skeletal muscle [14]. Here, the mitochondrial-  
148 localized manganese-dependent superoxide dismutase (SOD2) protein expression increased only in  
149 WT (+119%) but not in *ncfl*\* (+26%) in response to HIIT, driven by a higher baseline SOD2 content  
150 in *ncfl*\* vs. WT mice (Figure 5A). A similar tendency was observed for catalase expression in WT  
151 (+74%,  $p = 0.06$ ) vs. *ncfl*\* (26%) (Figure 5B). Neither genotype nor exercise training affected  
152 thioredoxin reductase 2 (TRX2), gp91phox, or nNOS protein expression (Figure 5C-E). Thus, NOX2  
153 deficiency reduces the HIIT adaptations in specific antioxidant enzymes but not globally in redox-  
154 signaling proteins.

155

### 156 **2.4 HIIT-induced muscle HKII expression was NOX2-dependent**

157 Exercise training may improve muscle insulin sensitivity partially by increasing the protein content  
158 of glucose handling proteins [24]. Since antioxidants decrease exercise training-induced insulin  
159 sensitivity [22], we tested whether HIIT and/or NOX2 deficiency affected glucose handling proteins.  
160 Neither HIIT nor genotype affected glucose transport 4 (GLUT4) expression (Figure 5F). In contrast,  
161 hexokinase II, a rate-limiting glycolytic enzyme, increased +77% with HIIT in WT ( $p < 0.05$ ) but did  
162 not respond to HIIT in the *ncfl*\* mice (Figure 5G). No effects were observed on total TBCD4 or  
163 AKT2 protein abundance (Figure 5H-I).

164

## 165 **2.5 Lack of NOX2 activity impaired mitochondrial adaptations to HIIT.**

166 HIIT has been associated with an increase in both mitochondrial content and function [25]. ROS  
167 could regulate training-induced mitochondrial biogenesis [26, 27]. Moreover, pharmacological  
168 inhibition of NOX2 blunts exercise-stimulated mRNA levels of mitochondrial enzymes [14]. To test  
169 whether NOX2 activity is required for training-induced mitochondrial biogenesis, we first measured  
170 mitochondrial electron transport chain complex protein abundance and other mitochondrial proteins.  
171 The mitochondrial complex I marker was increased (+55%) in the trained group compared to the  
172 sedentary control in WT but not significantly increased in *ncfl*\* mice (Figure 6A). A genotype main  
173 effect was found for complex III and Complex IV markers (Figure 6C-D). Moreover, total PDH levels  
174 increased in the WT (+129%) but not in *ncfl*\* mice (+30%, Figure 6G).

175 Mitochondria in skeletal muscle undergo fission/fusion-events and can vary from highly fragmented  
176 to interconnected tubular networks [28]. Since exercise training has been associated with more  
177 elongated and fused mitochondria [29, 30], we estimated the change in the mitochondrial network in  
178 single quadriceps muscle fibers by immunofluorescent imaging of COX4 (Figure 7A-D).  
179 Mitochondrial fragmentation was reduced by HIIT training in WT but not *ncfl*\* mice (Figure 7B),  
180 and the cytosolic area occupied by mitochondria was increased by HIIT in WT but not in *ncfl*\* mice  
181 (Figure 7C). Consistent with a disturbed ability to fuse mitochondria in *ncfl*\* mice, we observed an  
182 increase of the inner membrane fusion protein OPA1 by HIIT in WT but not in *ncfl*\* mice, and a  
183 tendency towards the same for the mitochondrial outer membrane fusion protein mitofusin-2 (MFN2)  
184 ( $p=0.05$ ) (Figure 7D-F). Taken together, this shows that NOX2 is required for multiple aspects of  
185 mitochondrial adaptations to HIIT, with particularly strong effects on mitochondrial network  
186 morphology.

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### 190 3. DISCUSSION

191 ROS are proposed to act as signaling molecules mediating myocellular exercise training adaptations  
192 [9]. However, the exact source of ROS involved is still debated and may differ depending on the  
193 intensity of exercise. A recent study showed that adaptive gene expression to acute *in vivo* and *in*  
194 *vitro* exercise models was blunted by NOX2 inhibitors [14]. Our current study supports and extends  
195 on these data by showing that the functional improvement in maximal exercise capacity, a number of  
196 exercise-responsive proteins and most strikingly, mitochondrial morphological adaptations were less  
197 responsive to HIIT training in mice lacking functional NOX2 complex. The specific adaptations are  
198 briefly discussed below followed by some closing reflections on their potential connectivity.

199

#### 200 3.1 Running performance

201 Some [31-33] but not all studies [23, 27] have found that ROS scavengers impair the training-induced  
202 improvements in maximal exercise capacity in rodents and humans. The lack of consistency in  
203 previous studies has been attributed to differences in antioxidant supplementation efficacy, specificity  
204 and/or training regimens. Here, using a genetic loss-of-function model, we observed that an acute  
205 bout of HIIE was sufficient to induce p47roGFP biosensor oxidation in skeletal muscle, indicating  
206 that HIIE activated NOX2 *in vivo*, and that lack of NOX2 activity in skeletal muscle was associated  
207 with a blunted improvement in running capacity after a 6-week HIIT period. An immediate concern  
208 is that the decreased running capacity in *ncfl*\* vs. WT mice after HIIT could have contributed to the  
209 decreased protein response to HIIT. However, we find this unlikely since 1) there was no difference  
210 in running capacity before training and the mean difference in maximal running speed after training  
211 was only ~5%, arguing that these mice were exposed to a similar HIIT intensity and volume and 2)  
212 some training-responsive proteins such as GLUT4 responded equally to HIIT in both genotypes.  
213 Overall, these data suggest that NOX2 is likely a major contributor to the previously proposed ROS-  
214 mediated increase in endurance exercise capacity in rodents and humans [11].

