

26 **ABSTRACT**

27 Decreased expression of the mitochondrial protein frataxin (FXN) causes Friedreich's ataxia
28 (FRDA). FRDA is a neurodegenerative disease also characterized by systemic metabolic alterations
29 that increase the risk of developing type 2 diabetes thus aggravating FRDA prognosis. Brown adipose
30 tissue (BAT) is a mitochondria-enriched and anti-diabetic tissue that, in addition to its
31 thermoregulatory role, turns excess energy into heat to maintain energy balance. Here we report that
32 the FXN knock-in/knock-out (KIKO) mouse shows reduced energy expenditure and VO_2 ,
33 hyperlipidemia, decreased insulin sensitivity and enhanced circulating levels of leptin, recapitulating
34 diabetes-like signatures. FXN deficiency leads to alteration of mitochondrial structure and oxygen
35 consumption, decreased lipolysis and lipid accumulation in BAT. Transcriptomic data highlighted a
36 blunted thermogenesis response, as several biological processes related to thermogenesis (e.g.
37 response to temperature stimuli, mitochondrial gene transcription, triglyceride metabolism,
38 adipogenesis) resulted affected in BAT of KIKO mice upon cold exposure. Decreased adaptation to
39 cool temperature in association with limited PKA-mediated lipolysis and downregulation of the
40 expression of the genes controlling mitochondrial metabolism and lipid catabolism were observed in
41 KIKO mice. T37i brown adipocytes and primary adipocytes with FXN deficiency showed reduced
42 thermogenesis and adipogenesis markers respectively recapitulating the molecular signatures
43 detected in KIKO mice.

44 Collectively our data point to BAT dysfunction in FRDA and suggest BAT as a promising target to
45 overcome metabolic complications in FRDA.

46

47 **INTRODUCTION**

48 Friedreich's Ataxia (FRDA) is an inherited autosomal recessive neurodegenerative disorder. It is
49 caused by mutation in the gene encoding mitochondrial protein frataxin (FXN) (Abraham et al., 2015).
50 The primary function of FXN is to direct the mitochondrial synthesis of iron-sulfur clusters (Fe/S),
51 which are essential parts of several mitochondrial enzymes, including mitochondrial respiratory chain
52 complexes and aconitase (Maio and Rouault, 2015).

53 Deficiency in mitochondrial respiration, mitochondrial iron accumulation and oxidative stress are
54 claimed as the main pathogenic factors in FRDA. Mitochondrial dysfunction mostly affects heart
55 (Koeppen et al., 2015) and cerebellum at the level of dentate nucleus (Koeppen et al., 2011;Koeppen
56 and Mazurkiewicz, 2013).

57 FRDA is characterized by a variable phenotype. Besides neurological symptoms, cardiomyopathy
58 (Koeppen et al., 2015) and systemic metabolic alterations occur (Cnop et al., 2013), which can
59 predispose to diabetes development and cause premature death. In particular, patients with FRDA
60 experience a greater risk of abnormal glucose homeostasis, in the form of both insulin resistance and
61 glucose intolerance (Isaacs et al., 2016). Increased blood cholesterol and triglycerides levels have
62 been observed in FRDA patients (Raman et al., 2011;Tamarit et al., 2016). To date, the major cause
63 of diabetes mellitus occurrence in FRDA patients seems to be related to impairment of mitochondria
64 that in pancreatic β -cell are fundamental in generating signals that trigger and amplify insulin
65 secretion (Cnop et al., 2012).

66 Brown adipose tissue (BAT) is a high oxidative tissue extremely rich in mitochondria that highly
67 expresses the thermogenic protein uncoupling protein 1 (UCP1). BAT has emerged as a key regulator
68 of glucose, lipid and insulin metabolism (Chondronikola et al., 2014;Chondronikola et al.,
69 2016;Hankir and Klingenspor, 2018). Actually, BAT activity requires the uptake of substrates from
70 the circulation, mostly free fatty acids (FAs), but also glucose, successfully leading to hypolipidemic
71 and hypoglycemic effects which can significantly improve insulin sensitivity and exert a protective
72 role in the pathogenesis of type 2 diabetes (Sidossis and Kajimura, 2015). FAs liberated from

73 intracellular TGs through the action of lipases are also critical for BAT thermogenesis (Blondin et
74 al., 2017). Interestingly, abnormal accumulation of intracellular lipids has been observed in patients'
75 cells as well as in animal models (Puccio et al., 2001;Coppola et al., 2009;Tamarit et al., 2016;Stram
76 et al., 2017), pointing to a possible inefficient lipolysis.

77 Importantly, it has been discovered that decreased activity of BAT is associated with insulin
78 resistance and diabetes (Flachs et al., 2013;Sacks and Symonds, 2013). Despite this potential clinical
79 importance, the regulation of BAT in FDRA patients is not well-investigated.

80 In this work we show that FXN deficiency significantly affects lipolytic and thermogenic pathways
81 as well as thermogenic adipocyte differentiation suggesting that BAT impairment could be at center
82 stage of type 2 diabetes development in FRDA patients.

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85 **RESULTS**

86 *Alteration of basal metabolic parameters in KIKO mice*

87 Herein, we used KIKO mouse that represents a suitable *in vivo* model for studying neurodegeneration
88 as well as metabolic complications in FRDA (Coppola et al., 2009;McMackin et al., 2017). We firstly
89 monitored body weight and a trend to gain weight was observed in 6-months KIKO mice that
90 becomes significant at 8 months of age (**Fig. 1A**). In contrast, food and water intake were never found
91 changed (**data not shown**). We have performed bio-clinical analyses and, among the tested
92 parameters, a significant increase of triglycerides and cholesterol levels was detected in KIKO mice
93 both at 6- and 8-months of age (**Fig. 1B**). Other metabolic parameters such as fasting glycaemia (**Fig.**
94 **1B**) as well as markers of general organ functions (total plasma proteins), including kidney
95 (creatinine, urea) and liver (albumin) resulted unaltered (**data not shown**), suggesting that
96 hyperlipidemia represents an early event in FRDA. Even though fasting glycaemia appeared
97 unaffected, oral glucose tolerance test (OGTT) carried out in 8 months old KIKO mice revealed that
98 glycaemia remained higher than WT mice at 120 min from glucose administration (**Fig 1C**).

99 The circulating levels of adipose tissue-secreted cytokines, i.e. adipokines, nicely reflect body
100 metabolic state and are considered valid markers to monitor insulin resistance (Li et al., 2013;Adams-
101 Huet et al., 2014). Through Luminex® Multiplex Assay, we found that KIKO mice underwent a
102 prominent raise of plasma leptin levels at 8 months of age (**Fig. 1D**), while other adipokines such as
103 adiponectin and FGF21 remained unchanged (**Fig. 1E and 1F**). The increase in leptin, and more
104 specifically the leptin to adiponectin ratio (LAR), is considered a useful tool to evaluate insulin
105 resistance (Finucane et al., 2009). Accordingly, 8 months-old KIKO mice showed increased LAR
106 compared to WT mice (**Fig. 1G**). We have then characterized metabolic profile of KIKO mice by
107 performing indirect calorimetry. Before measurements, all mice were acclimatized for 48 h into
108 individual metabolic chambers at 25 °C, with free access to food (standard diet) and water. The
109 respiratory exchange ratio (RER), which is expressed as VCO_2/VO_2 , was similar in WT and KIKO
110 mice and around 1.0 indicating carbohydrates as the predominant fuel source (**data not shown**).

111 At 6 months, the recorded oxygen consumption (VO_2) was lower in KIKO than WT mice, with KIKO
112 mice showing a further decrease of VO_2 at 8 months (**Fig. 2A**). Moreover, KIKO mice showed a
113 decrease of resting energy expenditure (REE) at 6 months of age (**Fig. 2B**). This parameter was
114 altered at higher extent at 8 months of age pointing to a progressive dysfunction in basal BAT
115 thermogenic capacity (**Fig. 2B**).

116

117 *KIKO mice show altered mitochondrial function and lipid accumulation in BAT*

118 BAT activity strongly depends on mitochondrial lipid oxidation to produce heat (Hankir and
119 Klingenspor, 2018). As dysfunctional FXN affects mitochondrial oxidative capacity, we supposed
120 that BAT oxidative metabolism could be altered. This prompted us at firstly analyzing BAT
121 mitochondria at ultrastructural level through transmission electron microscopy. In WT mice typical
122 mitochondria (abundant, large, and rich in cristae) were present in BAT, while in KIKO mice the
123 mitochondria appeared lower in number and enlarged with disorganized and thickened cristae (**Fig.**
124 **3A**). We then compared the basal oxygen consumption in mitochondria isolated from BAT and as
125 expected the oxygen consumption was lower in KIKO than WT mice (**Fig. 3B**).

