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# Thyroid Hormone Receptor $\alpha$ Regulates Autophagy, Mitochondrial Biogenesis, and Fatty Acid Use in Skeletal Muscle

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1 **Thyroid hormone receptor- $\alpha$  regulates autophagy, mitochondrial biogenesis, and fatty**  
2 **acid utilization in skeletal muscle**

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14 **Short title:** TR $\alpha$ 1 mutation impairs muscle autophagy

15 **Key words:** TR $\alpha$ 1 mutation; autophagy; mitochondrial function; lipid metabolism; muscle

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24

25

## 26 **Abstract**

27 Skeletal muscle (SM) weakness occurs in hypothyroidism and resistance to thyroid hormone  
28 alpha (RTH $\alpha$ ) syndrome. However, the cell signaling and molecular mechanism(s) underlying  
29 muscle weakness under these conditions is not well understood. We thus examined the role of  
30 thyroid hormone receptor alpha (TR $\alpha$ ), the predominant TR isoform in SM, on autophagy,  
31 mitochondrial biogenesis and metabolism to demonstrate the molecular mechanism(s) underlying  
32 muscle weakness in these two conditions.

33 Two genetic mouse models, *TR $\alpha$ 1<sup>PV/+</sup>* mice which expresses mutant Thra1PV gene  
34 ubiquitously, and *SM-TR $\alpha$ 1<sup>L400R/+</sup>* mice, which expresses *TR $\alpha$ 1<sup>L400R</sup>* in a muscle-specific manner,  
35 were used in this study. Gastrocnemius muscle from *TR $\alpha$ 1<sup>PV/+</sup>*, *SM-TR $\alpha$ 1<sup>L400R/+</sup>*, and their control  
36 mice was harvested for analyses.

37 We demonstrated that loss of TR $\alpha$ 1 signaling in gastrocnemius muscle from both the genetic  
38 mouse models led to decreased autophagy as evidenced by accumulation of p62 and decreased  
39 expression of lysosomal markers (LAMP1, and LAMP2) and lysosomal proteases (cathepsin B  
40 and cathepsin D). The expression of PGC1 $\alpha$ , TFAM, and ERR $\alpha$ , key factors contributing to  
41 mitochondrial biogenesis as well as mitochondrial proteins were decreased, suggesting that there  
42 was reduced mitochondrial biogenesis due to the expression of mutant TR $\alpha$ 1. Transcriptomic and  
43 metabolomic analyses of SM suggested that lipid catabolism was impaired, and was associated  
44 with decreased acylcarnitines and tricarboxylic acid cycle (TCA cycle) intermediates in the SM  
45 from the mouse line expressing SM-specific mutant TR $\alpha$ 1. Our results provide new insight into  
46 TR $\alpha$ 1-mediated cell signaling, molecular, and metabolic changes that occur in SM when TR action  
47 is impaired.

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## 51 **Introduction**

52 Hypothyroidism is a common chronic condition that can occur in as many as 5% of the adult  
53 population in some countries (2,3). Patients with hypothyroidism have decreased resting energy  
54 expenditure and muscle strength (4,5). Additionally, thyroid hormone (TH) has profound effects  
55 on SM architecture, metabolism, contractile function, myogenesis and regeneration (6-8).  
56 Interestingly, TH stimulates both glycolysis and/or fatty acid  $\beta$ -oxidation in myocytes (9,10). It  
57 also induces the transition from slow twitch to fast twitch muscle fibers as well as the metabolic  
58 changes associated with that transition (6,10,11). Thus, it can stimulate SM activity based upon  
59 muscle fiber type and/or fuel availability. TH also increases autophagy/mitophagy and  
60 mitochondrial biogenesis (11). However, the effects of hypothyroidism on these processes is not  
61 well understood.

62 Thyroid hormone receptor alpha1 (TR $\alpha$ 1) is the pre-dominant receptor isoform in SM, and  
63 is thought to mediate most of TH's actions in SM (12). Animal and human studies on the actions  
64 of TH in SM have examined hypo- or hyperthyroidism at the systemic level (13). Thus, little is  
65 known about the direct effects of TH on transcription, metabolism, and mitochondria on SM *in*  
66 *vivo* when its actions are impaired during hypothyroidism or resistance to thyroid hormone alpha  
67 (RTH $\alpha$ ). Patients with RTH $\alpha$  have been reported to harbor TR $\alpha$  mutations, (TR $\alpha$ 1E403X,  
68 TR $\alpha$ 1F397fs406X, or TR $\alpha$ 1A397PfsX7) (14-16). Although patients were heterozygotes, they  
69 exhibited classical features of hypothyroidism in tissues that expressed high levels of TR $\alpha$  such  
70 as heart, skeletal muscle, and bone (14-16). Furthermore, these mutations exhibited dominant  
71 negative action and interfered with the transcriptional activity of wildtype TR $\alpha$  (14-16). The  
72 isoform-specific role of TR $\alpha$ 1 on metabolism and mitochondrial activity has been examined in  
73 several tissues (12,13) but little is known about TR $\alpha$ 1's role in SM. Accordingly, we examined  
74 two genetic mouse models in which dominant negative mutant TR $\alpha$ 1s were expressed  
75 ubiquitously or in a skeletal muscle-specific manner. These mutant TR $\alpha$ 1s both had impaired  
76 hormone binding to TH, decreased transcriptional activity, and dominant negative activity on

77 normal TR $\alpha$  isoforms (17,18). These abnormalities due to mutant TR $\alpha$ 1 were not rescued by TH  
78 administration (19). We examined the effects of inhibiting TR $\alpha$ 1 activity on SM on autophagy,  
79 mitochondrial turnover, lipid metabolism, and muscle fiber phenotype to better understand the  
80 molecular pathogenesis underlying the impaired energy metabolism observed in SM of patients  
81 with hypothyroidism and RTH $\alpha$  syndrome.

## 82 **Materials and Methods**

### 83 **Animal models**

84 *TR $\alpha$ <sup>AMI/+</sup>* mice were generated and genotyped as described earlier (18). *TR $\alpha$ <sup>AMI/+</sup>* mice allow  
85 the conditional expression of a TR $\alpha$ 1 point mutant, in which the leucine present in the AF2 is  
86 converted into an arginine (L400R). The *TR $\alpha$ 1<sup>L400R</sup>* reading frame flanked by the upstream  
87 *PGKNeoRpolyA* and the downstream *IRESTaulacZ* cassettes, was introduced in the mouse  
88 *THRA* locus by homologous recombination under its natural promoter. The upstream  
89 *PGKNeoRpolyA* cassette contains a simian virus 40 (SV40) polyadenylation signal, which  
90 arrests most transcription and prevents any TR $\alpha$ 1<sup>L400R</sup> translation. Because the upstream  
91 *PGKNeoRpolyA* cassette is flanked by two tandem *loxP* sequences, the expression of *TR $\alpha$ <sup>AMI</sup>*  
92 allele was induced only after CRE/*loxP*-mediated DNA recombination (18). To generate muscle-  
93 specific *TR $\alpha$ <sup>AMI/+</sup>* mice (referred as *SM-TR $\alpha$ 1<sup>L400R/+</sup>* mice thereafter), *TR $\alpha$ <sup>AMI/+</sup>* mice then were  
94 crossed with human skeletal actin (HSA)-cre mice, which enabled the expression of *TR $\alpha$ 1<sup>L400R</sup>*  
95 specifically in the muscle, while the *TR $\alpha$ <sup>AMI/+</sup>* mice served as control (referred as *Control*  
96 thereafter). Three to five-month old male mice were used for this study. This animal study was  
97 carried out in accordance with the European Community Council Directive of September 22,  
98 2010 (2010/63/EU) regarding the protection of animals used for experimental and other scientific  
99 purposes. The research project was approved by a local animal care and use committee  
100 (C2EA015) in Lyon and subsequently authorized by the French Ministry of Research.

101 Mice harbouring the mutant Thra1PV gene (*TR $\alpha$ 1<sup>PV/+</sup>* mice) and *wild-type* littermates were  
102 prepared and genotyped as described earlier (17). Three to four month old female mice were

103 used for this study. This animal study was carried out according to the protocol approved by the  
104 National Cancer Institute Animal Care and Use Committee.

105 Animals were housed in hanging polycarbonate cages under a 12-hour light/12-hour dark  
106 cycle at 23°C with food and water available ad libitum. Animals were sacrificed in CO<sub>2</sub> chambers  
107 and gastrocnemius muscle were collected in liquid N<sub>2</sub> and subsequently used for protein  
108 isolation.

### 109 **Western Blot**

110 A total of 20–40 mg gastrocnemius muscle were used to generate protein lysate, and the  
111 following western blotting procedure was performed as described (10). Visualization was  
112 performed using the enhanced chemiluminescence system (GE Healthcare). Densitometric  
113 analyses of western blot images were performed by using Image J software (NIH, Bethesda, MD,  
114 United States). Phosphorylated protein was normalized to total protein, and others were  
115 normalized to GAPDH or  $\beta$ -actin. Antibodies information listed in Supplementary Table 1(1).

### 116 **RNA-seq and pathway analysis**

117 RNA was isolated from gastrocnemius muscle tissue using QIAzol (Qiagen, Hilden,  
118 Germany), followed by clean-up using Invitex Mini Kit (Invitex, San Francisco, CA) following the  
119 manufacturer's protocol. RNA-seq and pathway analysis on pooled samples of each *Control*  
120 (n=4) and *SM-TR $\alpha$ 1<sup>L400R/+</sup>* (n=4) was performed by NovogeneAIT Genomics Singapore Pte Ltd.  
121 Briefly, Alignments were parsed using Tophat program and differential expressions were  
122 determined through DESeq2/edgeR. GO enrichment were implemented by the ClusterProfiler.

### 123 **Metabolomics**

124 In accordance with previously established mass spectrometry (MS)-based methods,  
125 gastrocnemius muscle samples were used for quantitative determination of targeted metabolite  
126 levels for acylcarnitine species and organic acids by the Metabolomics Core Facility of Duke-  
127 NUS Medical School (10). Muscle tissue was homogenized in 50% acetonitrile and 0.3% formic  
128 acid. For acylcarnitine and organic acid extraction, 100  $\mu$ L of tissue homogenate was extracted

129 using methanol. The acylcarnitine extracts were derivatized with 3 M hydrochloric acid in  
130 methanol, dried, and reconstituted in methanol for analysis in liquid chromatography/mass  
131 spectrometry (LC/MS). Organic acid extracts were derivatized to form butyl esters using 3 M HCl  
132 in butanol. Samples were then reconstituted in 80% aqueous methanol and 4  $\mu$ L of this solution  
133 was used for analysis. The heatmap of lipidomic profiling was presented as mean value of each  
134 group using normalized percentage value for each lipid species, with smallest mean defined as  
135 0%, and largest mean defined as 100%. All the analysis was done using GraphPad Prism 8.

136

### 137 **Statistical Analysis**

138 The comparison between *SM-TR $\alpha$ 1<sup>L400R/+</sup>* and *Control* mice, and *TR $\alpha$ 1<sup>PV/+</sup>* mice with its *wild-*  
139 *type* littermates were conducted using unpaired parametric Student's t-test in GraphPad Prism.  
140 Statistical significance was declared at a  $p^*p < 0.05$ ,  $**p < 0.01$ ,  $****p < 0.0001$ .

141

## 142 **Results**

### 143 **TR $\alpha$ 1 mutation decreased autophagy in gastrocnemius muscle**

144 In the current study, we used two mouse models expressing dominant-negative mutations in  
145 TR $\alpha$ 1: *SM-TR $\alpha$ 1<sup>L400R/+</sup>* knock-in mice which were heterozygous for a dominant negative  
146 *TR $\alpha$ 1<sup>L400R</sup>* point mutation (conversion of a Leucine to an Arginine in the AF2 domain) that was  
147 conditionally expressed in SM; and *TR $\alpha$ 1<sup>PV/+</sup>* knock-in mice which were heterozygous for a  
148 dominant negative TR $\alpha$ 1<sup>PV</sup> frameshift mutation that was ubiquitously expressed. *TR $\alpha$ 1<sup>PV/+</sup>* mice  
149 faithfully recapitulated the phenotype exhibited by patients with mutations of the *THRA* gene  
150 (17).

151 We previously observed that TH stimulated autophagy in SM (11), so we examined autophagy  
152 in the gastrocnemius muscle from *SM-TR $\alpha$ 1<sup>L400R/+</sup>* and *Control* mice. The protein levels of LC3B-  
153 II and p62 were elevated in *SM-TR $\alpha$ 1<sup>L400R/+</sup>* mice compared to *Controls* (Fig. 1A). Similarly, p62  
154 accumulated in gastrocnemius muscle from *TR $\alpha$ 1<sup>PV/+</sup>* mice (Fig. 1B). Since p62 accumulates

155 when autophagic flux is decreased (20), the increase in p62 protein levels suggested there was  
156 decreased autophagy in both mouse strains. We also assessed p62 level in wildtype C57BL/6  
157 mice injected with normal saline or T<sub>3</sub>, and found that T<sub>3</sub> decreased p62 in wildtype mice  
158 (Supplementary Fig. 1(1)), confirming that T<sub>3</sub> negatively regulates p62 protein. We next assessed  
159 the expression of lysosomal marker proteins such as lysosomal-associated membrane protein 1  
160 (LAMP-1) and lysosomal-associated membrane protein 2 (LAMP-2), and the lysosomal  
161 proteases, cathepsin B and cathepsin D. There was decreased expression of these lysosomal  
162 proteins in SM from *SM-TRα1<sup>L400R/+</sup>* mice compared to *Control* mice (Fig. 2A-B). Similar  
163 decreases in LAMP1, cathepsin B and cathepsin D protein expression also were seen in SM  
164 from *TRα1<sup>PV/+</sup>* mice (Fig. 2D). We next investigated the phosphorylation of TFEB (Fig. 2C and  
165 2E), a key transcription factor for lysosomal biogenesis genes that is inhibited by mTOR  
166 phosphorylation at Ser142 (21). Decreased total TFEB and/or increased phosphorylated TFEB  
167 protein levels led to an increased p-TFEB/TFEB ratios that were higher in *SM-TRα1<sup>L400R/+</sup>* and  
168 *TRα1<sup>PV/+</sup>* mice than controls (Fig. 2C and 2E). Taken together, these results showed that the  
169 expression of mutant TRα1 in SM decreased autophagy and decreased lysosomal protein  
170 expression.