215

### 216 **3.2 Antioxidant capacity**

217 Chronic adaptations to exercise training are thought to result from the cumulative stimulatory effect  
218 of repeated acute exercise bouts on gene transcription [34]. Three weeks of HIIT in humans improve  
219 antioxidant capacity in plasma of humans [35]. This is consistent with the proposal that ROS are  
220 essential mediators of the hormetic increase in endogenous antioxidant defense in response to training  
221 [36]. A previous study found that NOX2 was required for some of the acute antioxidant defense gene  
222 transcription responses to muscle activity since pharmacological blockade of NOX2 *in vivo* or  
223 electrically stimulated contraction *in vitro* blocked exercise-responsive gene transcription of SOD2  
224 and glutathione peroxidase transcription [14]. This was suggested to be regulated by a NOX2-  
225 dependent NF- $\kappa$ B pathway [37]. Whether NF- $\kappa$ B was a contributing factor for the lack of HIIT  
226 response in current study was not investigated.

227

### 228 **3.3 Glucose handling enzymes**

229 Both GLUT4 and HKII are known to respond to HIIT in humans [38]. A key finding in our study was  
230 that HKII, arguably one of the most exercise training-responsive proteins in man, did not increase  
231 after training in the NOX2-deficient *nxf1*\* mice. A ROS-dependence is consistent with previous  
232 studies showing that antioxidants blunt electrically stimulated contraction-induced HKII mRNA and  
233 activity in muscle cells [39, 40] and training-induced HKII mRNA in mice [41]. In contrast, the  
234 increase in GLUT4 expression with HIIT did not appear to depend on NOX2 activity.

235

### 236 **3.4 Mitochondrial protein expression**

237 Endurance exercise training and HIIT are known to increase the protein content, volume, and function  
238 of skeletal muscle mitochondria [42]. Previous studies reported that the antioxidants Vitamin C and  
239 E reduced exercise training-induced mitochondrial biogenesis markers such as COX4 and CS, likely

240 by downregulation of PCG1-alpha and TFAM in human and murine skeletal muscle [22, 23, 26, 31].  
241 Interestingly, exercise-induced TFAM and citrate synthase mRNA levels in mouse skeletal muscle  
242 were also blocked by pharmacological inhibition of NOX2 [14]. Presently, we observed that the well-  
243 established mitochondrial content marker COX4 [43], as well as the mitochondrial metabolic  
244 capacity-markers PDH and mitochondrial complex I, increased after HIIT in WT but not *ncfl*\* mice.  
245 We speculate that the general tendency towards increased base-line expression of mitochondria-  
246 related proteins in *ncfl*\* vs. WT mice might increase resting fat oxidation, consistent with the  
247 observed lower RER and leaner phenotype in trained *ncfl*\* vs. WT mice.

248

### 249 **3.5 Mitochondrial network morphology**

250 Endurance exercise training is associated with an elongated mitochondrial network [29] and  
251 decreased mitochondrial fragmentation [30] in the trained musculature in rodents and humans [44].  
252 HIIT has been reported to increase the respiratory capacity of mitochondria [25, 45] but the HIIT-  
253 associated changes in mitochondrial morphology have, as far as we are aware, not been investigated  
254 in any species. Our data show for the first time that these responses also occur with HIIT.  
255 Furthermore, the HIIT-induced mitochondrial network remodeling exhibited the strongest  
256 quantitative impairment of any endpoint in mice lacking NOX2 activity. The genotype difference in  
257 mitochondrial network was accompanied by a blunted HIIT-induced increases of the mitochondrial  
258 fusion related proteins OPA1 and MFN2 in *ncfl*\* vs WT muscle. We cannot dismiss the changes in  
259 fusion-related proteins are independent of the changes in mitochondrial content. This might provide  
260 clues to the underlying mechanism but requires further investigation.

261

### 262 **3.7 Are the different molecular findings connected?**

263 Many of the proteins that differed significantly between WT and *ncfl*\* mice displayed a similar  
264 expression pattern with an increased relative expression in untrained *ncfl*\* mice and a lower relative

265 responsiveness to HIIT in *ncfl*\* mice. Importantly, the changes were confined to subsets of proteins  
266 and not observed for e.g. GLUT4, TBC1D4 or Akt2 expression. Based on the literature many of these  
267 changes could be mechanistically connected. Thus, the mitochondrial adaptations to HIIT were  
268 impaired both in terms of network morphology and mitochondrial protein expression. The baseline  
269 upregulation of several antioxidant enzymes, including SOD2 and catalase could suggest increased  
270 mitochondrial ROS production. Mitochondrial ROS production has previously been associated with  
271 mitochondrial fragmentation and higher resting fat oxidation [46-48], but their contribution here is  
272 unclear since genotype differences in these parameters were only observed in the trained state. Worth  
273 noting, however, we did observe a significant decrease in baseline RER in *ncfl*\* vs. WT mice [20],  
274 indicating increased resting fat oxidation in *ncfl*\* mice. Hexokinase II is known to shuttle to and from  
275 mitochondria [49]. Interestingly, acute disruption of HKII binding to mitochondria, using a peptide-  
276 inhibitor in a perfused mouse heart ischemia-reperfusion injury model, markedly reduced cardiac  
277 recovery and increased ROS during ischemia and reperfusion [50]. Similar links have been  
278 established in other tissues, including skeletal muscle [51]. Thus, we speculate that HKII may  
279 somehow be connected to both the increased base-line fat oxidation and changes in mitochondrial  
280 morphology/function. The mechanistic connections between the observed molecular changes in *ncfl*\*  
281 vs WT muscle should be measured in the future.