126 Accumulation of lipid droplets (LDs) and an increased lipogenesis have been previously described in
127 fibroblasts of FRDA patients and cardiomyocytes from KIKO mice (Coppola et al., 2009). In line
128 with these data, we found that BAT of KIKO mice has higher lipid droplets dimension with respect
129 to WT mice (**Fig. 3C**). We have tested whether this event was dependent on defective lipolysis that
130 importantly contributes to LDs turnover. BAT lipolysis strongly relies on cAMP-dependent protein
131 kinase A (PKA) that coordinates lipolytic machinery activation through hormone/phospho-dependent
132 mechanisms. To this end, we evaluated the level of the PKA phosphorylated substrates as well as the
133 lipases directly responsible for LDs degradation. As reported in the immunoblot in **Fig. 3D**, PKA
134 substrates were reduced in their phosphorylated levels, suggesting a general inhibition of PKA
135 activity. Among the PKA substrates the hormone-sensitive lipase (HSL) is included, as it is activated
136 via the phosphorylation of Ser660. While the basal levels of HSL remained unaltered, the phospho-

137 active levels of HSL were lower in KIKO compared to WT mice (**Fig. 3D, E**). The levels of
138 phosphorylation at Ser565 (phospho-inactive form) were instead increased, indicating an impaired
139 basal HSL activity. According to what reported in other studies on mouse model and patients (Shan
140 et al., 2013; Sahdeo et al., 2014), we also found a significant decrease of the transcription factor Nrf2
141 (**Fig. 3D, E**) that, besides being an up-stream modulator of the expression of antioxidant genes and
142 protecting against oxidative stress, positively regulates enzymes involved in mitochondrial fatty acids
143 oxidation (Esteras et al., 2016).

144

145 *KIKO mice display affected expression of genes related to lipid utilization and thermogenesis*

146 Considering the alteration of mitochondrial respiration and lipid catabolism observed in BAT, we
147 hypothesized a compromised thermoregulatory activity in KIKO mice. To test this, we next exposed
148 mice to cool temperature (4°C) for 12 h and a lower body temperature was measured in KIKO mice
149 both at thermoneutrality as well at 4°C (**Fig. 4A**). Remarkably, BAT of cold-exposed mice showed a
150 that KIKO mice maintained a higher LD size with respect to WT mice (**Fig. 4B**). In particular, the
151 measurement of LD size at RT confirmed their higher dimension in KIKO mice compared to WT
152 mice (**Fig. 4C**). Cold exposure promoted a marked reduction of LDs diameter both in WT and KIKO
153 mice; however, in cold-exposed KIKO mice LDs were larger than WT mice (**Fig. 4C**).

154 To more deeply decipher the cause(s) of cold intolerance, we performed BAT transcriptome profiling
155 using an ultra-deep unbiased RNA sequencing (RNA-seq) approach. The RNA samples analyzed
156 were prepared from BAT of WT and KIKO mice maintained at RT or exposed to cold for 12 hrs. A
157 summary of the results obtained through RNAseq is reported in the volcano plots depicted in **Fig.**
158 **5A**. Through pair-wise differential gene expression, we found that about 200 genes were up-regulated
159 ($\text{Log}_2\text{FC} > 0.58$; $p < 0.05$) upon cold exposure both in WT and KIKO mice. In order to determine which
160 biological processes or pathways were overexpressed, the list of up-regulated genes was used as the
161 input for a functional enrichment analysis. By using the plugin ClueGo in the Cytoscape 3.7.1
162 platform, as expected we found that the response to temperature stimulus was the biological processes

163 significantly up-regulated either in WT or KIKO mice exposed to cold with respect to their controls,
164 even though KIKO mice showed a lower enrichment with respect to WT mice (**Fig. 5B**). The
165 biological processes found overrepresented in WT mice were positive regulation of cold induced
166 thermogenesis, triglyceride metabolic process (including lipolytic genes), monocarboxylic acid
167 transport (including fatty acid transporters) and mitochondrial gene expression (**Fig. 5B, left panel**),
168 in line with the notion that fatty acids transport and degradation, and expression of mitochondrial
169 proteins accompany thermogenesis. Interestingly, genes mapping to categories pertaining purine
170 nucleotide and monocarboxylic acid biosynthetic processes (including fatty acids biosynthesis genes)
171 were found overexpressed in KIKO mice exposed to cold compared to KIKO mice at RT (**Fig. 5B,**
172 **right panel**). Moreover, in cold-exposed KIKO mice we found an enrichment of reactive oxygen
173 species (ROS) biosynthetic process and cellular response to xenobiotic stimulus (**Fig. 5B, right**
174 **panel**).

175 The analysis of KEGG and Wiki pathways showed that genes belonging to PPAR signaling, notably
176 induced during thermogenesis, were significantly overrepresented both in cold-exposed WT and
177 KIKO mice; however, the number of genes found in this pathway was lower in KIKO than WT mice
178 (**Fig. 5C**). The same trend was observed for p53 signaling pathway. Among the top enriched
179 pathways, WT mice also had adipogenesis, peroxisome and beta-oxidation (**Fig. 5C, left panel**); by
180 contrast, RNA degradation, biosynthesis of fatty acids and ferroptosis were among the enriched
181 pathways found in BAT of cold-exposed KIKO mice (**Fig. 5C, right panel**). Ferroptosis is a more
182 recently recognized cell death caused by iron overload (Dixon et al., 2012; Stockwell et al., 2017),
183 suggesting that in KIKO mice the thermogenic response of BAT is blunted and likely accompanied
184 by iron/ROS-induced cell death.

185 To complement our analysis, we alternatively examined transcriptomic data through Funrich v3.0
186 and found that the response to cold was the biological process significantly and similarly enriched in
187 WT and KIKO mice upon cold exposure (**Fig. 5D**). Brown fat cell differentiation and other biological
188 processes related to in lipid metabolism were found overrepresented both in WT and KIKO mice.

189 However, in KIKO mice a lower percentage of genes pertaining to these processes was found
190 compared to WT mice (**Fig. 5D**). In **Fig. 5E**, a heatmap including some representative genes strictly
191 involved in adipocyte differentiation, heat production and thermogenic-related metabolic re-
192 adaptation is illustrated. The heatmap shows that KIKO mice undergo a lower up-regulation of these
193 genes compared to WT mice, arguing that cold intolerance of KIKO mice could be ascribed to
194 defective activation of adipocytes differentiation, thermogenic pathway, impaired BAT lipid
195 catabolism and/or oxidative capacity.

196 Transcriptomic results were confirmed by performing qPCR analyses. In particular, we confirmed
197 the downregulation of FXN mRNA in KIKO mice and found a significant downregulation of the
198 expression of other mitochondrial genes such as Tfam, Mtco1, Nd1, Atp6 and Nd4 (**Fig. 6A**). Upon
199 cold exposure, WT mice showed up-regulation of Ucp1, Tfam, Nd1 and Atp6. In KIKO mice an
200 increase of Ucp1, Tfam, Nd1 and Atp6 was also achieved; however, the level of upregulation was
201 lower than WT mice.

202 We also confirmed the alterations in the expression level of genes related to lipid metabolism and
203 thermogenesis in KIKO mice. Actually, as showed in **Fig. 6B**, the expression of genes implicated in
204 fatty acids transport (i.e. Cd36, Fabp4, Cpt1b) and thermogenesis (i.e. Dio2) resulted downregulated
205 in KIKO mice. A slight but significant decrease of the lipase responsible for the first step of
206 triglyceride lipolysis Atgl and its enhancer Abdh5 was also found, indicating an alteration of the
207 triglyceride catabolism and lipid signaling. Upon cold exposure, overall these genes resulted up-
208 regulated both in WT and KIKO mice; however, in KIKO mice Cd36, Abdh5, Fabp4, Cpt1b and
209 Dio2 resulted up-regulated at minor extent (**Fig. 6B**).

210 Through immunohistochemical analyses we confirmed the alteration of PKA lipolytic pathway in
211 BAT of KIKO mice. Indeed, a decrease of PKA kinase activity was occurring already at RT and a
212 further reduction of phospho-PKA substrates was found at 4°C with respect to WT mice (**Fig. 6C**).
213 In particular, at RT the BAT of WT mice shows brown adipocytes with a diffused and stronger
214 staining with respect to BAT of KIKO mice. Upon cold exposure, the immunostaining of phospho-

215 PKA substrates was predominantly nuclear, in line with the notion that PKA can migrate into the
216 nucleus during thermogenesis (Rim et al., 2004) and phosphorylate a number of transcriptional
217 regulators (e.g. Ppargc1a) and transcription factors triggering adipogenesis and thermogenesis as well
218 as mitochondrial oxidative activity and biogenesis (e.g. CREB) (Cannon and Nedergaard,
219 2004;Baldelli et al., 2014). Notably, the nuclear staining of phospho-PKA substrates was markedly
220 reduced in BAT of cold-exposed KIKO mice (**Fig. 6C**). Moreover, in these mice we found an increase
221 of LD-associated protein perilipin-1 (**Fig. 6D**), which is an inhibitor of triglyceride degradation by
222 HSL (Grahn et al., 2013), thus highlighting the dysfunction of lipolysis in BAT of KIKO mice.