171 The mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK)  
172 signaling are two major pathways that control autophagy, and both are regulated by TH (11,22).  
173 We found increased phosphorylation of two mTOR targets, eukaryotic translation initiation factor  
174 4E (eIF4E)-binding protein 1 (4EBP1) and p70S6 kinase (p70S6K) (Fig. 3A-C), suggesting an  
175 increase in mTOR signaling in the SM from *SM-TRα1<sup>L400R/+</sup>* mice. There also was decreased  
176 phosphorylation of AMPK and adipose triglyceride lipase (ATGL) at Ser406 (Fig. 3D-F), a site  
177 that is phosphorylated by AMPK (23) in the SM from *SM-TRα1<sup>L400R/+</sup>* mice. Similarly, there was  
178 increased phosphorylation of 4EBP1 and decreased phosphorylation of ATGL in the SM from  
179 *TRα1<sup>PV/+</sup>* mice (Supplementary Fig. 2(1)). These findings showed that expression of mutant TRα1  
180 in SM led to increased mTOR and decreased AMPK signaling.



181 **TR $\alpha$ 1 mutation decreased mitochondrial turnover in gastrocnemius muscle**

182 TH increased mitochondrial turnover by increasing mitophagy and mitochondrial biogenesis  
183 (11,22,24). We examined several mitophagy markers in SM from *SM-TR $\alpha$ 1<sup>L400R/+</sup>* and *Control*  
184 mice. There was no change in the expression of PINK1; however, protein levels of Drp1 and  
185 parkin were decreased in *SM-TR $\alpha$ 1<sup>L400R/+</sup>* mice (Fig. 4), suggesting that mitophagy may have  
186 been impaired in *SM-TR $\alpha$ 1<sup>L400R/+</sup>* mice. In SM from *TR $\alpha$ 1<sup>PV/+</sup>* mice, the protein levels of parkin  
187 was unchanged but Drp1 was decreased (Supplementary Fig. 3(1)). The decreases in Drp1  
188 expression also suggested that mitochondrial fission might be impaired in both mouse strains.  
189 We also assessed Drp1 levels in wildtype C57BL/6 mice injected with normal saline or T<sub>3</sub>, and  
190 found that T<sub>3</sub> did not affect Drp1 expression in wildtype mice (Supplementary Fig. 4(1)). Thus, it  
191 is possible that Drp1 expression is regulated at physiological levels of T<sub>3</sub> but is not further  
192 increased during hyperthyroidism.

193 Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 $\alpha$ ),  
194 mitochondrial transcription factor A (TFAM) and estrogen-related receptor alpha (ERR $\alpha$ ) are key  
195 factors involved in mitochondrial biogenesis (24,25). PGC1 $\alpha$ , TFAM, and ERR $\alpha$  protein levels  
196 were decreased in the SM from *SM-TR $\alpha$ 1<sup>L400R/+</sup>* mice (Fig. 5A), suggesting that *TR $\alpha$ 1<sup>L400R</sup>*  
197 decreased mitochondrial biogenesis. Mitochondrial proteins such as translocase of outer  
198 mitochondrial membrane 20 (TOMM20), voltage-dependent anion channel 1 (VDAC1), and  
199 cytochrome c oxidase subunit 4I1 (COXIV) also were decreased in *SM-TR $\alpha$ 1<sup>L400R/+</sup>* mice (Fig.  
200 5B-C). Similarly, *TR $\alpha$ 1<sup>PV/+</sup>* mice exhibited decreased protein levels of ERR $\alpha$ , TOMM20, VDAC1  
201 and COXIV in SM (Fig. 5D). These results suggested that mutant TR $\alpha$ 1 decreased mitochondrial  
202 biogenesis and content in SM.

203 **Dysregulated mitochondrial metabolism in muscle from *SM-TR $\alpha$ 1<sup>L400R/+</sup>* mice**

204 We previously showed that TH increased  $\beta$ -oxidation of fatty acids by inducing hepatic  
205 lipophagy and CPT1 $\alpha$  expression (26,27). We thus examined carnitine palmitoyltransferase I $\beta$   
206 (CPT1 $\beta$ ) protein expression and found that it was lower in SM of *SM-TR $\alpha$ 1<sup>L400R/+</sup>* mice

207 (Supplementary Fig. 5A(1)). The expression of CPT1 $\beta$  in muscle from *TR $\alpha$ 1<sup>PV/+</sup>* mice showed a  
208 similar trend but was not statistically significant (Supplementary Fig. 5B(1)). We next performed  
209 transcriptome pathway analyses of SM from *SM-TR $\alpha$ 1<sup>L400R/+</sup>* mice and *Control* mice, and found  
210 that lipid catabolism, lipid transport, and cellular response to hormone stimulus were the major  
211 down-regulated pathways in *SM-TR $\alpha$ 1<sup>L400R/+</sup>* mice (Supplementary Table 1(1)). Interestingly,  
212 pathways involved in collagen formation and SMAD signaling were upregulated (Supplementary  
213 Table 2(1)). We next performed metabolomic profiling of acylcarnitines and TCA cycle  
214 intermediates in SM from *SM-TR $\alpha$ 1<sup>L400R/+</sup>* mice. There were decreased levels of some, but not  
215 all, species of short, long, and very long chain acylcarnitines compared to *Control* littermates  
216 (Fig. 6A). The TCA cycle intermediates, citrate, fumarate, and malate also were decreased  
217 suggesting there likely was decreased TCA cycle flux (Fig. 6B). These results suggested that  
218 fatty acid utilization might have been impaired due to expression of the TR $\alpha$ 1 mutation. Finally,  
219 we examined the effect of TR $\alpha$ 1 mutation on SM fibre type. There was increased MHC-I and  
220 unchanged MHC2a protein levels in SM *TR $\alpha$ 1<sup>L400R/+</sup>* mice (Supplemental Fig. 6A(1)). In *TR $\alpha$ 1<sup>PV/+</sup>*  
221 mice, MHC-I protein expression remained the same while there was a decrease in MHC2a  
222 (Supplemental Fig. 6B(1)). Thus, the ratios of MHC-1/MHC2a were increased in SM from both  
223 mouse lines (Supplemental Fig. 6(1)).

## 224 **Discussion**

225 While there have been studies examining TR $\alpha$ 1-specific regulation of gene expression in  
226 the liver, small intestine, bone, and heart (28-31), little is known about TR $\alpha$ 1-specific regulation  
227 of transcription in SM. Additionally, although the roles of TH on the metabolome and autophagy  
228 recently have begun to be examined in SM (10,11), the specific role(s) of TR $\alpha$ 1 on these  
229 processes in SM is not known. In the current study, we addressed these issues by analyzing  
230 gastrocnemius muscle tissue obtained from genetically-altered mice that expressed *TR $\alpha$ 1<sup>L400R</sup>*  
231 specifically in SM and *TR $\alpha$ 1<sup>PV</sup>* ubiquitously. Both TR $\alpha$ 1 mutants had impaired hormone binding

232 to TH, decreased transcriptional activity, and dominant negative activity on normal TR isoforms  
233 expressed in SM (17,18). We found that expression of both TR $\alpha$ 1 mutants in SM led to  
234 accumulation of p62 as well as decreased expression of lysosomal membrane proteins and  
235 proteases (Figs. 1 and 2), suggesting that there was a late block in autophagy. We found  
236 evidence for increased mTOR activity as there was increased phosphorylation of mTOR targets,  
237 p70S6K, 4EBP1, and TFEB in SM that expressed TR $\alpha$ 1 mutants (Fig. 2C and 3). Increased  
238 mTOR activity phosphorylates TFEB (32), the master transcriptional regulator of lysosomal  
239 protein expression, to prevent nuclear localization and transcription of lysosomal proteins by  
240 TFEB (21). Additionally, we found decreased phosphorylation of AMPK and its downstream  
241 target, ATGL. The decreased phosphorylation of AMPK may be due to decreased ATP utilization  
242 in SM when TH action is inhibited (33) and also would decrease autophagy (34,35). These  
243 findings are consistent with our earlier findings that TH stimulated autophagy by activating AMPK  
244 and inhibiting mTOR signaling in metabolically active organs such as liver and SM (11,22).

245 Muscle-specific deletion of the crucial autophagic gene *Atg7* caused accumulation of  
246 dysfunctional mitochondria (36). Furthermore, deletion of *Atg7* in adult mice just prior to exercise  
247 led to damaged mitochondria and ROS damage (37). These results indicated that mitophagy  
248 had a fundamental role in maintaining muscle function during physical activity. Our previous  
249 studies demonstrated TH promoted mitochondrial quality control through mitophagy and  
250 mitochondrial biogenesis (22,24). In this connection, we found that mutant TR $\alpha$ 1 expression in  
251 SM led to decreased levels of Drp1 and Parkin expression (Fig. 4 and Supplementary Fig. 3(1)).  
252 Drp1-mediated fission appears to be a crucial prerequisite for mitophagy, and the upregulation  
253 of Drp1 is associated with mitophagy during the early differentiation of muscle cells (38), and  
254 TH-mediated stimulation of hepatic mitophagy (22,24). Expression of mutant TR $\alpha$ 1 in SM also  
255 decreased protein levels of PGC1 $\alpha$ , TFAM, and ERR $\alpha$  (Fig. 5), key transcription factors involved  
256 in mitochondrial biogenesis are regulated by TH (24), as well as decreased mitochondrial protein

257 levels of TOMM20, VDAC1 and COXIV (Fig. 5). Taken together, these findings suggested that  
258 there was decreased mitochondrial biogenesis and mitochondria content.

259 Hypothyroid patients are more dependent on muscle glycogen due to impaired ability to use  
260 fatty acids (39-41). A decrease in the amount of functional mitochondria in SM could lead to  
261 altered mitochondrial fuel utilization (42). To address this issue, we performed transcriptome  
262 pathway analyses of SM from *SM-TR $\alpha$ 1<sup>L400R/+</sup>* and *Control* mice. Interestingly, *SM-TR $\alpha$ 1<sup>L400R/+</sup>*  
263 mice displayed down-regulation of pathways involved in lipid catabolism (Supplementary Table  
264 2(1)). We observed decreased expression of CPT1 $\beta$  (Supplementary Fig. 5A(1)), which imports  
265 acylcarnitines into the mitochondria. Our observation that there were decreases in intracellular  
266 acylcarnitines combined with decreases in citrate, fumarate, and malate levels in SM from mice  
267 expressing *TR $\alpha$ 1<sup>L400R</sup>* further supports the notion that fatty acid utilization was impaired when  
268 dominant negative TR $\alpha$ 1 was expressed in SM (Fig. 6). Since the levels of long and very long  
269 chain acylcarnitine species were decreased, it is possible that the entry of free fatty acids into  
270 mitochondria for  $\beta$ -oxidation was impaired in SM expressing *TR $\alpha$ 1<sup>L400R</sup>* due to the reduction in  
271 CPT1 $\beta$  expression. Lipolysis of triglycerides from fat droplets and lipophagy are two major ways  
272 in which triglycerides are hydrolyzed to release of free fatty acids for  $\beta$ -oxidation by mitochondria.  
273 In this connection, we found decreased phosphorylation of ATGL (Fig. 3D and F) accompanied  
274 decreased autophagy (Fig. 1). Thus, decreased mitochondrial biogenesis, CPT1 $\beta$  expression,  
275 and autophagy shed light on why hypothyroid patients may have impaired fatty acid utilization in  
276 SM.

277 TH regulates SM fiber type to determine fuel utilization by converting slow twitch fibers that  
278 utilize fatty acids to fast twitch fibers that use glucose (10,11). TH directly regulates the  
279 expression of genes involved in SM contraction and glucose metabolism (43-45). We previously  
280 showed that TH increased the transcription of MHC2a and decreased MHC-I protein expression  
281 most likely via autophagy (11). Interestingly, we observed both increased MHC-I protein  
282 expression and decreased autophagy in SM from *TR $\alpha$ 1<sup>L400R/+</sup>* mice (Supplemental Fig. 6A(1)). In

283 *TRα1<sup>PV/+</sup>* mice, MHC2a protein expression was decreased whereas MHC-I remained the same  
284 (Supplemental Fig. 6B(1)); thus, the ratio of MHC1/MHC2a protein expression increased in both  
285 mouse strains. Currently, the precise molecular mechanism(s) determining SM fiber type  
286 expression in *SM-TRα1<sup>L400R/+</sup>* and *TRα1<sup>PV/+</sup>* mice remains unclear. Moreover, it is not known  
287 whether the aberrant fiber type switching is a consequence of decreased local or systemic TH  
288 action. Nonetheless, our findings suggest that expression of slow twitch MHC-1 muscle fibers  
289 were favored over fast twitch MHC2a fibers in the SM of both mouse models. Similarly, it was  
290 reported that MHC1 expression increased and MHC2a expression decreased in SM of  
291 hypothyroid patients (33). In this connection, TRα KO mice were reported to have decreased  
292 strength compared to wild type mice (46). Moreover, *Dio2* KO mice, which have impaired  
293 conversion of T<sub>4</sub> to T<sub>3</sub>, exhibited decreased strength and endurance as T<sub>3</sub> concentration in SM  
294 was reduced by 33% (47). However, another group found that *Dio2* KO mice had a markedly  
295 different phenotype than hypothyroid mice as they had a high rate of SM twitch contraction and  
296 resistance to fatigue due to down-regulation of PGC1α causing increased conversion to MHC2a  
297 muscle fiber type (48). The reason for the differences in findings between these two groups is  
298 not known since the KO mice were derived from the original parental line. The exercise behavior  
299 and lean mass in *SM-TRα1<sup>L400R/+</sup>* mice recently were reported by Nicolaisen et al (49). The time  
300 to exhaustion on treadmill and lean mass (measured by magnetic resonance imaging) were  
301 unchanged in *SM-TRα1<sup>L400R/+</sup>* mice compared with the littermate controls. Currently, we do not  
302 know the reason(s) *SM-TRα1<sup>L400R/+</sup>* mice maintained normal endurance running capacities. Since  
303 young adult animals were used in Nicolaisen's study, it is possible that the development of  
304 myopathy may occur when these mice become older. Additionally, TH mediates the muscle fiber  
305 switch from slow twitch to fast twitch muscle fibers in soleus muscle (10,11); thus, strength and  
306 sprinting would be affected more than endurance running. Performing studies in older mice or  
307 testing for skeletal muscle functions mediated by fast twitch muscles could be helpful in  
308 assessing the effects of the dominant negative mutant TRα on muscle function.