282

### 283 **3.8 Conclusion**

284 This study provided evidence that NOX2 is activated by HIIE and that the presence of functional  
285 NOX2 is required for long-term training adaptation, including increased muscle protein expression  
286 of antioxidant defense enzymes, mitochondrial enzymes and hexokinase II, and increased  
287 mitochondrial network volume and decreased mitochondrial fragmentation. This suggests that NOX2  
288 signaling modulates the exercise-response to HIIT. If and how these changes are interconnected  
289 should be clarified in future studies.

290

## 291 **MATERIALS AND METHODS**

292 **Animals.** 10-week-old female B10.Q wildtype (WT) and B10.Q. p47phox mutated (*ncfl\**) were  
293 maintained on a 12 hours light/dark cycle, group-housed with free access to water and standard rodent  
294 chow diet (Altromin no. 1324; Chr. Pedersen, Denmark).

295 All experiments were approved by the Danish Animal Experimental Inspectorate  
296 (2015–15–0201–00477).

297

298 **Maximal running capacity.** Running capacity was carried as previously described [52]. Briefly, mice  
299 were acclimated to the treadmill three times (10 min at 0.16 m/s) (Treadmill TSE Systems) a week  
300 before the maximal running tests. The maximal running test started at 0.16 m/s for 300 s with 10 %  
301 incline, followed by a step-wise increase (0.2 m/s every 60s) in running speed until exhaustion.  
302 Exhaustion was defined as the point at which instead of running on the treadmill, the mice fall back  
303 on the grid three times within 30 secs. Maximal running speed was determined as the last completed  
304 stage during the incremental test. All tests were performed blinded.

305

306 **Indirect calorimetry and body composition.** Body composition was assessed by MRI-scanning  
307 (EchoMRI-4, Echo Medical System LLC, Texas, USA) according to the manufacturer's instructions.  
308 Whole-body metabolism was assessed in a 16-chamber indirect calorimetry system after a 2-day  
309 acclimation period (PhenoMaster; TSE Systems, Frankfurt, Germany).

310

311 **Acute high-intensity interval exercise (HIIE).** Fed mice performed a single HIIE bout switching  
312 between 2 min running at 100% of the maximal running speed of each group, and then 2 min of active  
313 recovery running at 30% of the maximal running speed for a total of 60 min. Tissues were harvest  
314 immediately, one and four hours after exercise.

315

316 **HIIT intervention.** Both WT and *ncfl*\* mice were randomly assigned to the control or HIIT group.  
317 The HIIT training was carried as previously described [21]. Briefly, HIIT training involved treadmill  
318 running three days per week for six weeks. In each training session, mice switched between two min  
319 running at 100% of the maximal running speed of each group, and then 2 min of active recovery  
320 running at 30% of the maximal running speed for a total of 60 min. During the training period that  
321 HIIT group train, the control mice remained in their cages.

322

323 **In vivo gene transfer in adult skeletal muscle.** Tibialis anterior electroporation was performed as  
324 previously described [53]. Briefly, mice were anesthetized with 2-3% isoflurane. Hyaluronidase  
325 (H3884, Sigma) dissolved in sterile saline solution (0.36 mg/ml) was injected intramuscularly in  
326 tibialis anterior (TA) muscle, followed by 40 µg plasmid injection 1 hour later in re-anesthetized  
327 mice. Muscle electroporation was then performed by delivering 10 electrical pulses at an intensity of  
328 100 V/cm, 10-ms pulse duration, 200-ms pulse interval using a caliper electrode (#45-0101 Caliper  
329 Electrode, BTX Caliper Electrodes, USA) connected to an ECM 830 BTX electroporator (BTX  
330 Harvard Apparatus). The p47-roGFP biosensor used to determine NOX2 activity was a kind gift from  
331 Dr. George G. Rodney [16].

332

333 **Redox Histology.** Muscle freezing and sectioning was performed as previously reported [54]. In brief,  
334 TA muscles were dissected and embedded in optimum cutting temperature (OCT) medium from  
335 Tissue Tek, frozen in liquid nitrogen cooled isopentane and kept at -80 °C until processing. Redox  
336 histology was performed as previously described [55], Briefly, p47roGFP- transfected muscles  
337 were cut in 10 µm thickness followed by incubation in 50 µl of PBS containing 50 mM n-  
338 ethylmaleimide (NEM) for 10 min at 4°C. Sections were fixed in PBS-dissolved 4%

339 paraformaldehyde (50 mM NEM) for 10 min, washed three times in PBS (5 min) and mounted in  
340 mounting medium (Vectashield, USA).