223

224 *FXN deficiency leads to impaired thermogenesis and adipogenesis in cultured adipocytes*

225 To confirm that FXN deficiency affects lipid utilization and thermogenesis, we moved to an *in vitro*
226 system. In cultured T37i brown adipocytes, we downregulated FXN levels through RNA interference
227 by transfecting a pool of siRNA against FXN mRNA (FXN-). Transfection of T37i cells with a pool
228 of scramble siRNAs were used as control (Scr). To induce thermogenic cascade, T37i were treated
229 with isobutyl methylxanthine (IBMX), a nonspecific inhibitor of phosphodiesterase (PDE), which
230 enhances the intracellular cAMP levels, inducing a constitutive activation of PKA. As reported in
231 **Fig. 7A**, the analysis of LDs evidenced that lipolysis was likely blunted as consequence of FXN
232 deficiency as LDs dimension was larger in FXN- cells than Scr cells both in untreated and IBMX-
233 treated cells. **Fig. 7B** shows that FXN downregulation did lead to the inhibition of lipolysis, as
234 reduced levels of phospho-active HSL (p-HSL660), a known target of PKA activity, was elicited both
235 upon basal condition and IBMX treatment. In line with the transcriptomic data obtained in BAT of
236 KIKO mice, PPAR γ , a thermogenic transcription factor regulating mitochondrial biogenesis and lipid
237 catabolism, was reduced in FXN- cells under resting conditions and, in contrast to Scr cells, it
238 remained down-regulated upon IBMX treatment (**Fig. 7C**). Importantly, UCP1 protein was
239 efficiently induced by IBMX in Scr cells but not in FXN- cells. Mitochondrial proteins including
240 VDAC and TOM20 were also reduced in FXN- cells both prior and after IBMX treatment (**Fig. 7C**).

241 RT-qPCR analyses confirmed the impairment of thermogenic program, as the mRNA expression of
242 thermogenic markers including *Cox7a*, *Ppargc1a*, *Cidea* and *Cd36* were significantly reduced in
243 FXN- cells both under basal condition and upon IBMX treatment with respect to Scr cells (**Fig. 7D**).
244 In line with these data, reduced oxygen consumption was also recorded in FXN- cells (**Fig. 7E**). Since
245 RNAseq analyses pointed to a defective induction of adipogenic genes upon cold exposure, we then
246 attempted at evaluating whether adipocyte differentiation potential could be impaired in KIKO mice.
247 We hence isolated stromal vascular cells (SVCs) from subcutaneous adipose tissue, which is highly
248 enriched with primary adipocyte precursors. Upon certain circumstances (i.e. rosiglitazone
249 treatment), SVCs may differentiate in brown-like adipocytes displaying high mitochondrial mass and
250 expressing detectable levels of *Ucp1* (Aune et al., 2013). As reported in **Fig. 8A**, WT SVCs were
251 efficiently differentiated in adipocytes as a great number of cells show adipocyte morphology with
252 an evident accumulation of LDs. On the contrary, SVCs from KIKO mice show affected adipogenic
253 capacity as a lower number of adipocytes was obtained. As expected, RT-qPCR analyses evidenced
254 a marked reduction of FXN mRNA levels in KIKO with respect to WT adipocytes that was
255 accompanied by a significant down-regulation of genes related to adipogenesis, thermogenesis and
256 mitochondrial biogenesis including *Ppar γ* , *Ppargc1a*, *Ucp1*, *Cd36* and *Mtco1* (**Fig. 8B**).
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259 **DISCUSSION**

260 Abnormal glycemic control, increased cholesterol and triglycerides levels as well as type 2 diabetes
261 are more frequent in FRDA patients than in the general population and concur in the severity of
262 FRDA (extensively reviewed in (Tamarit et al., 2016)). In this work we demonstrated KIKO mice
263 show several diabetes-related hallmarks, including hyperlipidaemia, altered tolerance to glucose, and
264 elevated circulating level of leptin, a peptide hormone secreted by adipose tissue whose increase is
265 strictly related to insulin resistance and low-grade inflammatory states (Francisco et al., 2018).

266 Overall these metabolic changes make this model suitable to decipher the events contributing to
267 disease severity and find novel druggable target to overcome type 2 diabetes development in FRDA
268 patients.

269 In the last decade, the scientific community has put a spotlight on BAT as a crucial player in the
270 control of energy metabolism, being the main glucose and lipid clearance organ with the highest
271 mitochondrial fatty oxidation rate (Doh et al., 2005; Bartelt et al., 2011). We have reported that BAT
272 mitochondria of KIKO mice show a significant ultrastructural alteration, lower abundance and
273 oxygen consumption and decreased mRNA expression of electron transport chain complex subunits
274 compared to WT mice. Upon cold exposure KIKO mice show affected adaption to cold with an
275 overall less efficient up-regulation of canonical thermogenesis-related genes and pathways including
276 mitochondrial and lipid metabolism genes and PPAR signaling pathway.

277 It has been reported that FRDA patients show LDs accumulation in fibroblasts (Coppola et al., 2009).
278 LDs accumulation was observed also in FXN deficient cultured cardiomyocytes (Obis et al., 2014)
279 and heart of FRDA mouse models (Puccio et al., 2001; Stram et al., 2017). Hepatic steatosis in mice
280 with a liver-specific FXN ablation (Martelli et al., 2012) and altered lipid metabolism associated with
281 increased LDs in glial cells of the drosophila FRDA model (Navarro et al., 2010) were also observed.
282 Down-regulation of PPAR γ /PGC-1 α pathway and up-regulation of lipogenic genes have been
283 previously proposed among the mechanisms leading to LDs accumulation (Sutak et al., 2008; Coppola
284 et al., 2009). We have evidenced that FXN deficiency leads to LDs accumulation in BAT as well.

285 Notably, the size of LDs is the result of the balance between lipid demolition and deposition. Since
286 BAT requires plentiful FAs, which are its primary substrate, lipolysis is activated during BAT
287 thermogenesis (Cannon and Nedergaard, 2004;Nedergaard et al., 2011;Townsend and Tseng, 2014).
288 We have demonstrated that impaired lipid degradation could contribute to LDs accumulation, as
289 PKA-dependent lipolysis resulted significantly affected both under resting condition and upon cold
290 exposure. As consequence, a significant reduction of the active form of HSL and increase of the LD-
291 associated inhibitor of lipolysis perilipin was observed in BAT of KIKO mice.

292 Importantly, besides acting as fuels to sustain the electron transport chain flow, fatty acids are
293 involved in the regulation of UCP1, or directly activate UCP1-mediated energy dissipation and heat
294 production (Cannon and Nedergaard, 2004). The impaired lipid degradation observed in KIKO mice
295 likely limits the funneling of fatty acids into mitochondria with consequent affected capacity to
296 produce heat. Accordingly, fatty acids beta-oxidation was not disclosed among the enriched pathways
297 in BAT of cold-exposed KIKO mice and a diminished expression of the mitochondrial fatty acid
298 carrier Cpt1b was found. Notably, intracellular purine nucleotides exert an inhibitory action on UCP1
299 protein (Fromme et al., 2018). It was recently demonstrated that upon thermogenic stimuli, brown
300 adipocyte expression of enzymes implicated in purine metabolic remodeling is altered (Fromme et
301 al., 2018). In particular, an overexpression of genes implicated in purine nucleotide degradation was
302 found that was associated with a down-regulation of genes involved in purine nucleotide synthesis.
303 In BAT of KIKO mice exposed to cold we found an overrepresentation of genes pertaining purine
304 nucleotide biosynthetic process. Therefore, these data collectively indicate that UCP1, in addition to
305 being up-regulated at lesser extent than WT mice, is less efficient in impinging heat production.

306 To exclude that the alterations found in BAT of KIKO mice could be the results of systemic adaptive
307 responses to FXN deficiency, we validated our findings in cultured brown adipocytes downregulating
308 FXN. By igniting thermogenic program via the PKA agonist IBMX we confirmed that FXN
309 deficiency leads to the inhibition of lipolysis and affected up-regulation of genes related to the
310 thermogenic cascade.

311 Another process having a critical role in maintaining thermogenic efficiency of BAT is the
312 differentiation of resident brown fat adipocytes *de novo* that assures BAT regeneration and avoids its
313 loss (Birerdinc et al., 2013). Transcriptomic data evidenced brown fat cell differentiation and
314 adipogenesis as biological process and pathway affected upon cold exposure in BAT of our mouse
315 model. We confirmed such hypothesis by analyzing the adipogenic potential of stromal vascular cells
316 isolated from mouse adipose depots that resulted unable to fully differentiate in thermogenic
317 adipocytes in KIKO mice.