309 In summary, we showed that expression of dominant-negative TR $\alpha$ 1 mutations in SM in two  
310 different mouse models resulted in decreased autophagy, mitochondrial turnover, fatty acid  
311 utilization, and TCA cycle flux, as well as altered muscle fiber phenotype. These results provide  
312 novel insight into the molecular and metabolic mechanism(s) of TR $\alpha$ 1 that underlie the SM  
313 changes in energy metabolism observed in hypothyroid patients and individuals with RTH $\alpha$   
314 syndrome.

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#### 318 **Author contributions**

319 Conceptualization – J.Z. R.A.S., and P.M.Y.;

320 Methodology –J.Z., K.G., S.C.,and P.M.Y.;

321 Investigation –J.Z., K.G., J.P.H., A.L., X.Z., and C.R.H.;

322 Writing (Original Draft) – J.Z.; P.M.Y.;

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324 Funding Acquisition –J.Z., K.G., S.C., and P.M.Y.,

325 Resources –K.G., S.C., and P.M.Y.;

326 Supervision – P.M.Y.

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338

339 **Data Availability Statement:** Supplementary Figures(1) of Thyroid hormone receptor alpha  
340 regulates autophagy, mitochondrial biogenesis, and fatty acid utilization in skeletal muscle.

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## 518 **Figure legends**

519 **Figure 1.** Mutant TR $\alpha$ 1 expression impaired autophagic degradation. (A) Immunoblot and  
520 densitometric analysis showing protein levels of p62 and LC3B-II in gastrocnemius muscle from  
521 *Control* (n=4) and *SM-TR $\alpha$ 1<sup>L400R/+</sup>* (n=4) mice. (B) Immunoblot and densitometric analysis  
522 showing protein levels of p62 and LC3B-II in gastrocnemius muscle from *wild-type* (n=5) and  
523 *TR $\alpha$ 1<sup>PV/+</sup>* (n=5) mice. Data are shown as mean $\pm$ SD.

524 **Figure 2.** Mutant TR $\alpha$ 1 expression decreased levels of lysosomal markers. (A-C) Immunoblot  
525 and densitometric analysis showing protein levels of lysosomal markers LAMP1 and LAMP2 (A),  
526 lysosomal protease cathepsin D and cathepsin B (B), and phosphorylation of TFEB (C) in  
527 gastrocnemius muscle from *Control* (n=4) and *SM-TR $\alpha$ 1<sup>L400R/+</sup>* (n=4) mice. (D) Immunoblot and  
528 densitometric analysis showing protein levels of LAMP1, LAMP2, cathepsin D, and cathepsin B  
529 in gastrocnemius muscle from *wild-type* (n=5) and *TR $\alpha$ 1<sup>PV/+</sup>* (n=5) mice. (E) Immunoblot and  
530 densitometric analysis showing phosphorylation of TFEB in gastrocnemius muscle from *wild-*  
531 *type* (n=5) and *TR $\alpha$ 1<sup>PV/+</sup>* (n=5) mice. Data are shown as mean $\pm$ SD.

532 **Figure 3.** *TR $\alpha$ 1<sup>L400R/+</sup>* expression increased mTOR signaling and decreased AMPK signaling.  
533 (A-C) Immunoblot (A) and densitometric analysis (B-C) showing phosphorylation of mTOR  
534 targets p70S6 kinase and 4EBP1 and in gastrocnemius muscle from *Control* (n=4) and *SM-*  
535 *TR $\alpha$ 1<sup>L400R/+</sup>* (n=4) mice. (D-F) Immunoblot (D) and densitometric analysis (E-F) showing  
536 phosphorylation AMPK and ATGL in gastrocnemius muscle from *Control* (n=4) and *SM-*  
537 *TR $\alpha$ 1<sup>L400R/+</sup>* (n=4) mice. Data are shown as mean $\pm$ SD.

538 **Figure 4.** *TR $\alpha$ 1<sup>L400R/+</sup>* expression decreased protein levels of Drp1 and parkin. (A-D) Immunoblot  
539 (A) and densitometric analysis (B-D) showing protein levels of factors for mitochondrial quality

540 control, Drp1, parkin and PINK in gastrocnemius muscle from *Control* (n=4) and *SM-TRα1<sup>L400R/+</sup>*  
541 (n=4) mice. Data are shown as mean±SD.

542 **Figure 5.** Mutant TRα1 expression impaired mitochondrial biogenesis. (A-C) Immunoblot and  
543 densitometric analysis showing protein levels of key regulators for mitochondrial biogenesis,  
544 PGC1α, TFAM, and ERRα (A), mitochondrial proteins TOMM20 and VDAC1 (B), and COXIV  
545 (C) in gastrocnemius muscle from *Control* (n=4) and *SM-TRα1<sup>L400R/+</sup>* (n=4) mice. (D) Immunoblot  
546 and densitometric analysis showing levels of PGC1α, TFAM, ERRα, TOMM20, VDAC1, and  
547 COXIV in gastrocnemius muscle from *wild-type* (n=5) and *TRα1<sup>PV/+</sup>* (n=5) mice. Data are shown  
548 as mean±SD.

549 **Figure 6.** Mutant TRα1 expression impaired fatty acid and TCA cycle metabolism. The levels of  
550 acylcarnitines (A) and organic acids (B) in gastrocnemius muscle from *Control* (n=4) and *SM-*  
551 *TRα1<sup>L400R/+</sup>* (n=3) mice. Data are shown as mean±SD.

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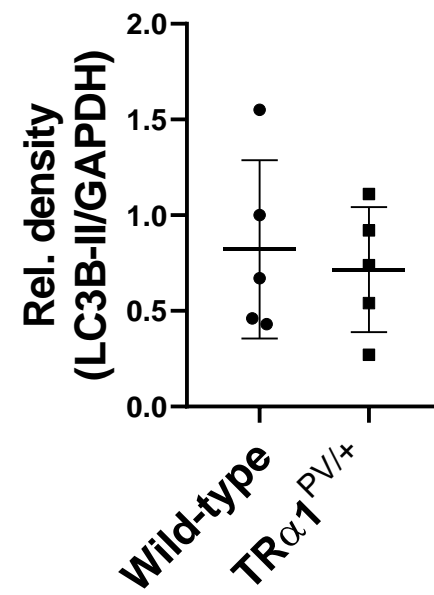
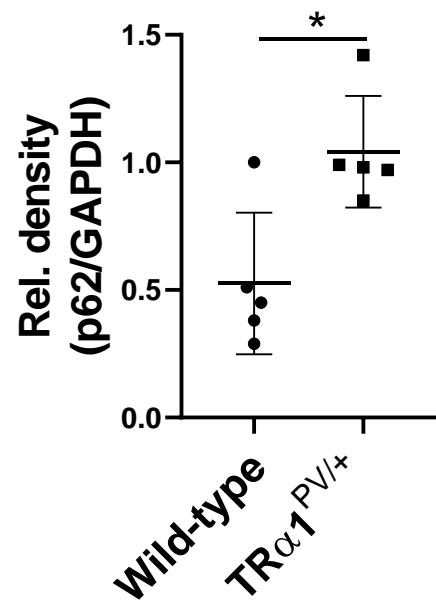
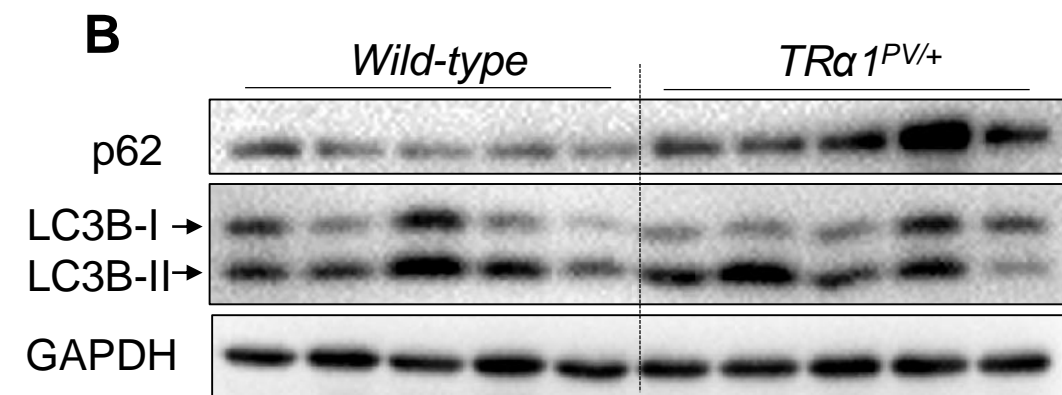
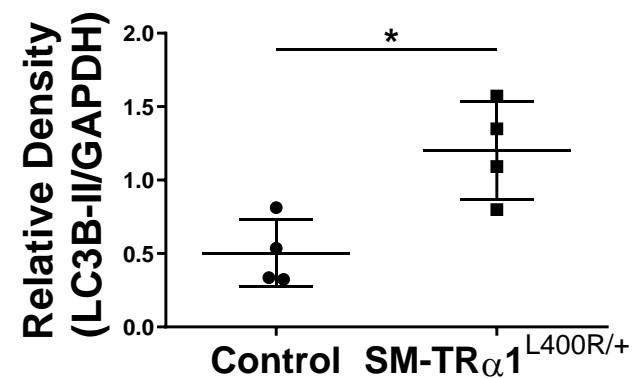
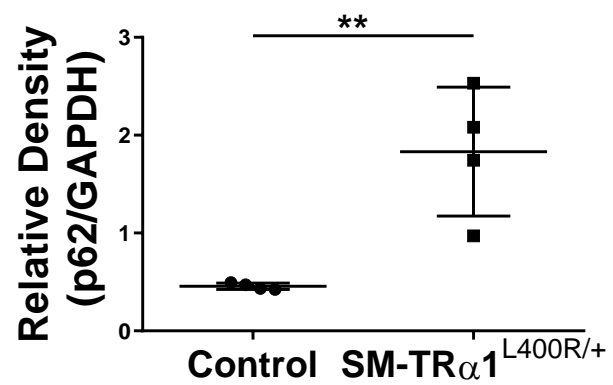
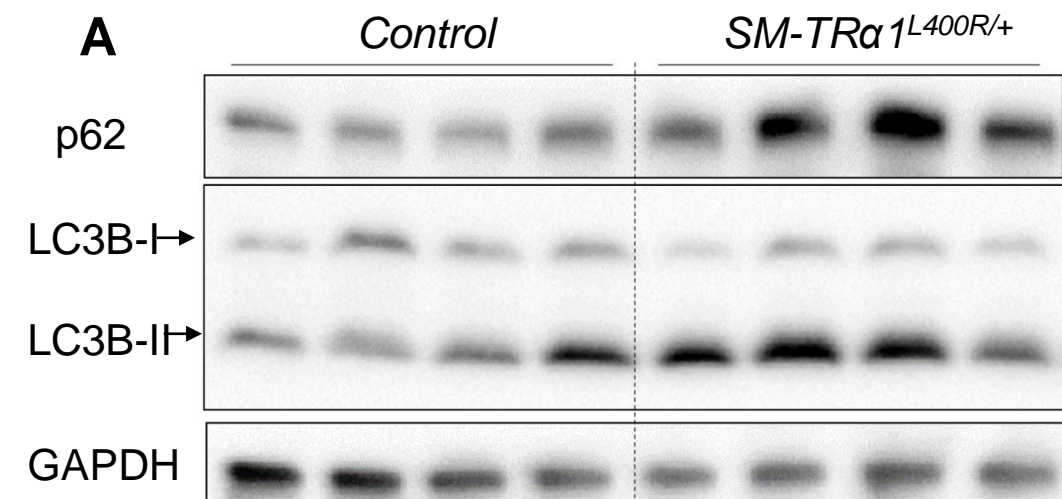
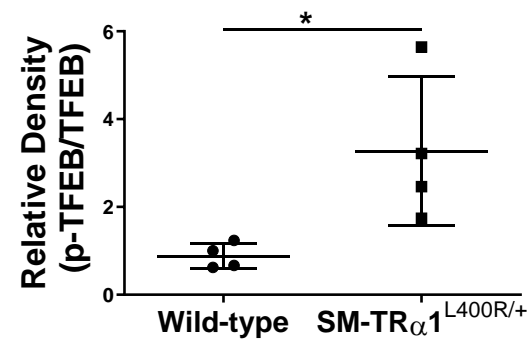
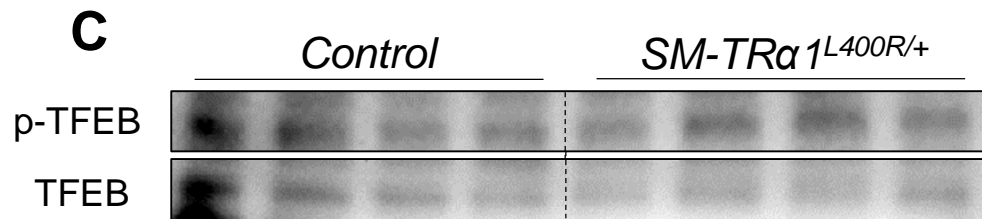
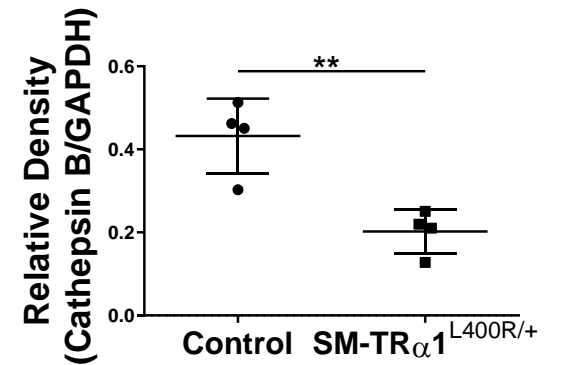
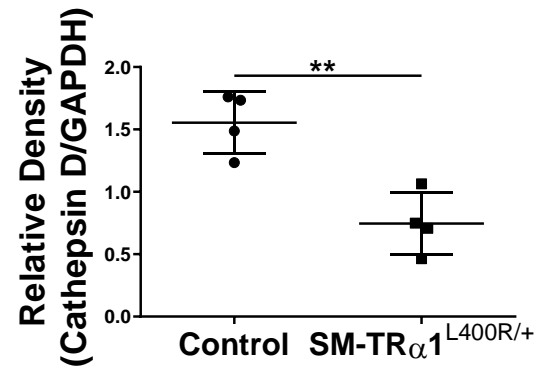
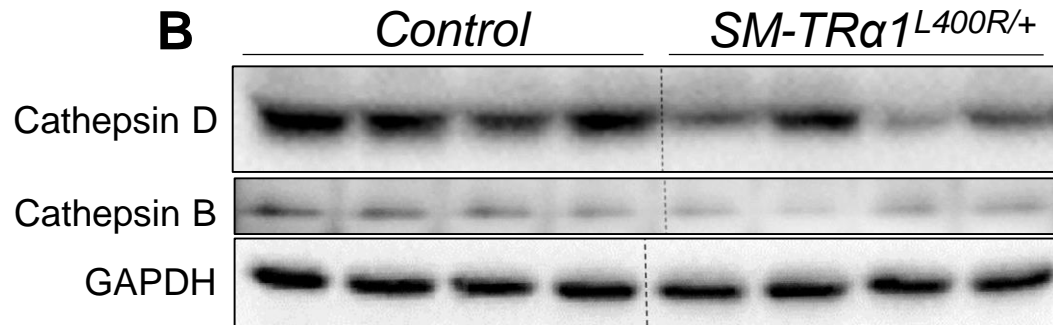
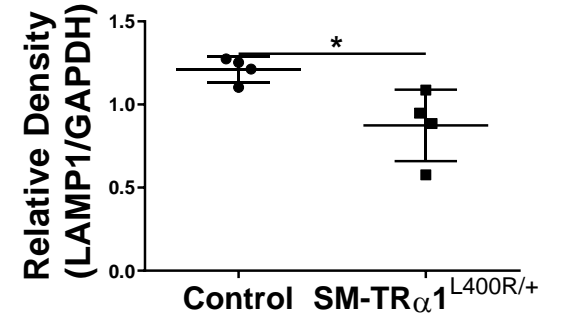
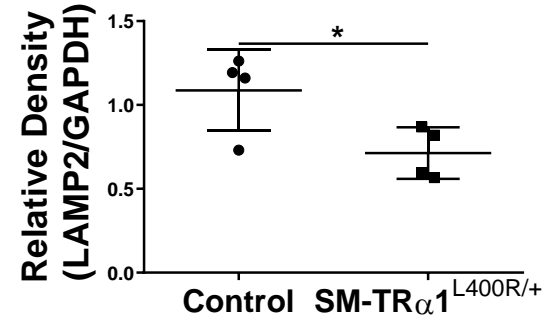
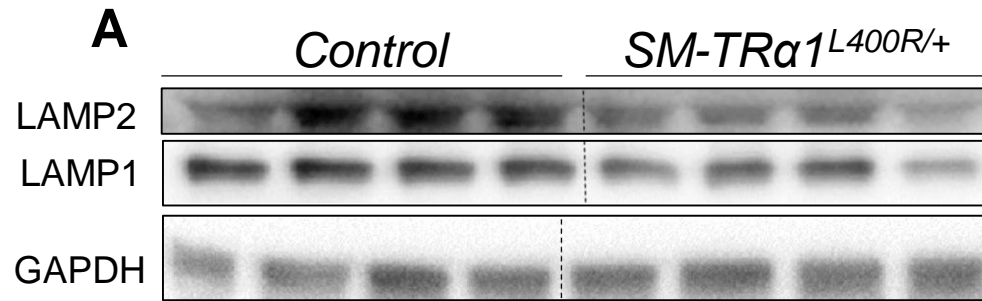