341

342 **Western Blotting.** Western blotting was performed as previously described [12]. Briefly, ~40 µg of  
343 quadriceps muscle and the whole soleus were lysed for 1 min at 30 Hz on a shaking bead-mill  
344 (TissueLyser II, Qiagen, Valencia, CA, USA) in ice-cold lysis buffer (0.05 mol/L Tris Base pH 7.4,  
345 0.15 mol/L NaCl, 1 mmol/L EDTA and EGTA, 0.05 mol/L sodium fluoride, 5 mmol/L sodium  
346 pyrophosphate, 2 mmol/L sodium orthovanadate, 1 mmol/L benzamidine, 0.5% protease inhibitor  
347 cocktail (P8340, Sigma Aldrich), and 1% NP-40). After rotating end-over-end for 30 min at 4°C,  
348 lysate supernatants were collected by centrifugation (18,327 g) for 20 min at 4°C. Lysate protein  
349 concentrations were determined using BSA standards (Pierce) and bicinchoninic acid assay reagents  
350 (Pierce). Total protein and phosphorylation levels of relevant proteins were determined by standard  
351 immunoblotting techniques, loading equal amounts of protein. The primary antibodies used; p-  
352 AMPK<sup>Thr172</sup> (Cell Signaling Technology (CST)), #2535S), p-p38 MAPK<sup>Thr180/Tyr182</sup> (CST, #9211),  
353 Hexokinase II (CST, #2867), GLUT4 p-ACC2 Ser<sup>212</sup> (Millipore, 03-303), (ThermoFisher Scientific,  
354 PA-23052), Rac1 (BD Biosciences, #610650), NOX2 (Abcam, #Ab129068), Catalase (SCBT, sc-  
355 271803), SOD2 (Millipore, 06-984), TRX2 (SCBT, sc-50336), actin (CST, #4973) total p38 MAPK  
356 (CST, #9212), alpha2 AMPK (a gift from D. Grahame Hardie, University of Dundee), total ERK 1/2  
357 (CST, #9102), TBC1D1<sup>ser231</sup> (Millipore #07-2268), Hexokinase II (CST, #2867) MFN2 (#9482), and  
358 OPA1 (BD Biosciences #612606). A cocktail antibody (Abcam, #ab110413) was used as  
359 representative of the mitochondrial electron chain complexes (Oxphos). The optimal protein loading  
360 was pre-optimized to ensure measurements in the linear dynamic range for each antibody. Bands were  
361 visualized using a ChemiDoc imaging system (Bio-Rad, USA). Total protein staining (Coomassie)  
362 was used as a loading control rather than house-keeping proteins, as previously recommended [56].

363 Grayscale levels range for the blots shown was minimally and linearly adjusted across entire blots,  
364 as recommended by the American Society for Biochemistry and Molecular Biology.

365

366 ***Single fiber immunostaining.*** Staining of single fibers was performed as previously described with  
367 slight modifications [30]. Briefly, ~20  $\mu$ g of quadricep muscles were fixed by immersion in ice-  
368 cooled 4% formaldehyde for 4h and long-term stored in 50% glycerol (diluted in PBS) at -20 °C.  
369 Muscles were teased into single fibers with fine forceps and transferred to immunobuffer (50 mM  
370 glycine, 0.25% bovine serum albumin (BSA), 0.03% saponin and 0.05% sodium azide in PBS) After  
371 isolation of a minimum of 30 muscle fibers were incubated overnight with an anti-COX4 antibody  
372 (#16056, Abcam, Cambridge, UK) in immunobuffer containing 0.5% saponin and, after 3 washes  
373 with immunobuffer, single muscle fibers were incubated for 2h with a secondary antibody conjugated  
374 with Alexa Fluor 488 (Invitrogen, UK). A negative control was performed with fibers not exposed to  
375 the primary antibody. The muscle fibers were mounted in Vectashield mounting medium.

376

377 ***Imaging acquisition.*** All confocal images were collected using a 63x 1.4 NA oil immersion objective  
378 lens on an LSM 780 confocal microscope (Zeiss) driven by Zen 2011. Image acquisition was  
379 performed blinded. For the p47roGFP biosensor images, raw data of the 405- and 488-nm laser lines  
380 were exported to ImageJ as 16-bit TIFFs for further analysis. Data are represented as normalized  
381 fluorescence ratio (405/488 nm) and normalized to the WT untrained group.

382

383 ***Statistical analyses.*** Results are shown as means  $\pm$  S.E.M. Statistical testing was performed using *t*-  
384 tests, one-way or two-way (repeated measures when appropriate) ANOVA as applicable. Sidak's post  
385 hoc test was performed when ANOVA revealed significant main effects. Statistical analyses were  
386 performed using GraphPad Prism 8.

387



388 **Author Contributions**

389 CHO and TEJ designed research; CHO, LB, ZL, LAC, JRK, SHR, TEJ performed research; RH  
390 provided the *ncfl*\* mice and intellectual input, CHO analyzed data; CHO and TEJ wrote the paper;  
391 all authors commented on the draft, TEJ Funding Acquisition.

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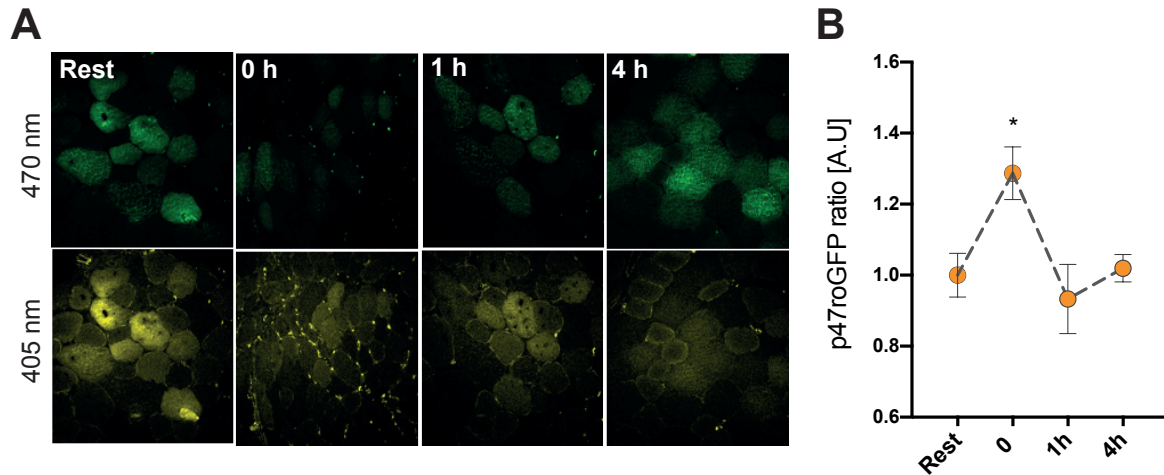
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578 **FIGURES AND LEGENDS**