318 It is important to notice that a less proficient up-regulation of p53 signaling pathway in KIKO mice
319 was observed in BAT upon cold exposure. p53 is a stress responsive transcription factor that was
320 reported to exert a positive regulatory effect on brown adipocyte differentiation (Al-Massadi et al.,
321 2016) and BAT thermogenesis (Molchadsky et al., 2013). This evidence corroborates our idea that
322 BAT dysfunction could at least in part depend on altered capacity of FXN deficient adipocyte
323 precursors to efficiently differentiate and express mitochondrial and thermogenic genes.

324 Even though not still adequately investigated in the present work, upon cold stress condition the
325 KEGG pathway of ferroptosis was significantly up-regulated in KIKO mice along with genes related
326 to ROS metabolism. This is not surprising as we found a decrease of the Nrf2 protein, and oxidative
327 stress, iron overload and ferroptosis have been claimed among the main pathogenic factors in FRDA
328 (Shan et al., 2013; Petrillo et al., 2017; Abeti et al., 2018; Cotticelli et al., 2019). Interestingly, Nrf2,
329 besides being an up-stream modulator of the expression of antioxidant genes and protection against
330 oxidative stress/ferroptosis (Faraonio et al., 2006; Dodson et al., 2019), positively regulates enzymes
331 involved in mitochondrial fatty acids oxidation (Esteras et al., 2016) and adipogenesis (Pi et al.,
332 2010; Hou et al., 2012). Based on this, Nrf2 inducers, already proposed as FRDA therapeutics (Petrillo
333 et al., 2017; Abeti et al., 2018) could be also advantageous for preserving BAT integrity and mitigating
334 metabolic disturbances of FRDA patients via a BAT-dependent manner. Accordingly, Nrf2 targeting
335 has been proposed as promising for treating type 2 diabetes (David et al., 2017).

336 BAT studies carried out in human fetuses and infants indicate that the tissue is widely distributed
337 during these developmental stages and that the thermogenic capacity of BAT develops with
338 gestational age reaching its maximum in infancy and early childhood when the demands for
339 thermogenesis can be expected to be especially high (Lidell, 2019). Several studies suggest that BAT
340 dysfunction during gestation and early childhood negatively influences metabolic health predisposing
341 to type 2 diabetes development later in life (Liang et al., 2016;Entringer et al., 2017;Lettieri-Barbato
342 et al., 2017). Therefore, it is possible to postulate that FRDA patients would have experienced BAT
343 dysfunction early in life leading to disruption of systemic metabolic homeostasis.

344 In conclusion, by deeply characterizing KIKO mice at metabolic level we have provided multiple
345 lines of evidence that FXN deficiency in mice leads to clinical-pathological features parallel to those
346 observed in diabetic patients. Among the metabolic parameters we have evidenced that the lipolytic
347 and thermogenic activities of BAT are reduced, thus providing the possibilities of targeting BAT that
348 might result in therapeutic benefits in FRDA.

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355 **MATERIALS AND METHODS**

356 **Animals**

357 Mouse experimentation was conducted in accordance with accepted standard of humane animal care
358 after the approval by relevant local (Institutional Animal Care and Use Committee, Tor Vergata
359 University) and national (Ministry of Health, license n°324/2018-PR) committees. Unless otherwise
360 stated, mice were maintained at $21.0 \pm ^\circ\text{C}$ and $55.0 \pm 5.0\%$ relative humidity under a 12 h/12 h
361 light/dark cycle (lights on at 6:00 AM, lights off at 6:00 PM). Food and water were given *ad libitum*.
362 Experiments were carried out according to institutional safety procedures.

363 Knock-in knock-out (KIKO) mice were purchased from Jackson Laboratories (#012329). Littermate
364 C57BL/6 mice (WT) were used as controls. Researchers were blinded to genotypes at the time of
365 testing. Only WT and KIKO male mice were used and divided in the following groups: 1) 6 months-
366 old WT and KIKO mice (n=12 each group); 2) 8 months-old WT and KIKO mice (n=12 each group);
367 For cold exposure experiments additional male WT and KIKO at 6-months of age were divided in
368 the following groups: 1) WT and KIKO mice maintained at room temperature (n=6 each group); 2)
369 WT and KIKO mice maintained at 4°C for 12 h (n=6 each group). Rectal temperature was measured
370 by high precision ($\pm 0.1 ^\circ\text{C}$) rectal probe for mice (RET-3, ThermoWorks, Alpine, UT, USA).

371

372 **Bio-clinical and adipokine analyses**

373 Prior to bio-clinical analyses, mice were starved for 2 h. After blood collection, bio-clinical analyses
374 were performed by colorimetric methods. In particular, glucose, cholesterol, triglycerides, creatinine,
375 total plasma proteins, albumin and urea were measured through the automatized KeyLab analyzer
376 (BPCBioSed, Italy) using specific assay kits (BPCBioSed).

377 Serum adiponectin, FGF21 and leptin levels were measured through a Mouse Magnetic Luminex
378 Screening Assay (R&D System, Minneapolis, MN, USA).

379 For the glucose tolerance test (OGTT), mice were subjected to fasting for 12 h, followed by oral
380 gavage with 2 g of dextrose/kg body mass. At the indicated time points, blood was collected from the

381 tail vein and glycaemia measured using a glucometer (Bayer Countur XT, Bayer Leverkusen,
382 Germany).

383

384 **Indirect calorimetry**

385 Indirect calorimetry performed using LabMaster (TSE Systems, Bad Homburg, Germany) as
386 previously described (Lettieri-Barbato et al., 2018). Oxygen consumption (VO₂), carbon dioxide
387 production (VCO₂) and Resting energy expenditure (REE) were recorded every 15 min for 24h, and
388 the data were averaged for each mouse.

389

390 **Histochemical analysis**

391 Formalin-fixed paraffin-embedded (BAT explants were cut in 3 µm sections and stained with
392 hematoxylin and eosin (H&E) prior microscope analysis. Lipid droplet diameters were measured in
393 three fields (300 droplets total at least) with ImageJ. For correlative purpose, an average score was
394 derived for each sample. Perilipin and phospho-PKA substrate levels were investigated by
395 immunohistochemistry on tissue sections. After antigen retrieval with Citrate Buffer (pH 6.0),
396 sections were incubated at room temperature with the following primary antibodies: rabbit polyclonal
397 antibody anti-Perilipin diluted 1:1000 (9349T, Cell Signalling Technology) and rabbit polyclonal
398 antibody anti-phospho-PKA substrates diluted 1:100 (9621S, Cell Signalling Technology). Negative
399 controls were obtained by omitting primary antibodies. Immunohistochemical reactions were
400 visualized by DAB as the chromogen from MACH 1 Universal HRP-Polymer Detection (Biocare
401 Medical, Concord, MA, USA).

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403 **Ultrastructural Analysis**

404 BAT samples from animal models were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for
405 the morphological study. After fixation, dehydration and impregnation, samples were included in
406 epoxy resins and acrylic, cut at the ultramicrotome and processed for the ultrastructural study of

407 brown adipocytes by electron microscopy. In particular, morphological aspects of the mitochondria,
408 such as the presence and density of cristae, were evaluated. Ultrastructural images were collected
409 with a Transmission Electron Microscope FEI Tecnai™ (Hillsboro, Oregon, USA), equipped with
410 a dedicated Imaging Software System.

411

412 **Cell and mitochondria oxygen consumption**

413 T37i cell line was gently provided by Prof. Marc Lombes (Inserm U693, Paris, France), cultured and
414 differentiated as described by Nakae et al., 2008 (Nakae et al., 2008). Differentiated T37i brown
415 adipocytes were transfected with a pool of 3 target-specific siRNAs against FXN mRNA FXN or
416 scramble siRNAs (Santa Cruz Biotechnology, Dallas, TX, USA) by using DeliverX Plus kit
417 (Affymetrix, Santa Clara, CA, USA). Treatment with isobutyl methyl xanthine (IBMX) was carried
418 out at concentration of 0.5 mM for 4 h. For lipid droplet detection, cells were stained with Nile Red
419 (0.25 µg/ml, 10 min). Staining with Hoechst 33342 (1 µg/ml, 10 min) was used to counterstain nuclei.
420 Images were visualized by Nikon Eclipse TE200 epifluorescence microscope (Nikon, Florence, Italy)
421 connected to a CCD camera. Images were captured under constant exposure time, gain and offset.
422 Stromal vascular cells (SVCs) were isolated from subcutaneous adipose tissue and differentiated in
423 brown-like adipocytes according to Aune et al (Aune et al., 2013). Oil Red O was used to detect
424 intracellular triglycerides content as previously described (Lettieri-Barbato et al., 2018).
425 Oxygen consumption was determined in cells and crude mitochondria and by using the Oxygraph
426 Plus oxygen electrode system (Hansatech Instruments Ltd., Norfolk, UK). Intact cells were
427 resuspended (1×10^6 /ml) in culture medium without FBS. Crude mitochondria were isolated from
428 BAT as previously described (Lettieri Barbato et al., 2015) and resuspended in an appropriate
429 mitochondrial activity buffer (70 mM sucrose, 220 mM mannitol, 2 mM HEPES buffer, 5 mM
430 magnesium chloride, 5 mM potassium phosphate, 1 mM EDTA, 5mM succinic acid, and 0.1% fatty
431 acid free bovine serum albumin, pH 7.4). Oxygen consumption rate was recorded at 37° C for 10 min
432 and normalized for protein concentration.