Figure 1



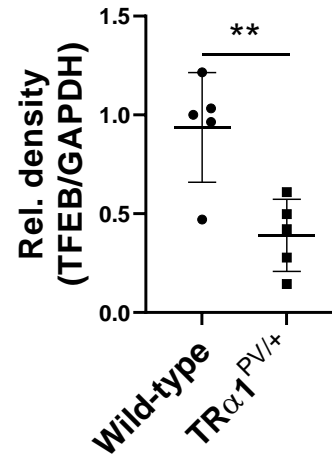
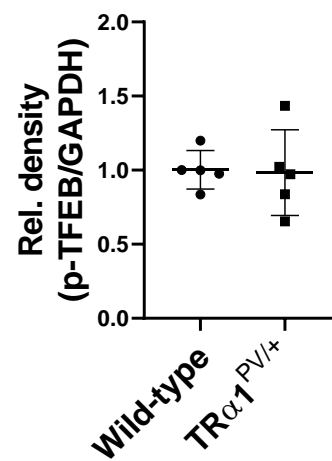
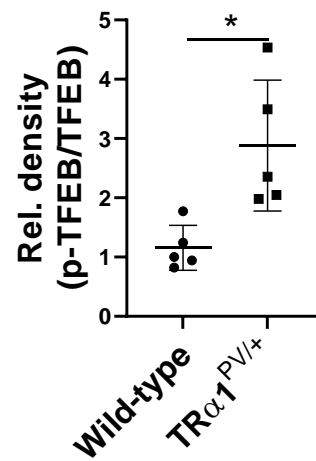
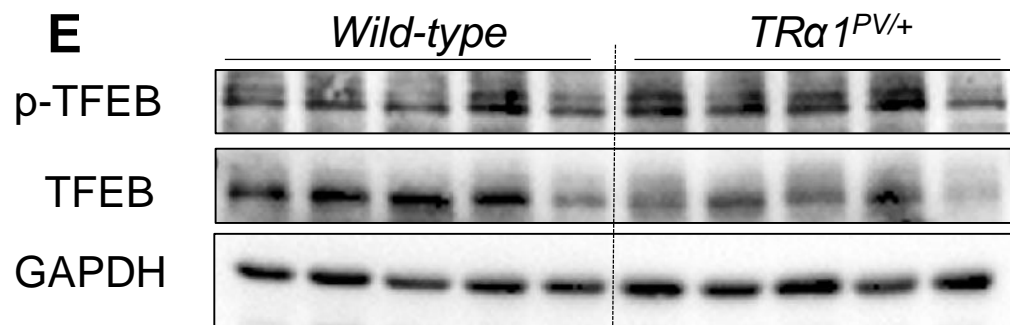
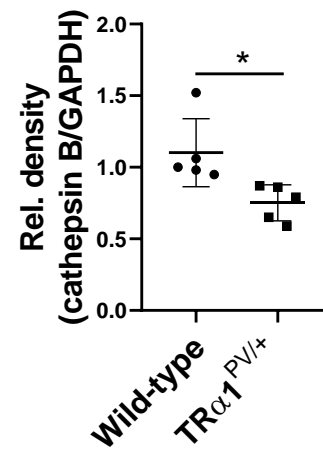
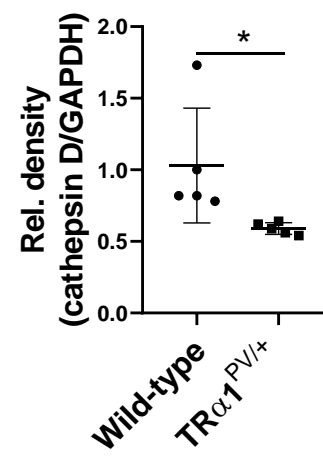
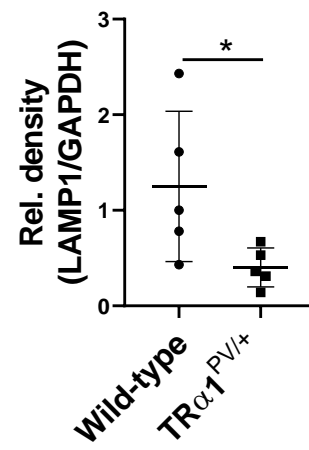
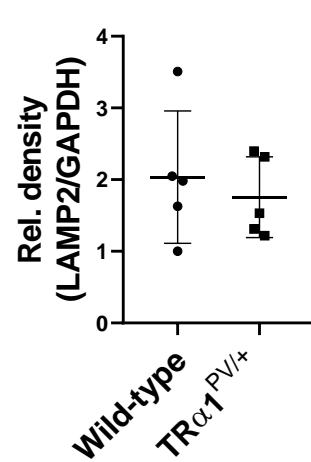
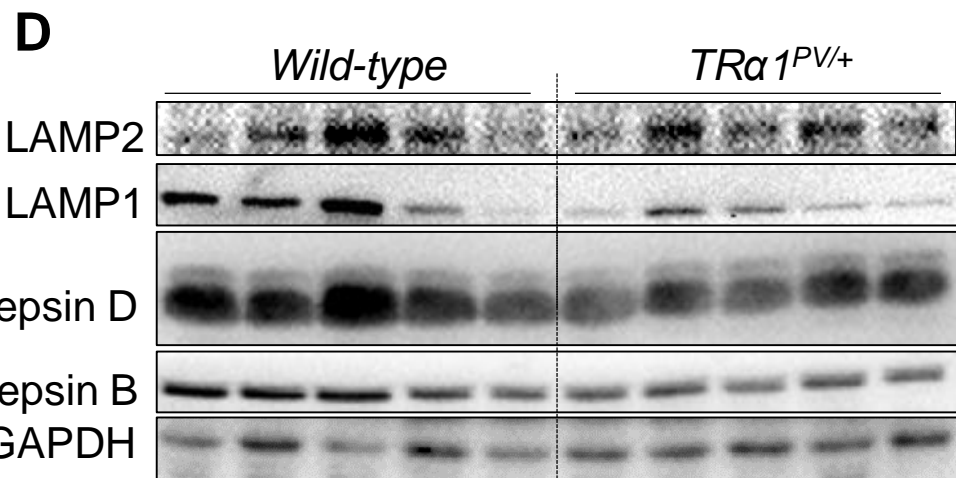


Figure 2

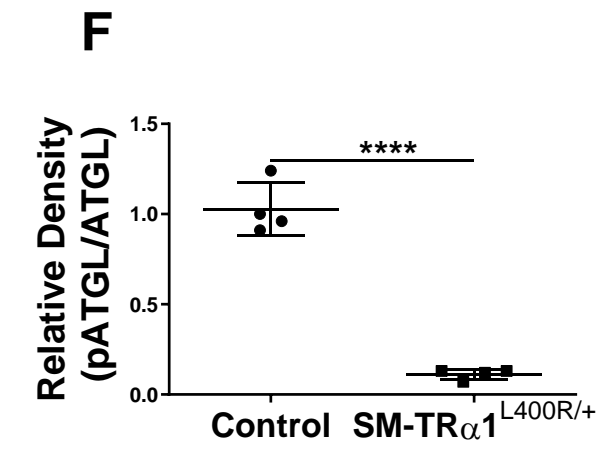
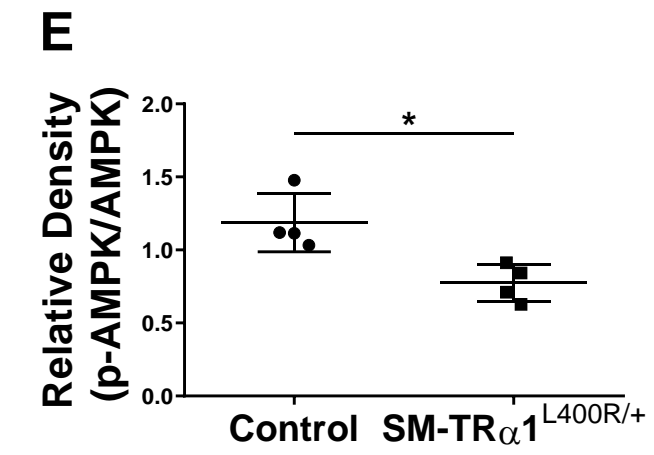
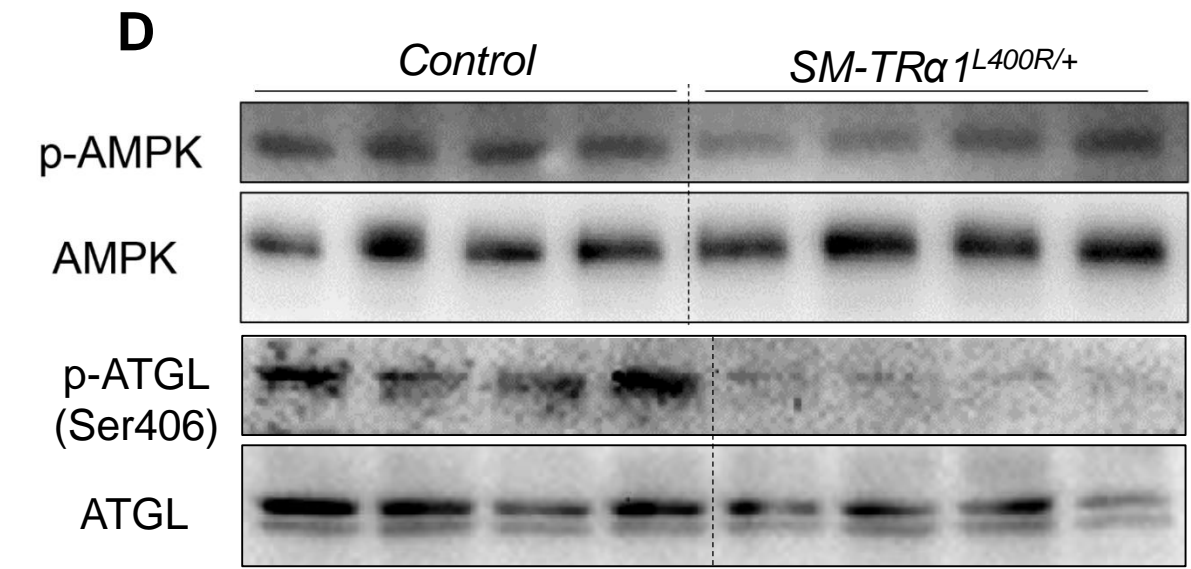
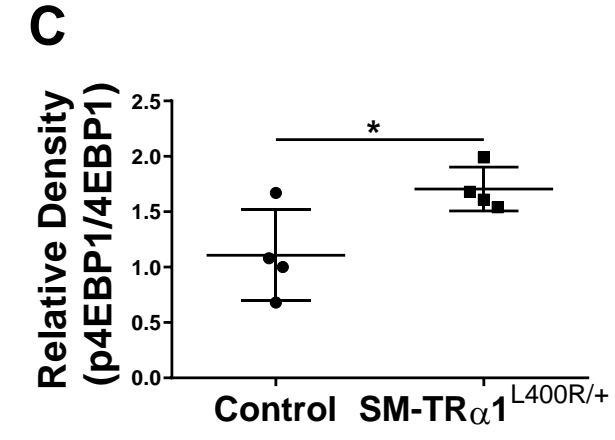
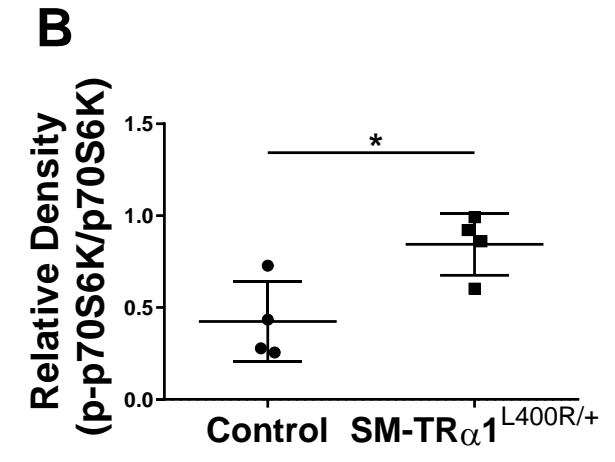
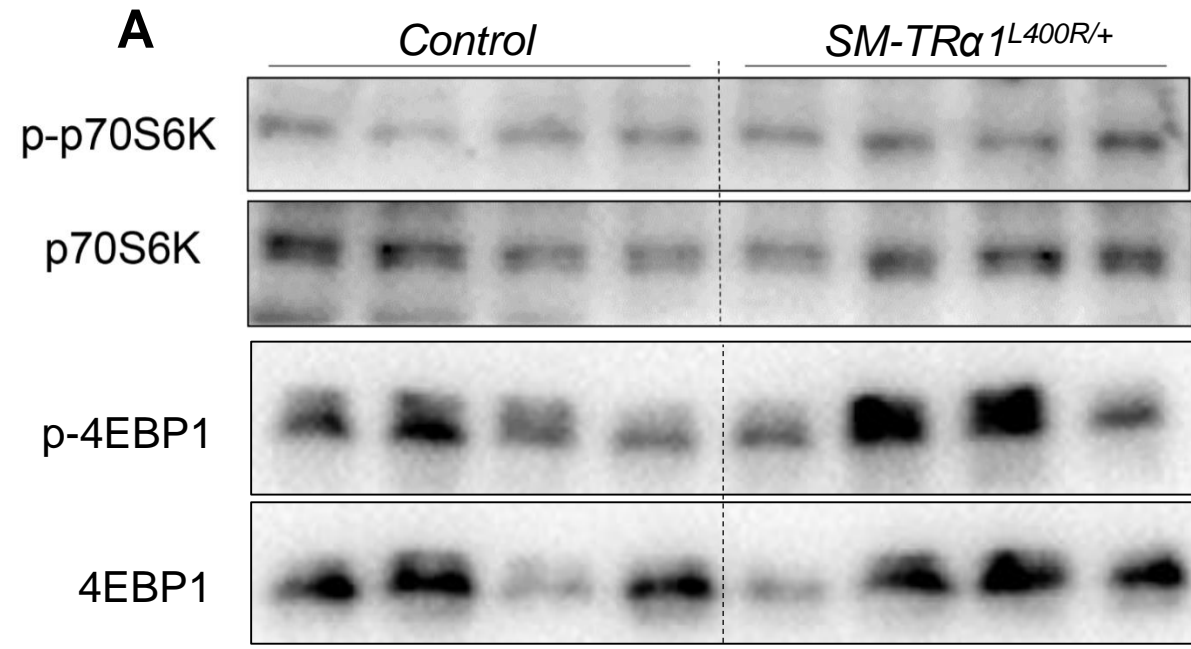