579 **Figure 1**



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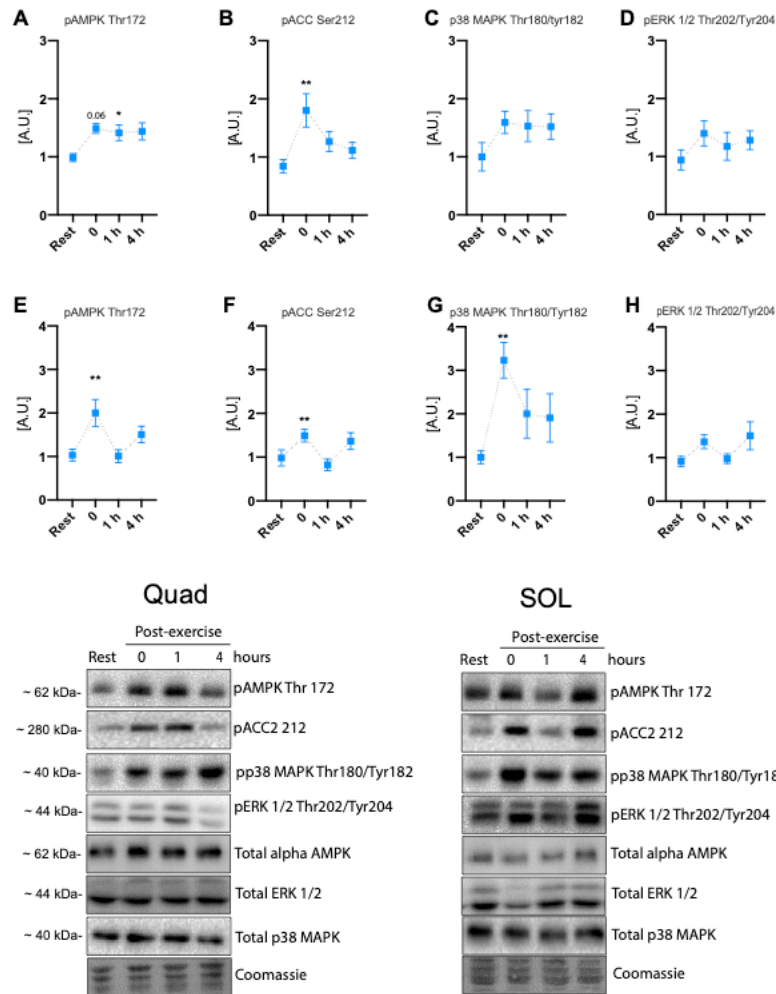
581 **Figure 1. High-intensity interval exercise activates NOX2 transiently in skeletal muscle.** A)  
582 Representative images and b) ratio metric quantification (405/470 nm) of p47roGFP biosensor signal  
583 at resting, exercise and post-exercise period in tibialis anterior muscle. \* denotes  $p < 0.05$  compared  
584 to the resting condition using a one-way ANOVA followed by a Holm-Sidak's multiple comparisons  
585 test. Values are mean  $\pm$  SEM (n=4 per group).

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587



588 **Figure 2**



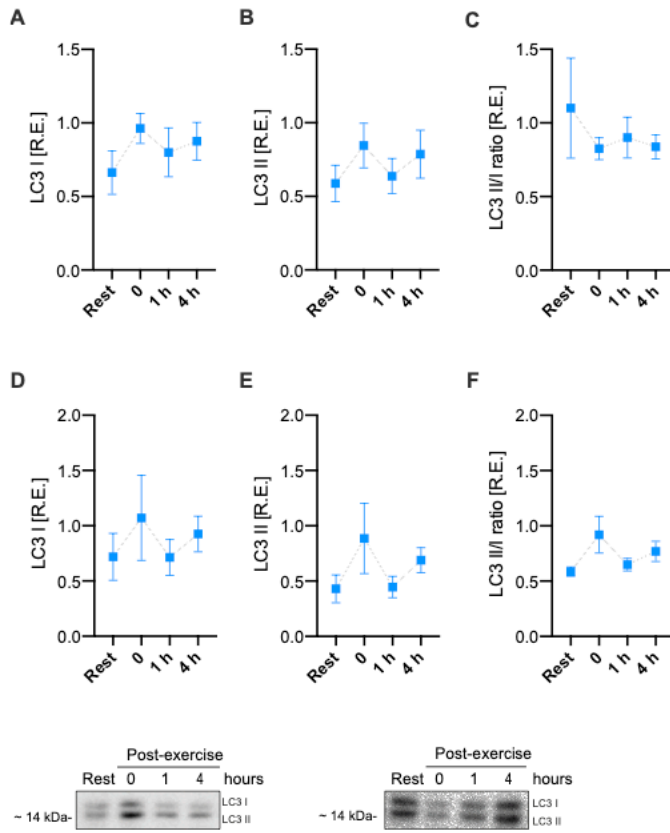
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590 **Figure 2. Acute cellular signaling induced by high-intensity interval exercise.** Exercise-  
 591 stimulated phosphorylation of A) pAMPK Thr172 B) pACC Ser212 C) p38 MAPK Thr180/Tyr182  
 592 D) pERK 1/2 Thr202/Tyr204 for quadriceps muscle and E) pAMPK Thr172 F) pACCC Ser212 G)  
 593 p38 MAPK Thr180/Tyr182 H) pERK 1/2 Thr202/Tyr204 for soleus muscle. \*\*\* denotes  $p < 0.05$  and  
 594  $p < 0.01$ , respectively, compared to the resting condition using one-way ANOVA followed by a Holm-  
 595 Sidak's multiple comparisons test. Values are mean  $\pm$  SEM (n=8 per group).