433 **RNA-seq data expression quantification and functional enrichment analysis**

434 The quality of the single-end reads was evaluated with *FastQC* v.0.11.5
435 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). All the *fastqc* files were filtered to
436 remove low quality reads and adapters with *Trimmomatic* v.0.36 (Bolger et al., 2014). The resulting
437 reads were mapped to the *Mus musculus* genome (GRCm38) with *HISAT2* v.2.1.0 (Kim et al., 2015)
438 using default parameters, while *Stringtie* v1.3.4d (Pertea et al., 2015) was applied to the BAM files
439 obtained with *HISAT2* to generate expression estimates and to quantify the transcript abundance as
440 transcripts per kilobase per million of mapped reads (TPM). The count matrices generated by
441 *Stringtie* were imported in *R* where differential expression analysis was performed using the *Deseq2*
442 package (Love et al., 2014) to compare the two different conditions. The functional annotation was
443 performed through the *AnnotationDbi* R library
444 (<http://bioconductor.org/packages/release/bioc/html/AnnotationDbi.html>).

445 Differential expressed genes were selected with threshold of $\text{Log}_2\text{FC} > 0.58$ ($p < 0.05$). Functional
446 enrichment analysis including GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway
447 was performed by using the ClueGo plugin of the Cytoscape v3.7.1. ClueGo settings were:
448 enrichment only, Benjamini-Hochberg false discovery rate (FDR) correction, GO Term Restriction
449 Level 3-8 and 3 genes/4% minimum, GO Term Connection (Kappa) minimum 0.4, GO Term
450 Grouping was on, with an initial group size of 3 and Group Merging set at 50%. Only pathway with
451 $pV < 0.05$ were shown. Funrich v3.0 tool (<http://funrich.org/index.html>) with default settings was
452 alternatively used for functional enrichment analyses.

453

454 **Real time PCR**

455 Total RNA was extracted using TRI Reagent® (Sigma-Aldrich). RNA (3 μg) was retro-transcribed
456 by using M-MLV (Promega, Madison, WI). qPCR was performed in triplicate by using validated
457 qPCR primers (BLAST), Applied Biosystems™ *Power™* SYBR™ Green Master Mix, and the
458 QuantStudio3 Real-Time PCR System (Thermo Fisher, Whaltam, MA, USA) as previously described

459 (Aquilano et al., 2016). mRNA levels were normalized to actin mRNA, and the relative mRNA levels
460 were determined through the $2^{-\Delta\Delta Ct}$ method.

461

462 **Immunoblotting**

463 Tissues or cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 12 mM
464 deoxycholic acid, 0.5% Nonidet P-40, and protease and phosphatase inhibitors). Five μ g proteins
465 were loaded on SDS-PAGE and subjected to Western blotting. Nitrocellulose membranes were
466 incubated with anti-HSL (4107, Cell Signalling Technology), anti-p-HSL660 (4126T, Cell Signalling
467 Technology), anti-p-HSL565 (4137P, Cell Signalling Technology), anti-UCP1 (CS-14670S, Cell
468 Signalling Technology) anti-p-PKA substrates (9621S , Cell Signalling Technology), anti-FXN (sc-
469 25820, Santa Cruz Biotechnology), anti-Nrf2 (sc-722, Santa Cruz Biotechnology), anti-PPAR γ (sc-
470 7196, Santa Cruz Biotechnology), anti-Tomm20 (sc-11415, Santa Cruz Biotechnology), anti-Vdac1
471 (sc-8828, Santa Cruz Biotechnology), anti-Tubulin (T9026, Sigma-Aldrich) primary antibodies at
472 1:1000 dilution. Successively, membranes were incubated with the appropriate horseradish
473 peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected by a FluorChem
474 FC3 System (Protein-Simple, San Jose, CA, USA) after incubation of the membranes with ECL
475 Selected Western Blotting Detection Reagent (GE Healthcare, Pittsburgh, PA, USA). Densitometric
476 analyses of the immunoreactive bands were performed by the FluorChem FC3 Analysis Software.

477

478 **Statistical analysis**

479 The results are presented as means \pm S.D. Statistical analyses were carried out by using the Student's
480 *t* test to compare the means of two groups. One-way ANOVA followed by Tukey's test was used for
481 comparing the means of more than two groups. Differences were considered to be significant at
482 $p < 0.05$.

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484

485

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689

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694

695 **CONFLICT OF INTEREST**

696 Authors declare no conflict of interest.

697

698 **AUTHOR CONTRIBUTIONS**

699 K.A. conceptualized and designed the study, wrote the manuscript

700 D.L.B. analyzed and interpreted the data, wrote the manuscript

701 R.T., designed and supervised the in vivo experiments and performed research

702 G.G., V.C., L.D.A., R.B. performed in vivo experiments and collected results

703 F.T., performed in vitro experiments

704 F.I., M.F. performed computational analyses

705 S.C., M.Fr. performed immunohistochemistry experiments and optical microscopy analyses;

706 S.C., M.Z. performed electron microscopy experiments and analyses.

707 S.R., S.M., M.M., M.F., R.F. contributed in interpreting the data and in planning the research.

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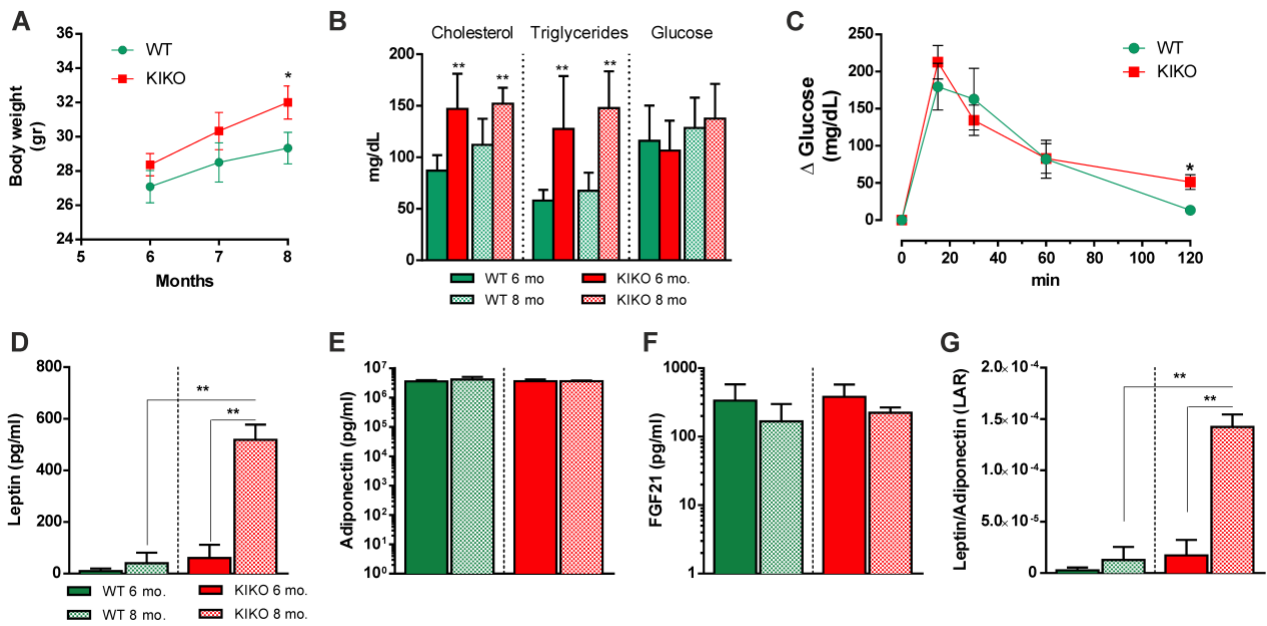
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712 **FIGURES AND FIGURE LEGENDS**

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716 **Fig. 1. Bio-clinical and adipokine analyses revealed dyslipidemia and insulin resistance in KIKO**
 717 **mice.**

718 (A) Body weight recorded at different ages (n=12 each group; *p<0.05 vs age-matched WT mice).