Figure 3



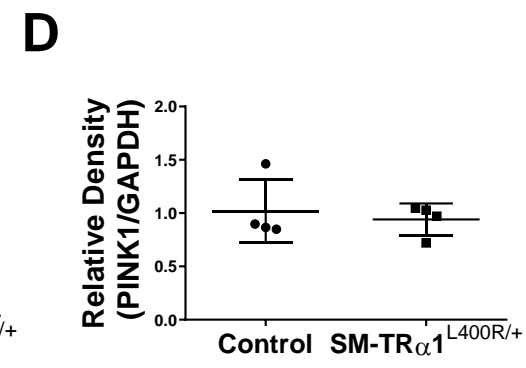
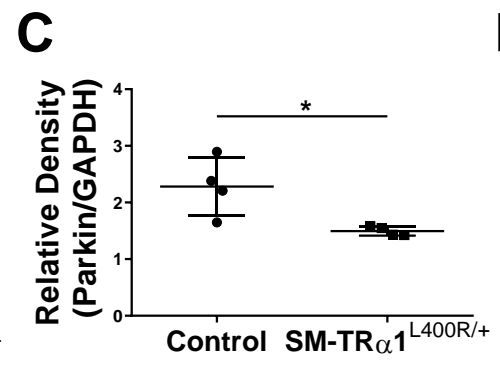
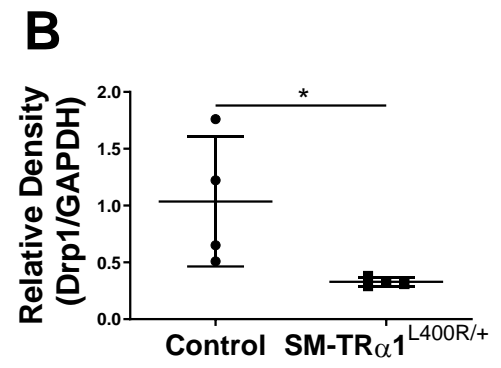
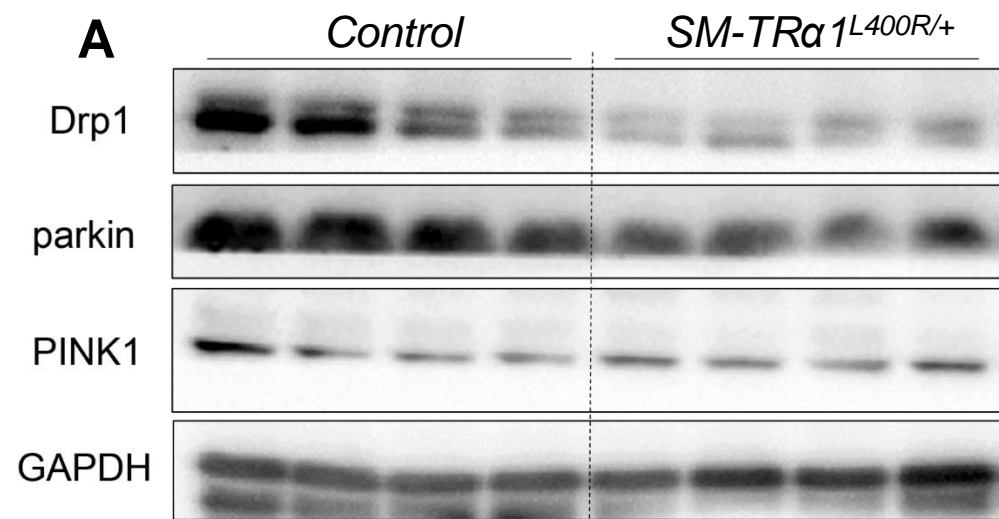
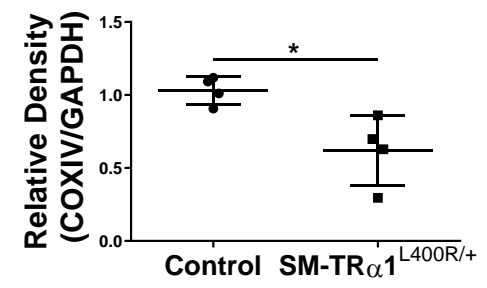
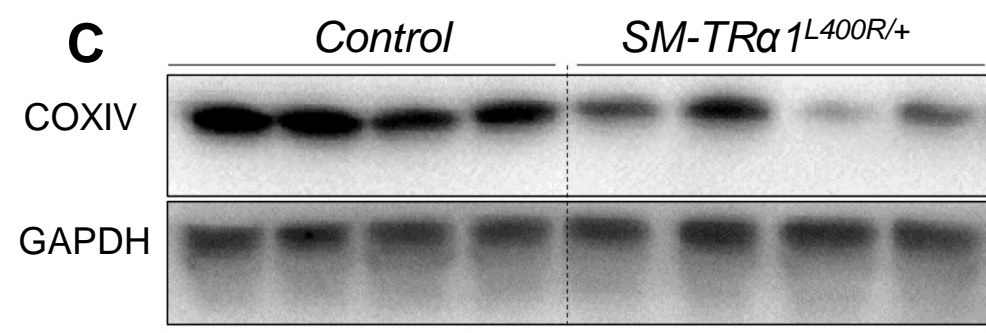
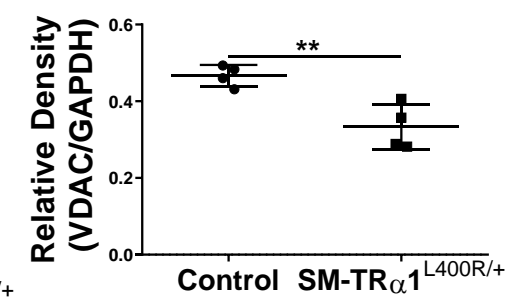
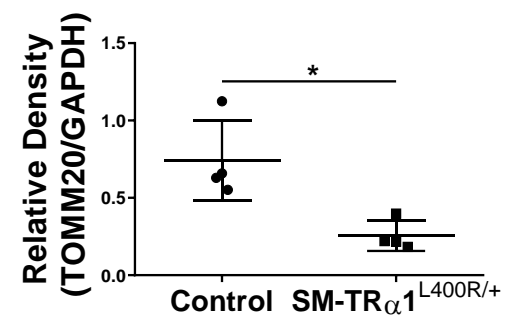
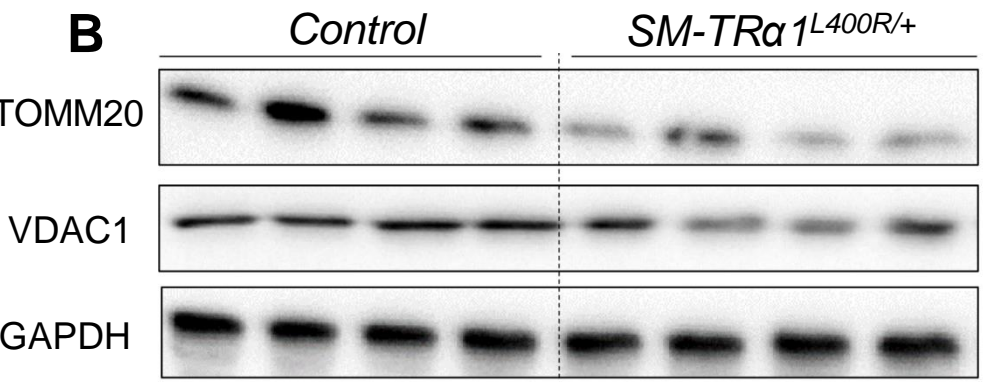
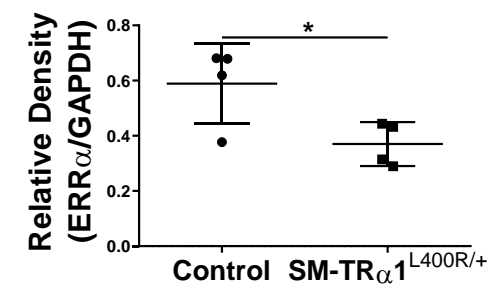
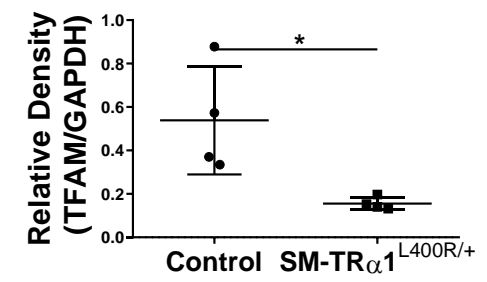
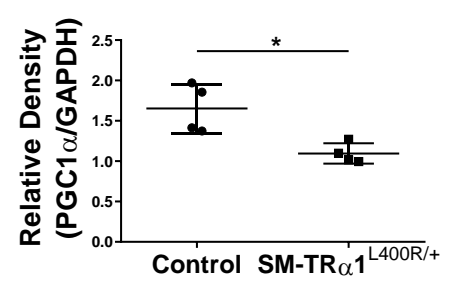
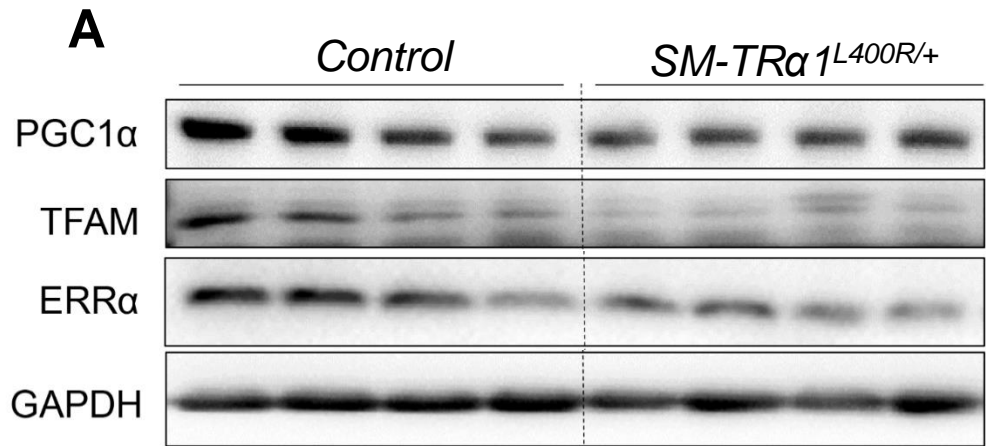
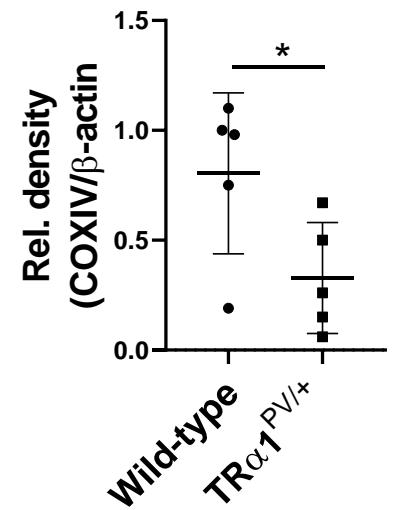
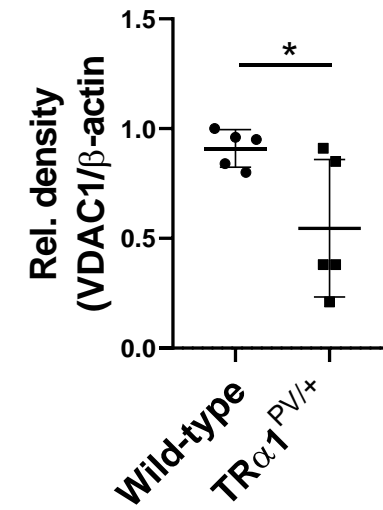
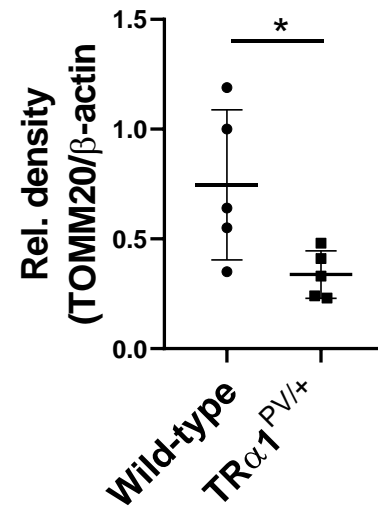
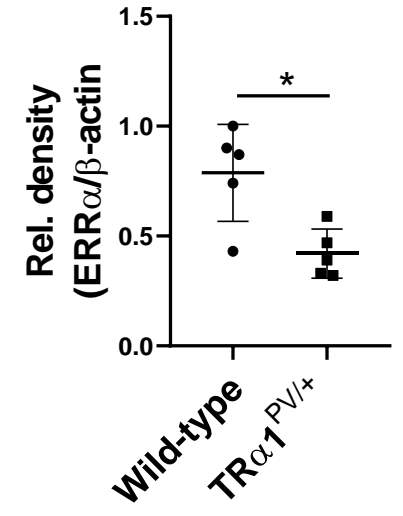
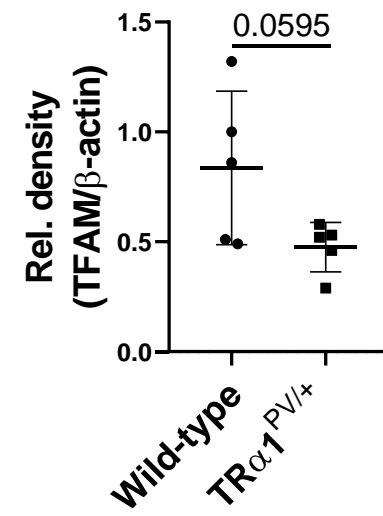
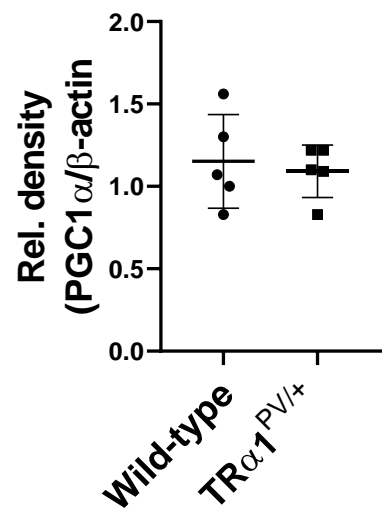
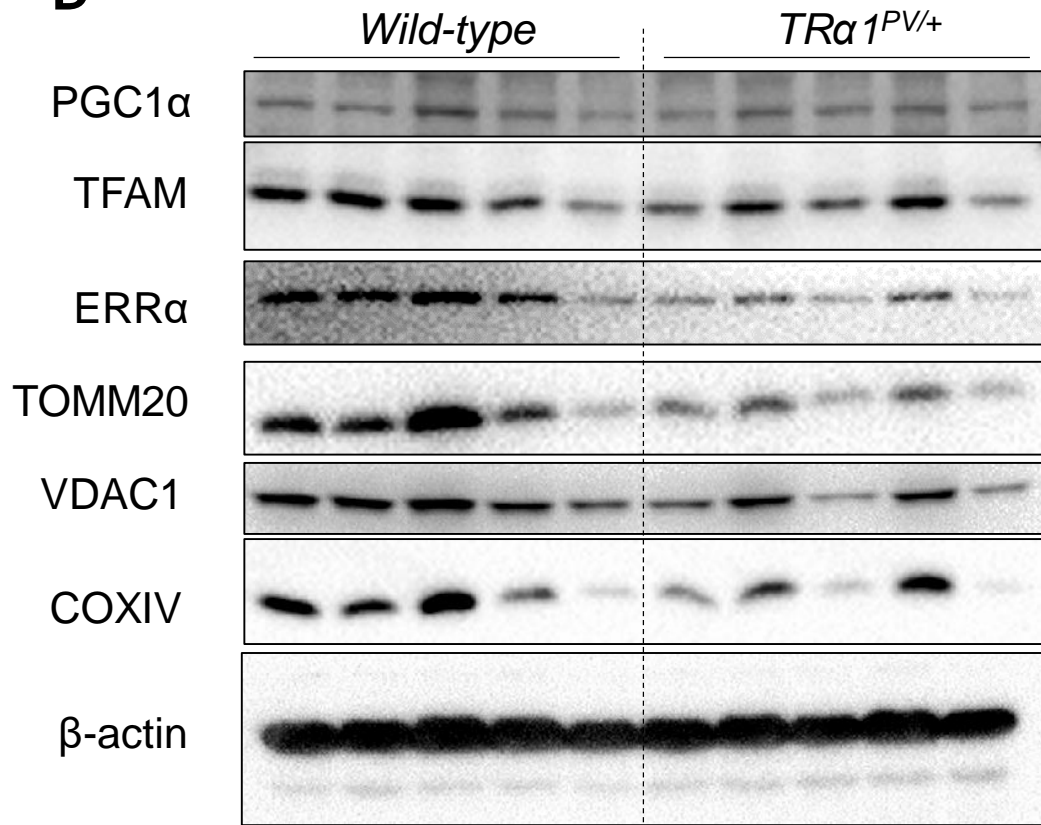