596

597

598 **Figure 3**



599

600 **Figure 3. Autophagy markers are not induced by acute high-intensity interval exercise.**

601 Exercise-stimulated changes in LC3-I, LC3-II, and LC3-II/LC3-I ratio. One-way ANOVA was  
602 performed. Values are mean  $\pm$  SEM (n=8 per group).

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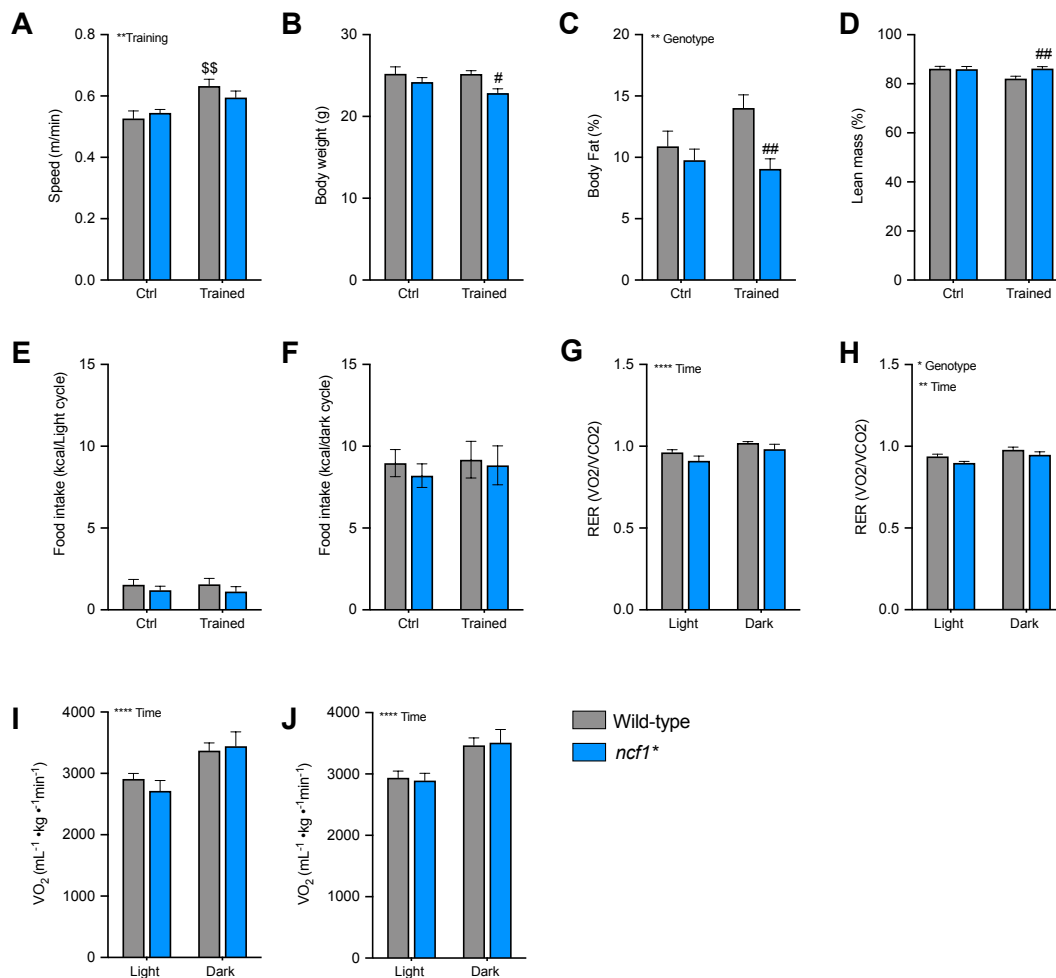
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608 **Figure 4.**