719 (B) Bio-clinical analyses of fasting glycaemia and lipidemia (triglycerides and cholesterol) carried
 720 out at different ages (n=12 each group; **p<0.001 vs age-matched WT mice).

721 (C) Oral glucose tolerance test (OGTT) carried out in 8-months old mice (n=6 each group; *p<0.05
 722 vs WT mice).

723 (D-G) Serum levels of leptin (D), adiponectin (E), FGF21 (F) and leptin to adiponectin ratio (G)
 724 determined in mice at different ages (n=6 each group; **p<0.01).

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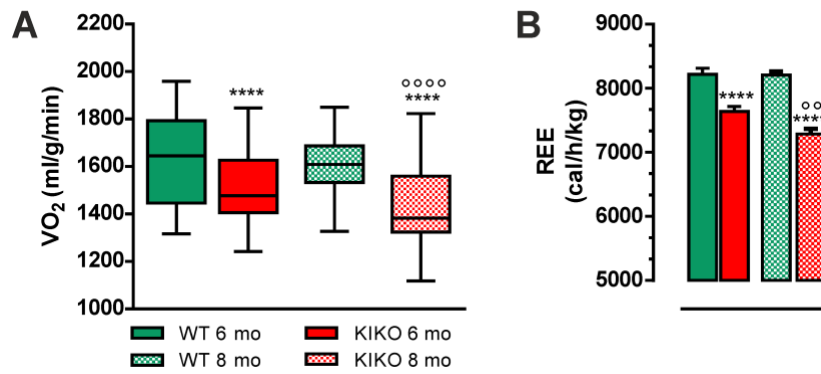
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749 **Fig. 2. Indirect calorimetry indicated decreased oxygen consumption and energy expenditure in**
750 **KIKO mice.**

751 (A, B) Oxygen consumption (VO₂) (A) and resting energy expenditure (REE) (B) measured by
752 indirect calorimetry at different ages (n=12; each group; ****p<0.0001 vs age-matched WT mice,
753 °°°°p<0.0001, °°p<0.01 vs 6-months KIKO mice).

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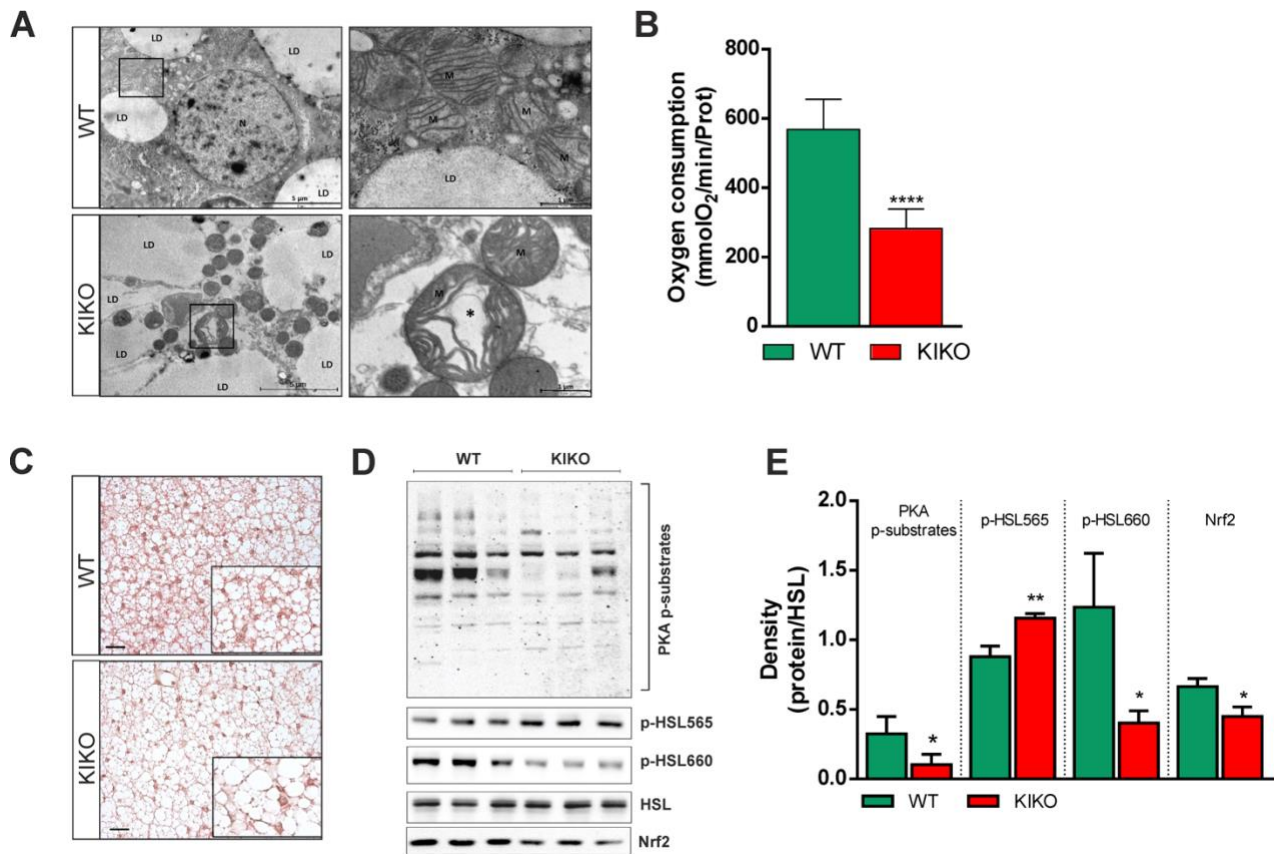
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Fig. 3. Mitochondria of KIKO mice show altered mitochondrial morphology and respiratory function.

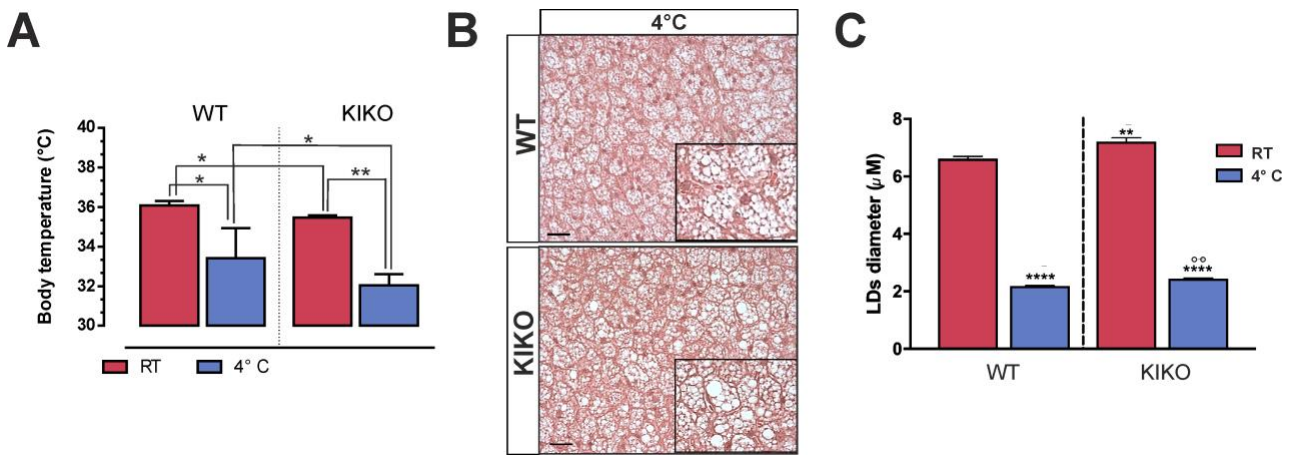
(A) Representative transmission electron microscopy pictures of BAT from 6-months old WT and KIKO mice are reported in *left panels* (5800x). Images with high power field (18500x) are reported in *right panels*. A representative mitochondrion of KIKO mice with altered ultrastructure was evidenced with an asterisks (*). LD, lipid droplets; N, nucleus; M, mitochondria.

(B) Oxygen consumption determined through a polarographic method on crude mitochondria isolated from BAT of 6-months old mice (n=6 each group; ****p<0.0001 vs WT).

(C) Representative BAT histology images after staining with H&E (n=3 each group). Scale bars, 25 μ m.

(D, E) Representative immunoblots (D) of total HSL, phospho-active (p-HSL660) and phospho-inactive HSL (p-HSL565), PKA phosphorylated substrates (PKA p-substrates) and Nrf2 in total BAT homogenates, and densitometric analyses of the immunoreactive bands (E). HSL was used as loading control (n=6 each group, *p<0.05, **p<0.01 vs WT).