Figure 4



**D**

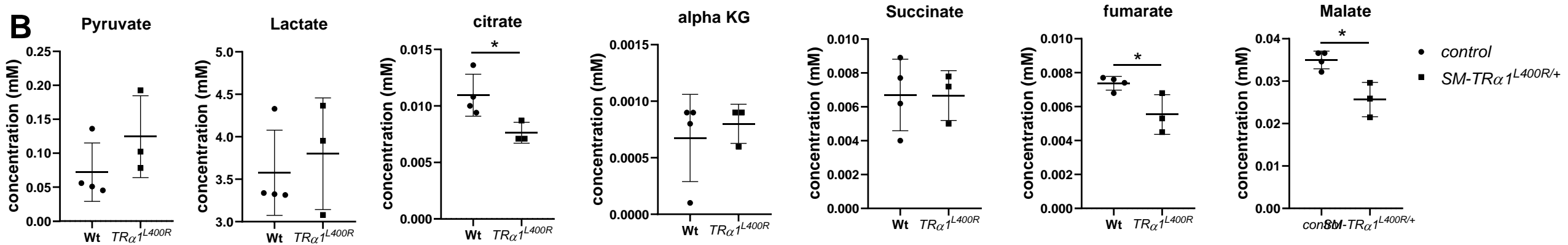
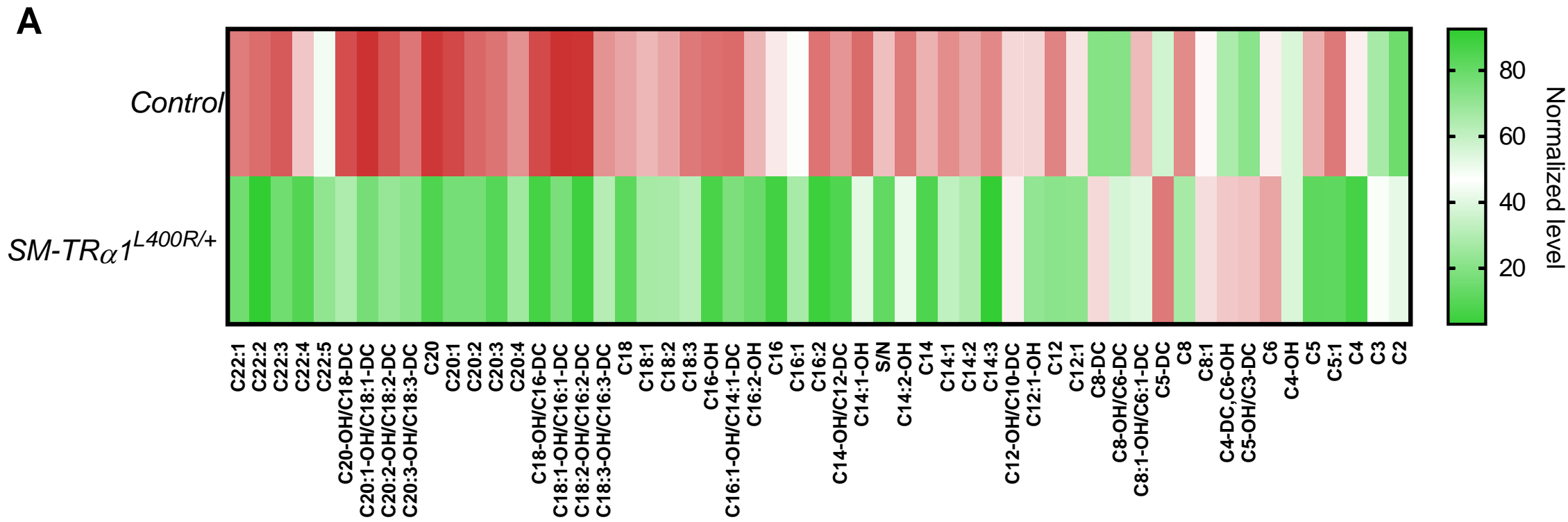
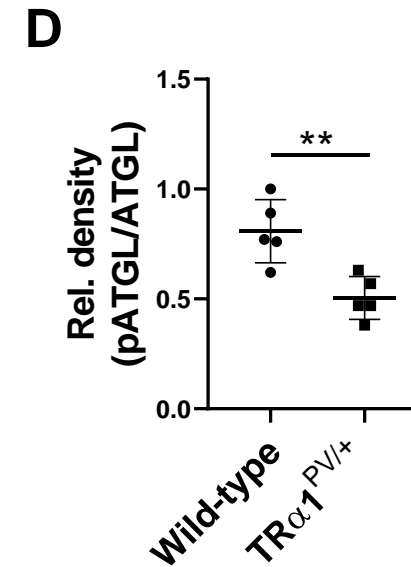
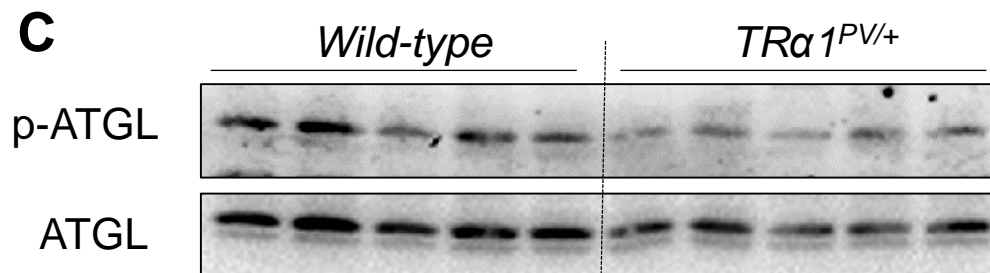
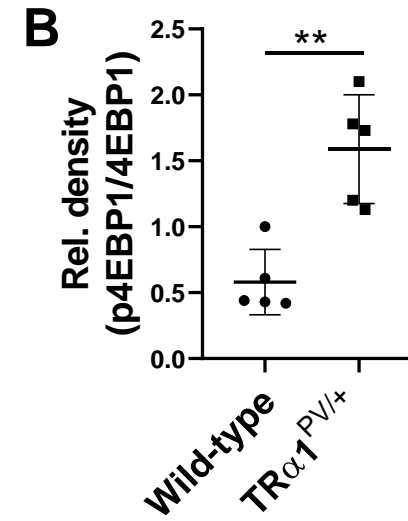
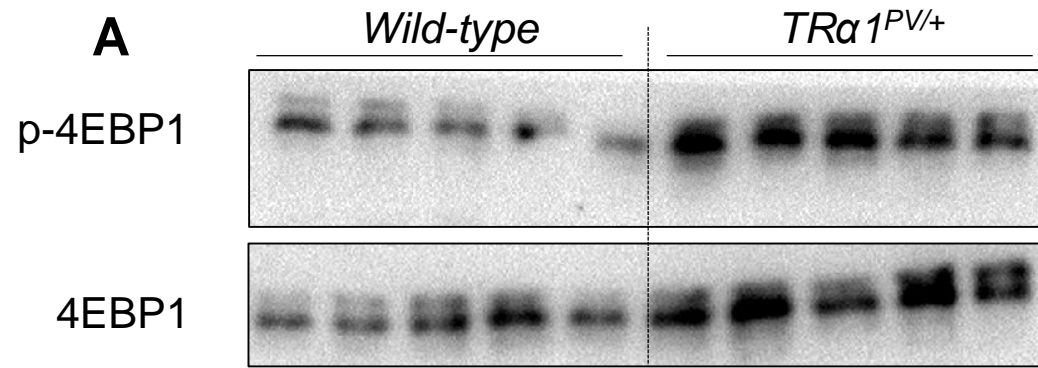
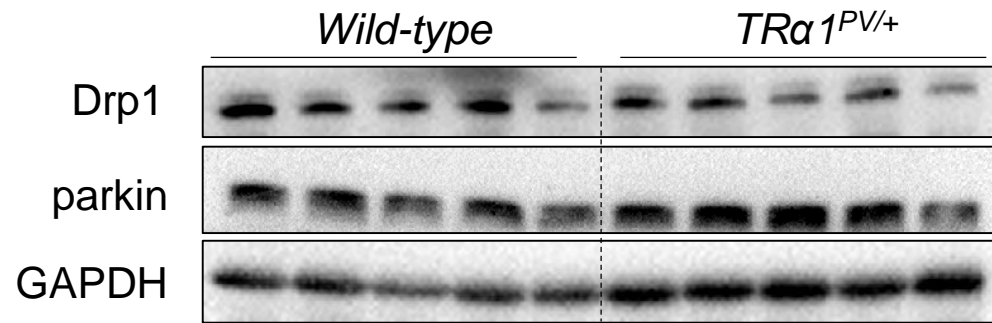
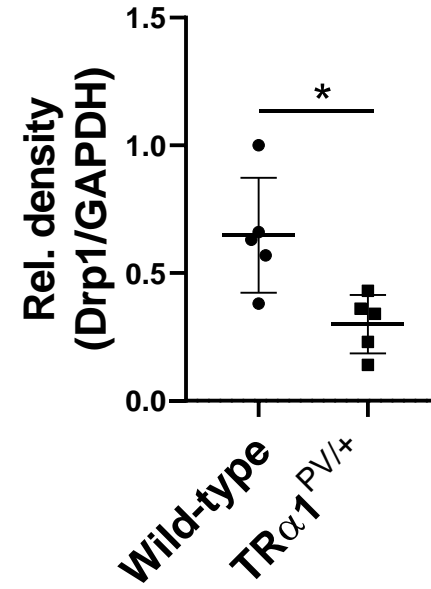
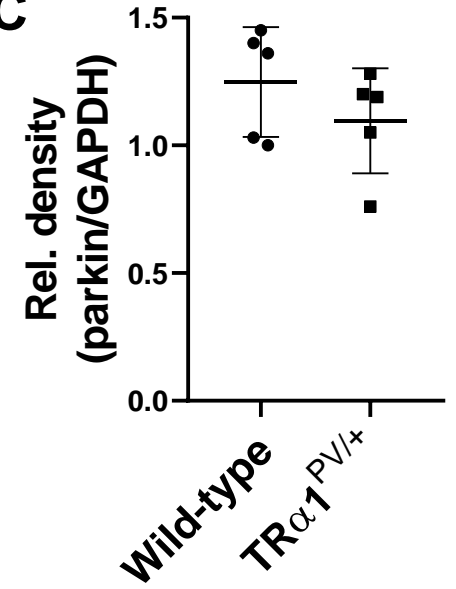
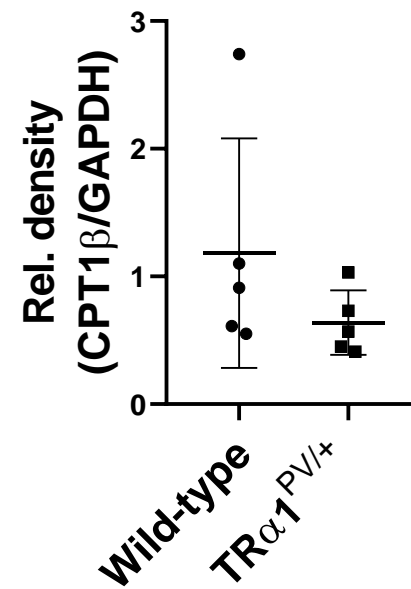
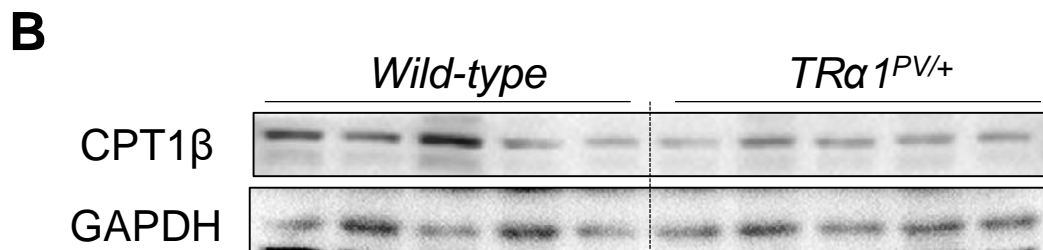
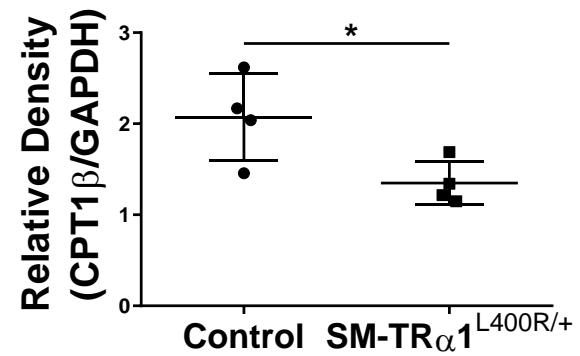
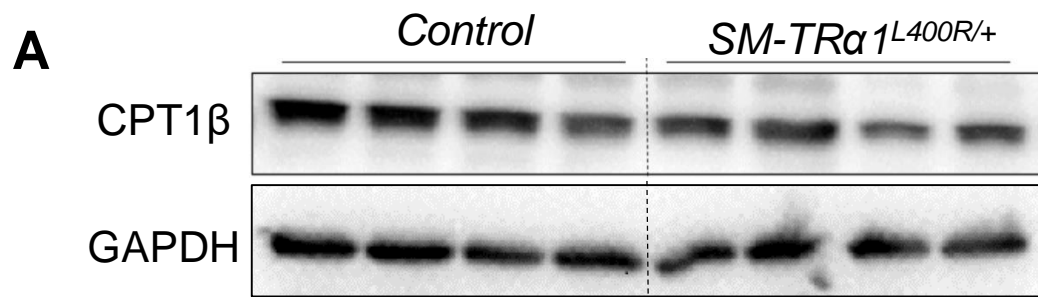
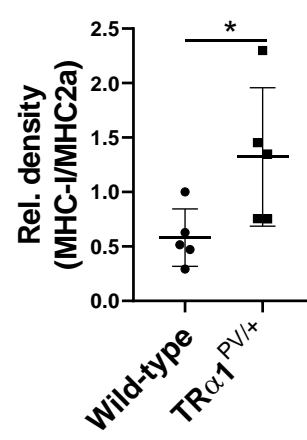
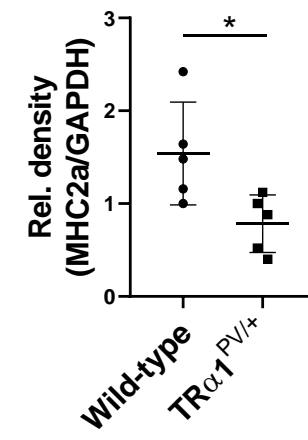
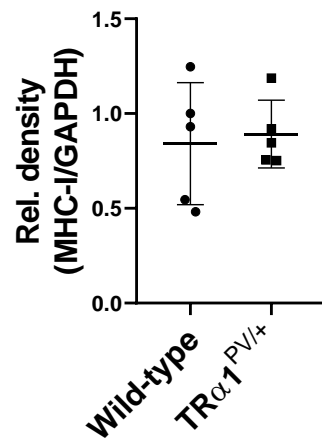
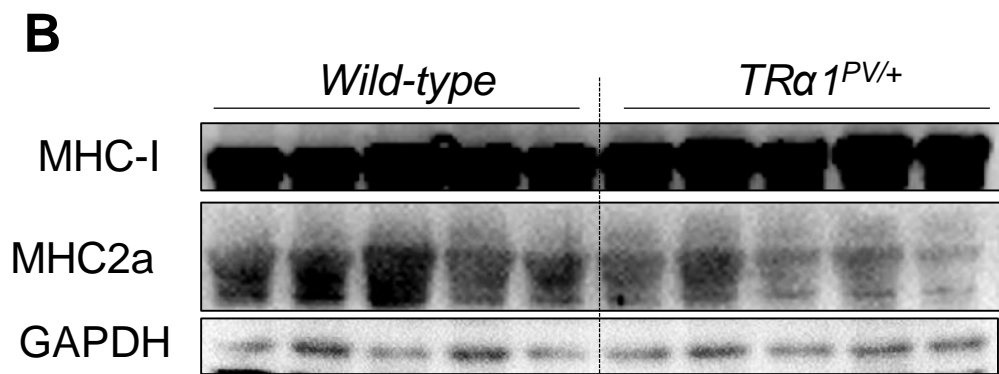
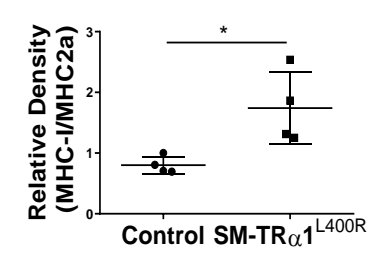
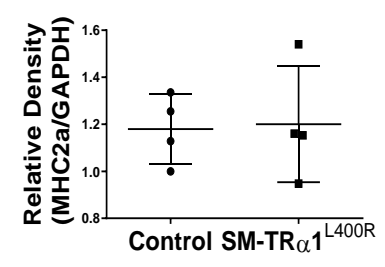
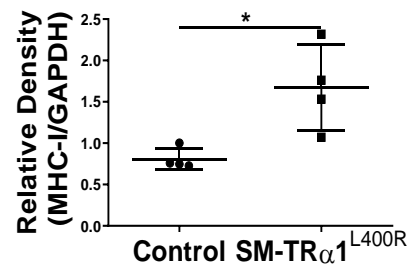
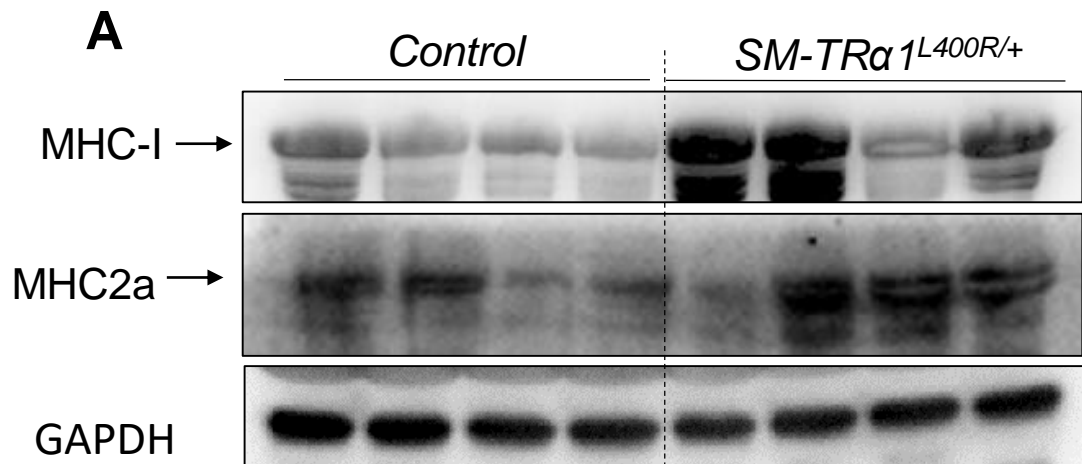


Figure 6