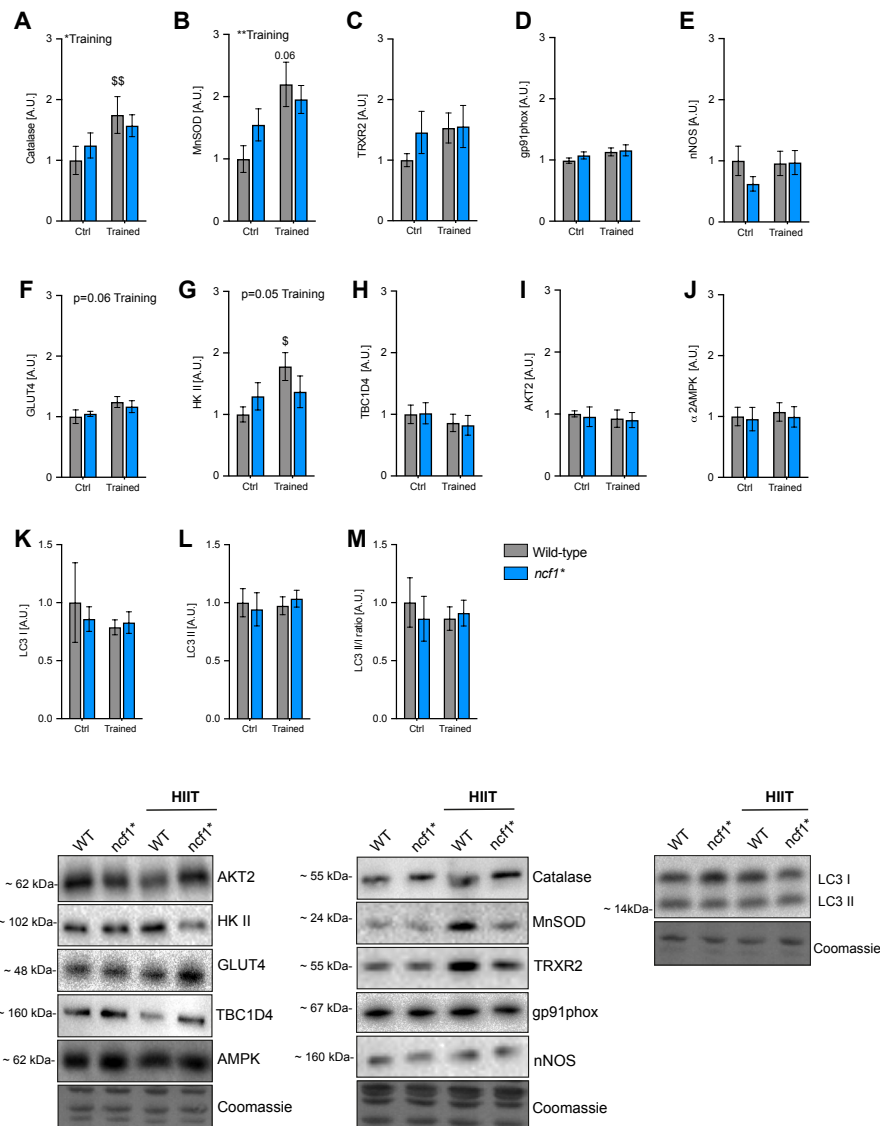


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610 **Figure 4. *ncf1\** mice show a lower responsiveness in running capacity after long-term HIIT.** 6-  
 611 weeks high-intensity interval training outcomes in A) maximal running speed B) Body weight C-D)  
 612 Body composition E) Food intake during the light and F) dark cycle Respiratory exchange ratio in G)  
 613 untrained and H) trained mice, oxygen uptake in I) Untrained and J) trained mice. \$\$\$ denotes p<0.01  
 614 vs. WT Ctrl group, ## denotes p<0.01 vs. WT trained mice. Two-way ANOVA was performed to test  
 615 for effects of training, genotype, and time, followed by a Holm-Sidak's *post hoc* test corrected for  
 616 multiple comparisons. Values are mean ± SEM (n=7-8).

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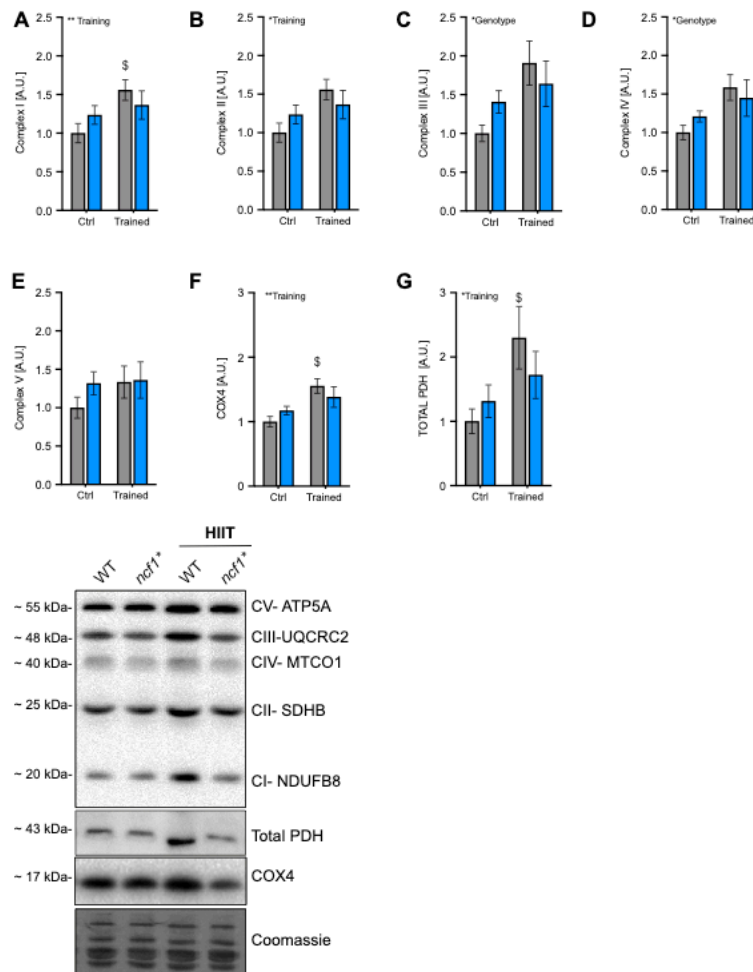
618 **Figure 5**



619

620 **Figure 5. Multiple exercise-training markers in *ncf1\** muscles are less responsive to 6-weeks of**  
 621 **HIIT.** A-E) High intensity interval training-induced changes in redox-related proteins, F-J) Glucose  
 622 handling related proteins and K-M) autophagy-related proteins. \$, \$\$ denotes  $p < 0.05$  and  $p < 0.01$ ,  
 623 respectively compared to WT ctrl Two-way ANOVA was performed to test for effects of training and  
 624 genotype, followed by a Holm-Sidak's *post hoc* test corrected for multiple comparisons. Values are  
 625 mean  $\pm$  SEM (n=7-8).