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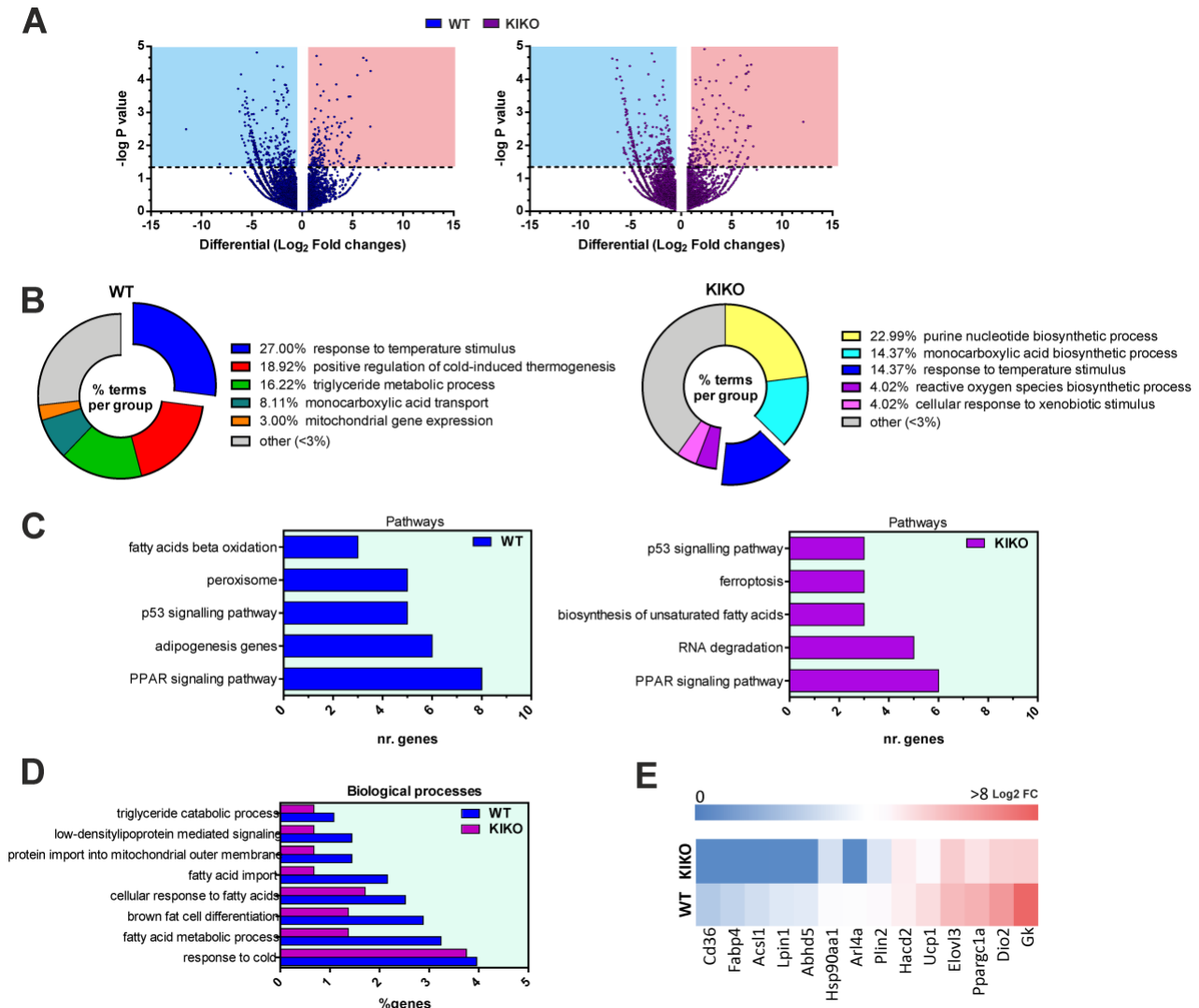
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Fig. 4. KIKO mice have reduced cold tolerance.

(A) Body temperature measured in 6-months old mice after 12 h cold exposure (n=6 each group; *p<0.05, **p<0.01).

(B) Representative histology images of BAT from mice exposed to cold after staining with H&E (n=3 each group). Scale bars, 25 μm.

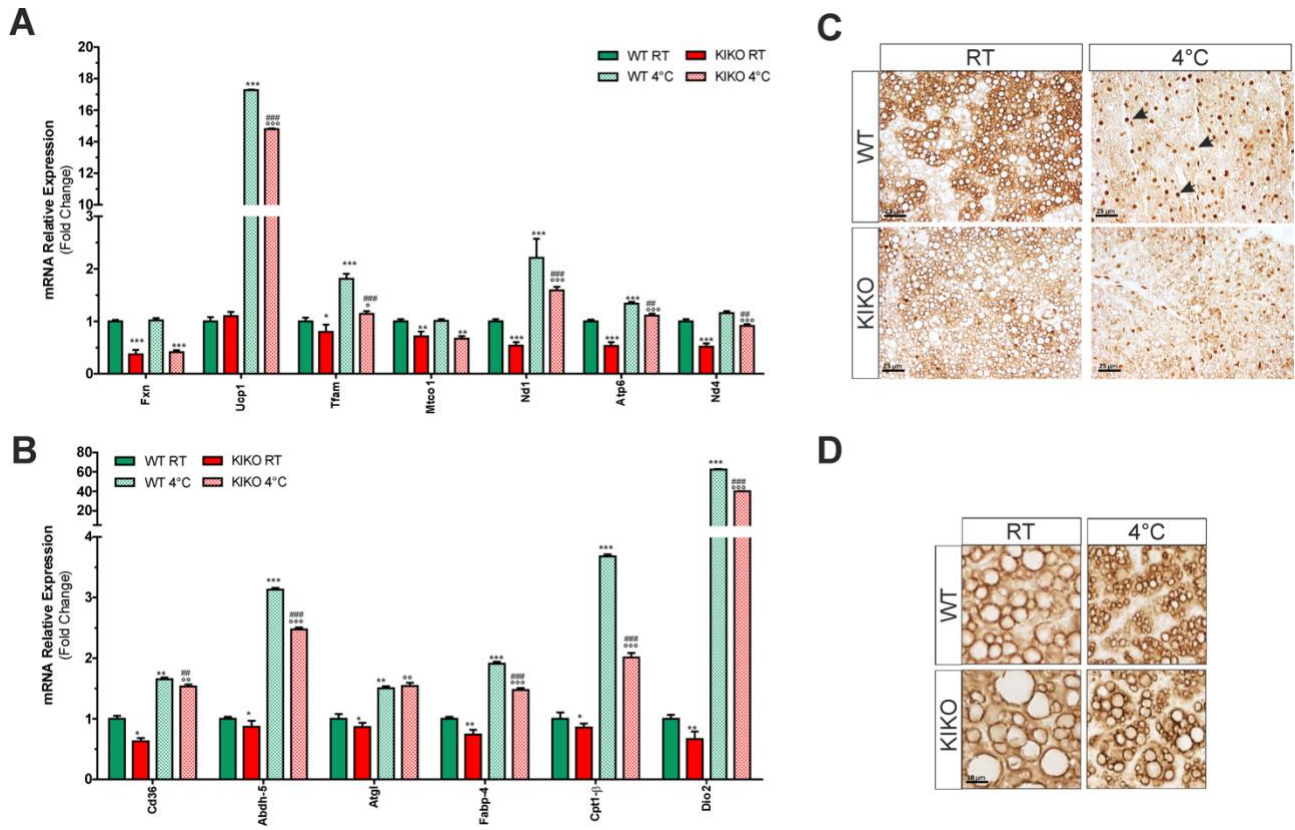
(C) Lipid droplets (LDs) diameter measurement in three fields for each BAT histology through ImageJ (300 droplets total at least for each sample). BAT of WT and KIKO mice at RT (see Fig. 4C) or exposed to cold (B) for 12 h were analyzed (n=3, ****p<0.0001 vs RT, **p<0.001 vs WT mice at RT, °°p<0.001 vs WT mice at 4°C).



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Fig. 5. RNAseq analysis revealed reduced expression of genes related to BAT activity in KIKO mice.