**A****B****C**





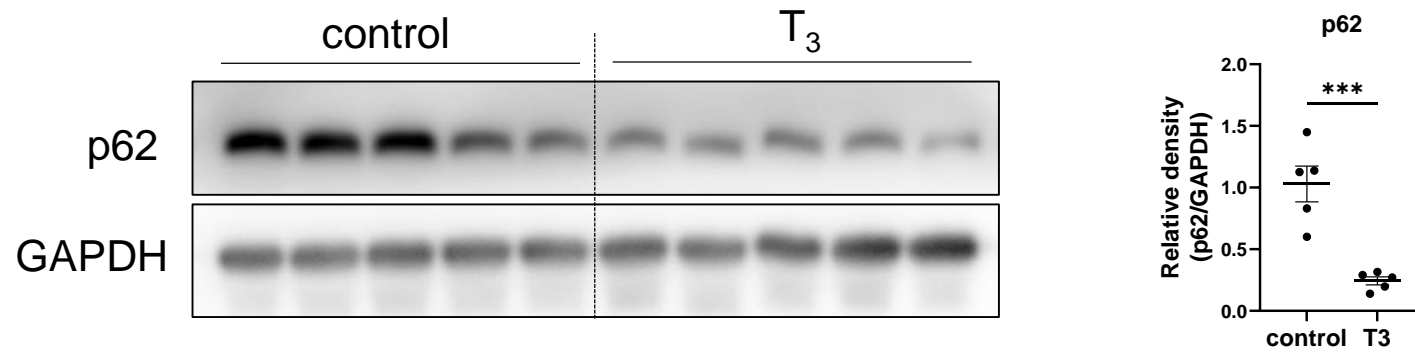


**Supplementary table 1. Antibody list**

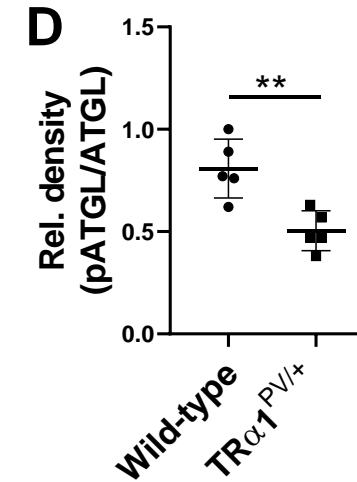
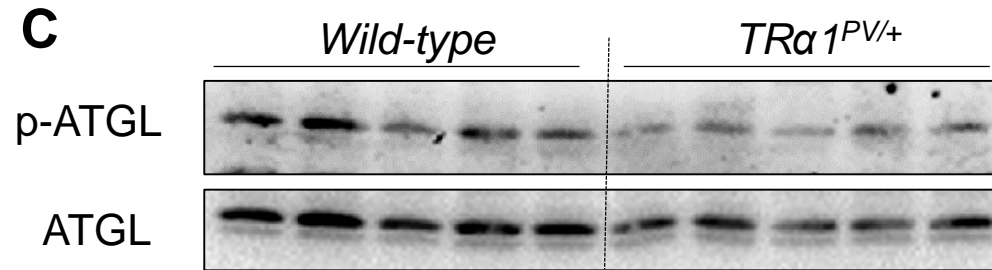
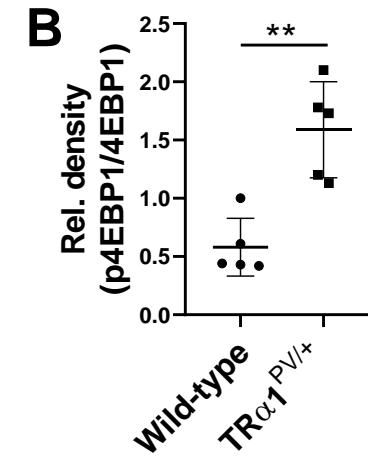
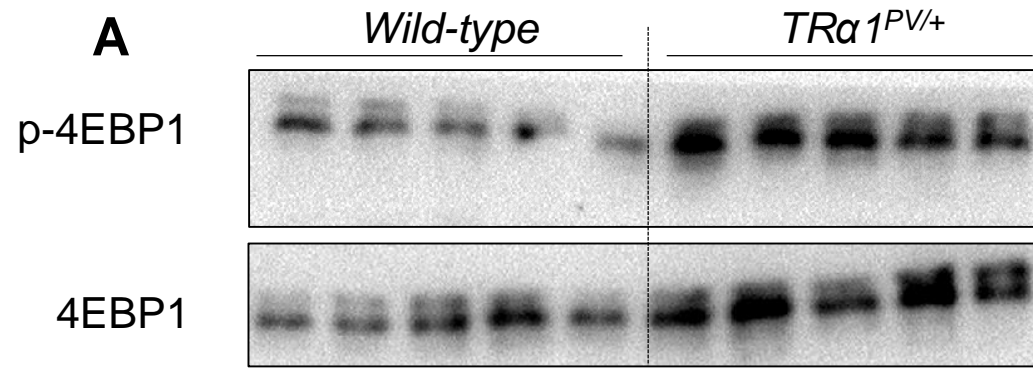
Antibody	Company	Catalogue number	Antibody	Company	Catalogue number
LC3B	Cell Signaling Technology	2775	cathepsin B	Santa Cruz Biotechnology	sc-13985
p62		5114	cathepsin D		sc-6486
AMPK		5831	$\beta$ -actin		sc-47778
p-AMPK (Thr172)		2535	p-ATGL (Ser406)	Abcam	ab135093
p-P70S6K (Thr389)		9205	ATGL		ab99532
P70S6K		9202	ERRa		07-662
4EBP1		9452	CPT1 $\beta$		ab134988
p-4EBP1 (Thr37/46)		2855	TFAM		ab131607
Tomm20		42406	PINK1		ab23707
COXIV		4850	TFEB		ab2636
Drp1		8570	p-TFEB (Ser142)	Millipore	ABE1971
parkin		4211	PGC1a	Sigma Chemical Co	SAB2500781
LAMP1		9091	LAMP2	Thermo Fisher Scientific	PA1-655
GAPDH		5174			
VDAC1		4661			