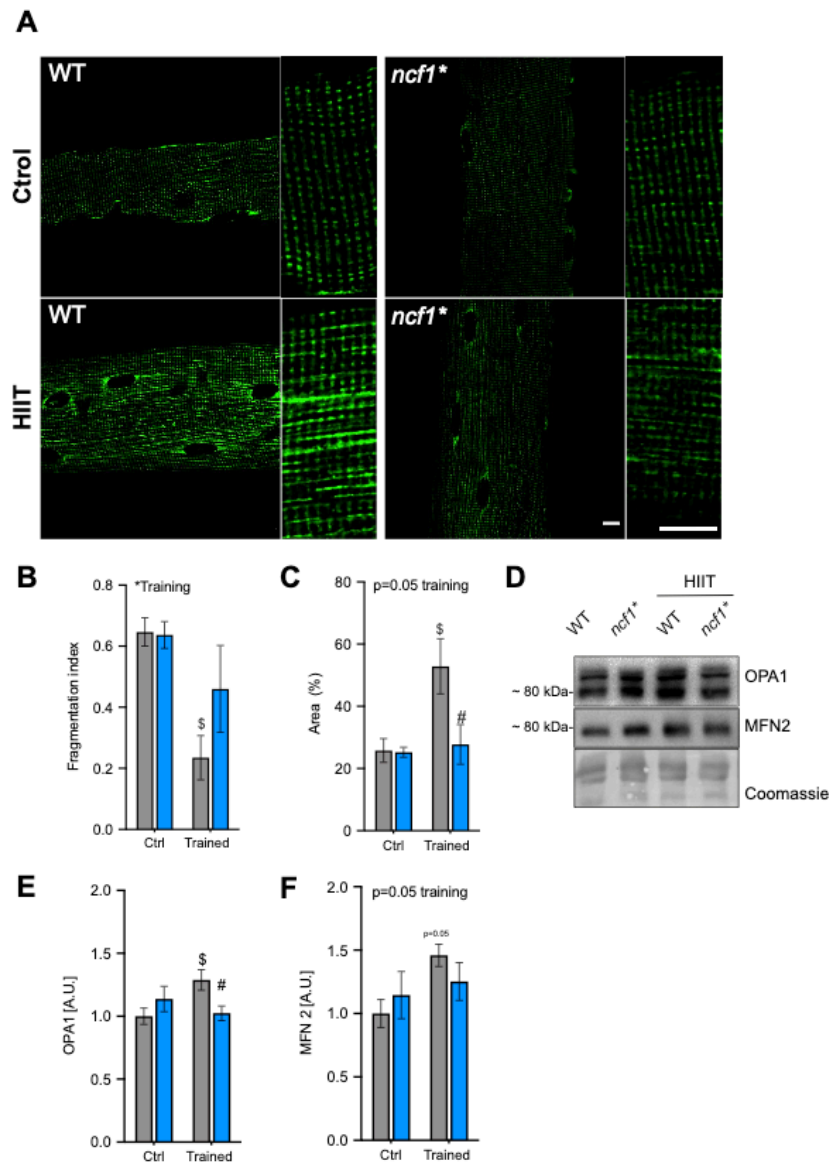
626 **Figure 6.**



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628 **Figure 6. Mitochondrial-related proteins increase to a smaller extent after HIIT in mice lacking**  
 629 **functional NOX2 activity.** Mitochondrial electron chain complexes A) Complex I B) Complex II  
 630 C) Complex III D) Complex IV B) Complex V F) COX4 and G) PDH. \$ denotes p<0.05 vs. WT Ctrl  
 631 group. Two-way ANOVA was performed to test for effects of training, and genotype, followed by a  
 632 Holm-Sidak's *post hoc* test corrected for multiple comparisons. Values are mean ± SEM (n=7-8).

633 **Figure 7.**



634

635 **Figure 7. HIIT increases mitochondrial fragmentation and related proteins in skeletal muscle**  
 636 **of wildtype mice only.** **A)** Representative images from mitochondria-specific stains in quadriceps  
 637 single fibers. Bars indicate 10  $\mu$ m. **B)** Mitochondrial fragmentation index **C)** Mitochondrial area **D)**  
 638 representative and quantification of **E)** OPA1 and **F)** MFN2 protein abundance. \$ denotes  $p < 0.05$ ,  
 639 respectively compared to WT Ctrl. # denotes  $p < 0.05$  WT trained vs. *ncf1\** trained mice Two-way  
 640 ANOVA was performed to test for effects of training and genotype, followed by a Holm-Sidak's *post*  
 641 *hoc* test corrected for multiple comparisons. Values are mean  $\pm$  SEM (A-C,  $n=4$  and E-F,  $n=7-8$ ).