819 (A) Volcano plot representation of differential gene expression analysis in BAT of 6-months old WT
820 (*upper panel*) and KIKO mice (*lower panel*) exposed to cold for 12 h. Light blue and red squares
821 mark the genes with significantly decreased or increased expression respectively ($p < 0.05$, $n = 3$).
822 (B, C) Biological processes (B), KEGG and Wiki pathways (C) significantly up-regulated in BAT of
823 WT (*left panels*) and KIKO mice (*right panels*) upon cold exposure determined through ClueGo
824 plugin of Cytoscape v3.7 platform (Benjamini-corrected p values < 0.05).
825 (D) Comparative analysis of significantly up-regulated biological processes ($p < 0.05$) in WT mice and
826 KIKO mice determined through Funrich v3.0.
827 (E) Heatmap showing representative genes significantly up-regulated ($p < 0.05$) in WT mice compared
828 to KIKO mice.
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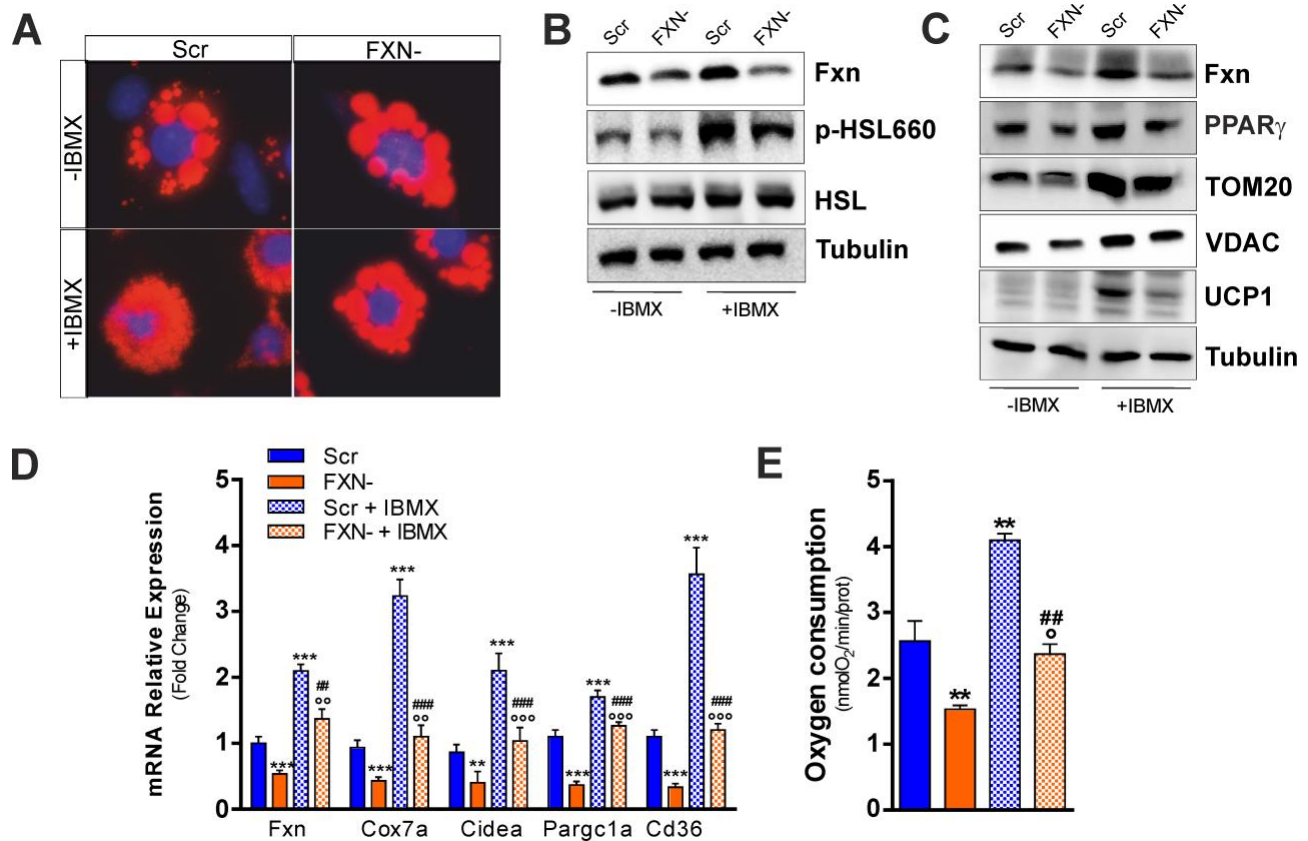
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832 **Fig. 6. The expression of genes related to mitochondrial respiration, thermogenesis and lipolytic**
833 **pathways are altered in BAT of KIKO mice.**

834 (A, B) RT-qPCR analysis of mitochondrial genes (A) and genes related to lipid metabolism (B) in
835 BAT of 6-months old mice (n=6; *p<0.05, **p<0.01, ***p<0.001 vs WT; °p<0.05, °°p<0.01,
836 °°°p<0.001 vs KIKO; ###p<0.01, ###°p<0.001 vs WT at 4°C).

837 (C, D) Representative immunohistochemical analyses of phospho-PKA substrates (C) and perilipin-
838 1 (D) (n=3 mice each group). Black arrows (C) indicate some nuclei showing positivity to the
839 immunostaining of phospho-PKA substrates.

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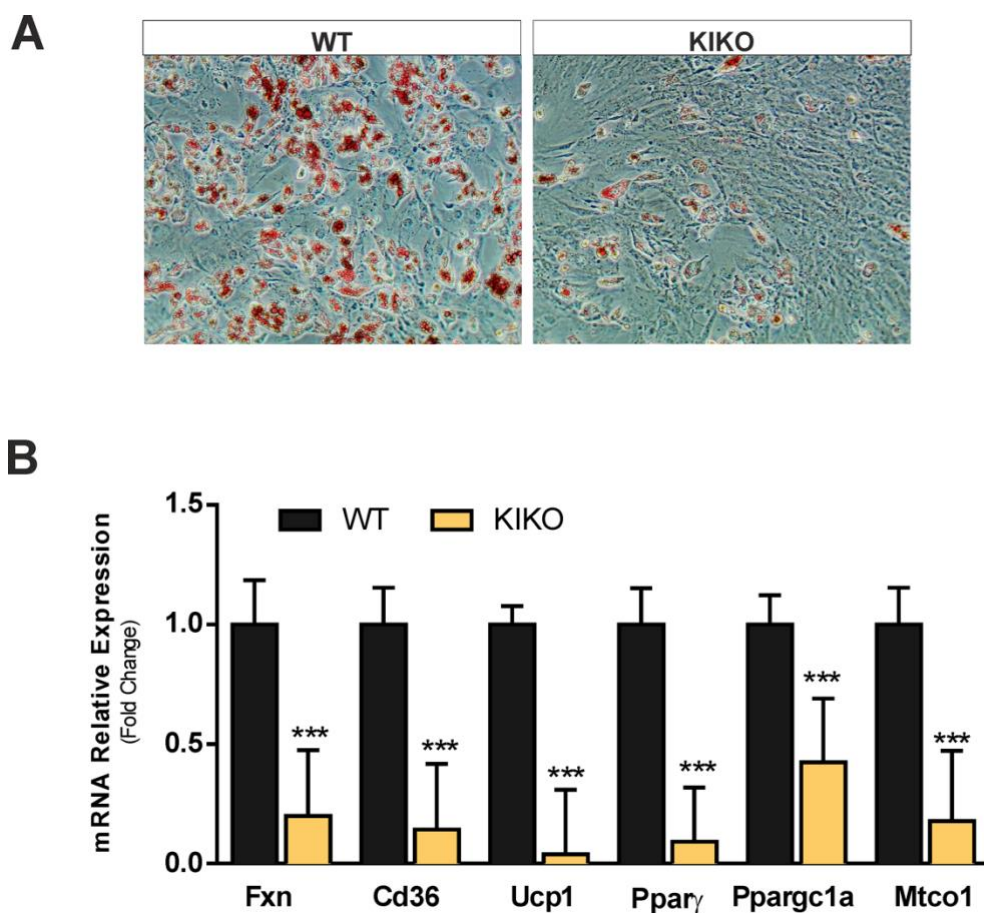
Fig. 7. FXN deficiency impairs thermogenic program also in a brown adipocytes cell line.

(A) Representative immunofluorescence analysis of lipid droplets after staining with the neutral lipid probe Nile Red in T37i brown adipocytes transfected with a pool of siRNAs targeting FXN mRNA (FXN-) or with a pool of Scr siRNAs (Scr) (n=4).

(B, C) Representative immunoblots of FXN, total and phospho-active HSL (p-HSL660) (B), PPAR γ , TOM20, VDAC and UCP1 (C) (n=4).

(D) RT-qPCR analysis of genes implicated in thermogenesis (n=4; **p<0.01, ***p<0.001 vs Scr; °°p<0.01, °°°p<0.001 vs FXN-; ###p<0.01, ####p<0.001 vs Scr + IBMX).

(E) Cellular oxygen consumption determined through a polarographic method (n=4, **p<0.01 vs Scr, °p<0.05 vs FXN-; ###p<0.001 vs Scr + IBMX).



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Fig. 8. KIKO mice show affected adipogenic potential.

(A) Representative images of stromal vascular cells (SVCs) obtained from mouse adipose depots of WT or KIKO mice, differentiated in adipocytes and stained with Oil Red O to detect lipid droplet-containing mature adipocytes (n = 3).

(B) RT-qPCR analysis of genes implicated in adipogenesis and thermogenesis in SVCs differentiated in adipocytes (n = 6; ***p < 0.001 vs WT).