Supplementary Table 2. Pathway analysis of differentially expressed genes in the gastrocnemius muscle from *SM-TRα1<sup>L400R/+</sup>* vs. *Control* mice

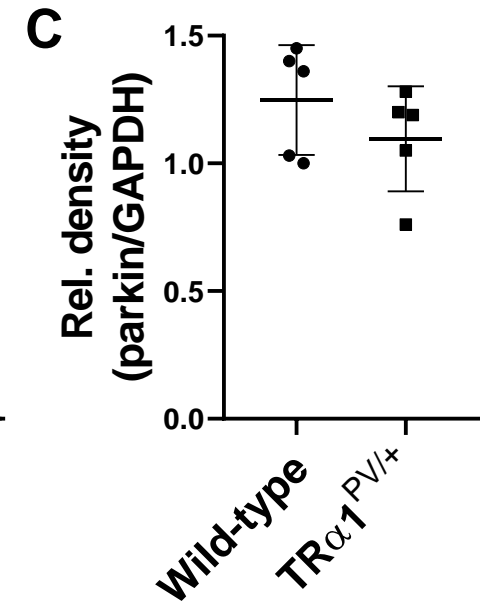
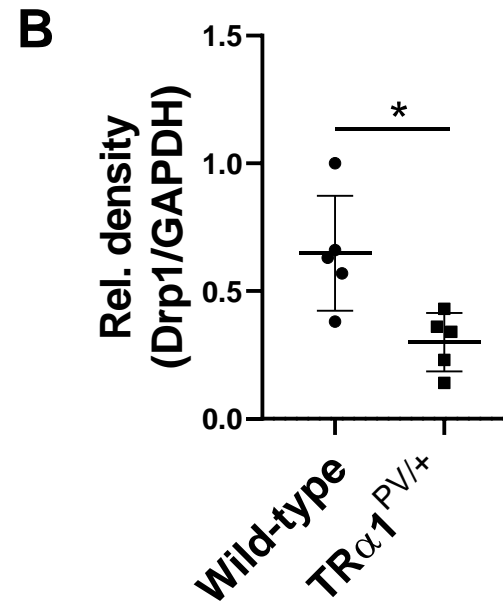
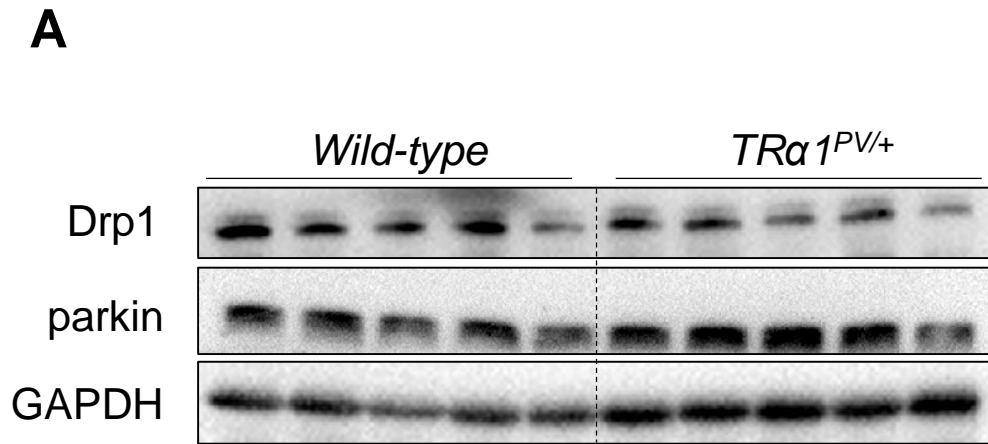
<i>SM-TRα1<sup>L400R/+</sup></i> <i>vs. Control</i>	Pathway ID	description	Adjusted p-value	Gene list
<b>Down-regulated</b>	GO:0046461	neutral lipid catabolic process	0.00074	Apob Ces1d Cps1 Apoa2 Apoc3 Apoa1
	GO:0032870	cellular response to hormone stimulus	0.001445	Aldob Rhoq Fgb Apob Acvr1 Nr6a1 Fyn Eif5a Nr3c1 Arg1 Rxrg Egr1 Junb Fos Cpeb2 Myo5a Uri1 Mef2c Plcb1 Cacna1h Jak2 Tsc1 Akt3 Cps1
	GO:0032368	regulation of lipid transport	0.012703	Acsl4 Mapk9 Apoa2 Abca7 Pla2g6 Apoa1 Apoc3 Atp8a1
	GO:0006639	acylglycerol metabolic process	0.001501	Apoa1 G6pc Agpat1 Apoc3 Cps1 Slc27a5 Acsl4 Apoa2 Apob Ces1d
	GO:0032409	regulation of transporter activity	0.013862	Myo5a Apoa2 Cbarp Camk2d Plcb1 Pla2g6 Mef2c Gpd1l Neto2 Kmt2a Cacng7 Wnk2
<b>Up-regulated</b>	GO:0005581	collagen	8.58E-08	Col4a2 Col5a2 Col11a2 Col11a1 Col1a1 Col5a1 Col5a3 Col15a1 Col6a1 Col6a3 Col12a1 Col3a1 Col4a1 Col14a1 Pcolce Col19a1 Col6a2 Col1a2
	GO:0071560	cellular response to transforming growth factor beta stimulus	2.31E-07	Nrep Peg10 Aspn Ankrd1 Smad5 Col1a1 Fbn1 Axin1 Smad2 Fn1
	GO:0046332	SMAD binding	1.33E-05	Ankrd1 Flna Acvr1 Mef2a Pml Smad7 Col5a2 Zeb2 Col3a1 Tcf12 Rnf111l Col1a2 Sma



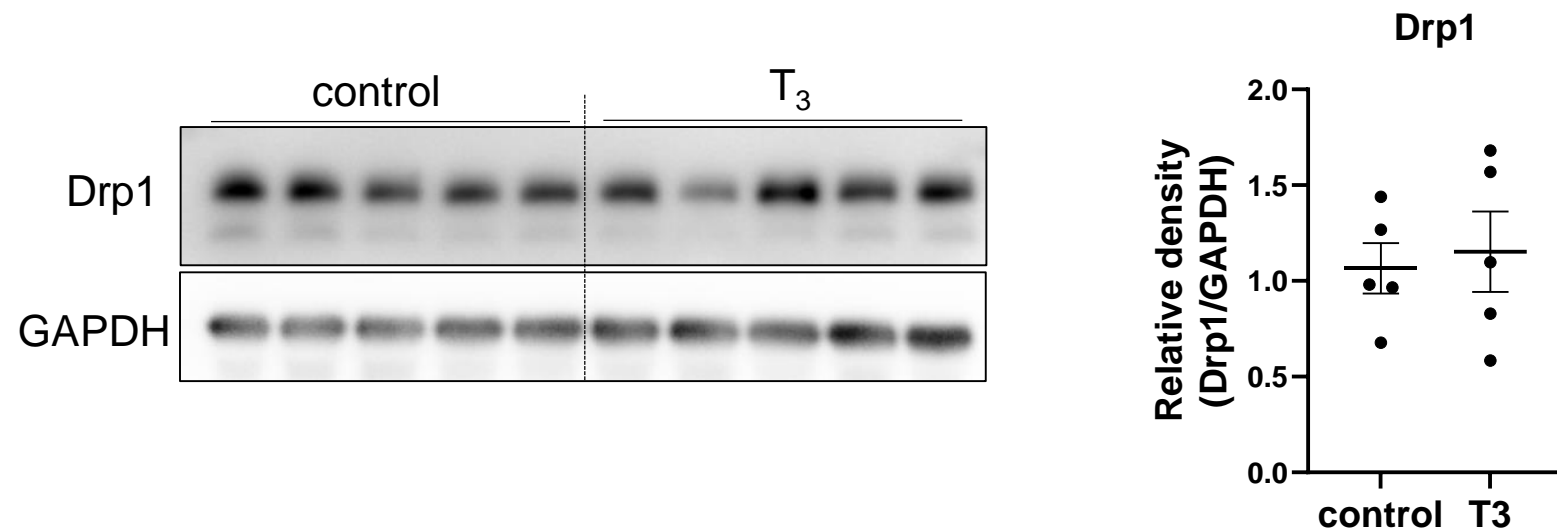
**Supplementary Figure 1.** T<sub>3</sub> decreased p62 in gastrocnemius muscle. Immunoblot and densitometric analysis of p62 in gastrocnemius muscle from control or T<sub>3</sub> injected wildtype male C57BL/6 mice. T<sub>3</sub> was given through intraperitoneal injection at a dose of 20 µg/100g body weight for 10 consecutive days.



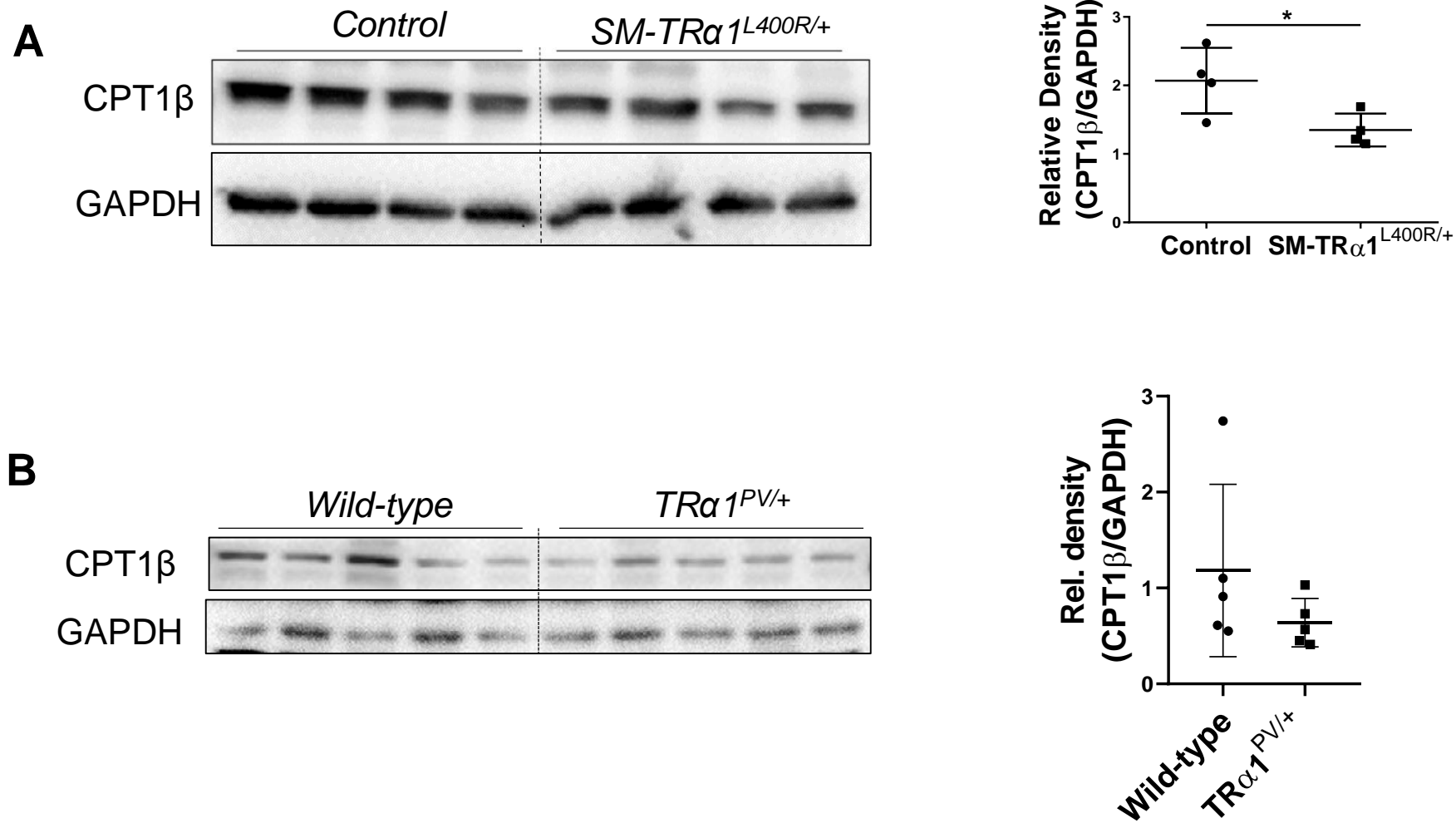
**Supplementary Figure 2.**  $TR\alpha 1^{PV}$  expression increased phosphorylation of 4EBP1, and decreased phosphorylation of ATGL. (A-B) Immunoblot (A) and densitometric analysis (B) showing phosphorylation of 4EBP1 and 4EBP1 in gastrocnemius muscle from *wild-type* (n=5) and  $TR\alpha 1^{PV/+}$  (n=5) mice. (C-D) Immunoblot (C) and densitometric analysis (D) showing phosphorylation ATGL in gastrocnemius muscle from *wild-type* (n=5) and  $TR\alpha 1^{PV/+}$  (n=5) mice. Data are shown as mean $\pm$ SD.



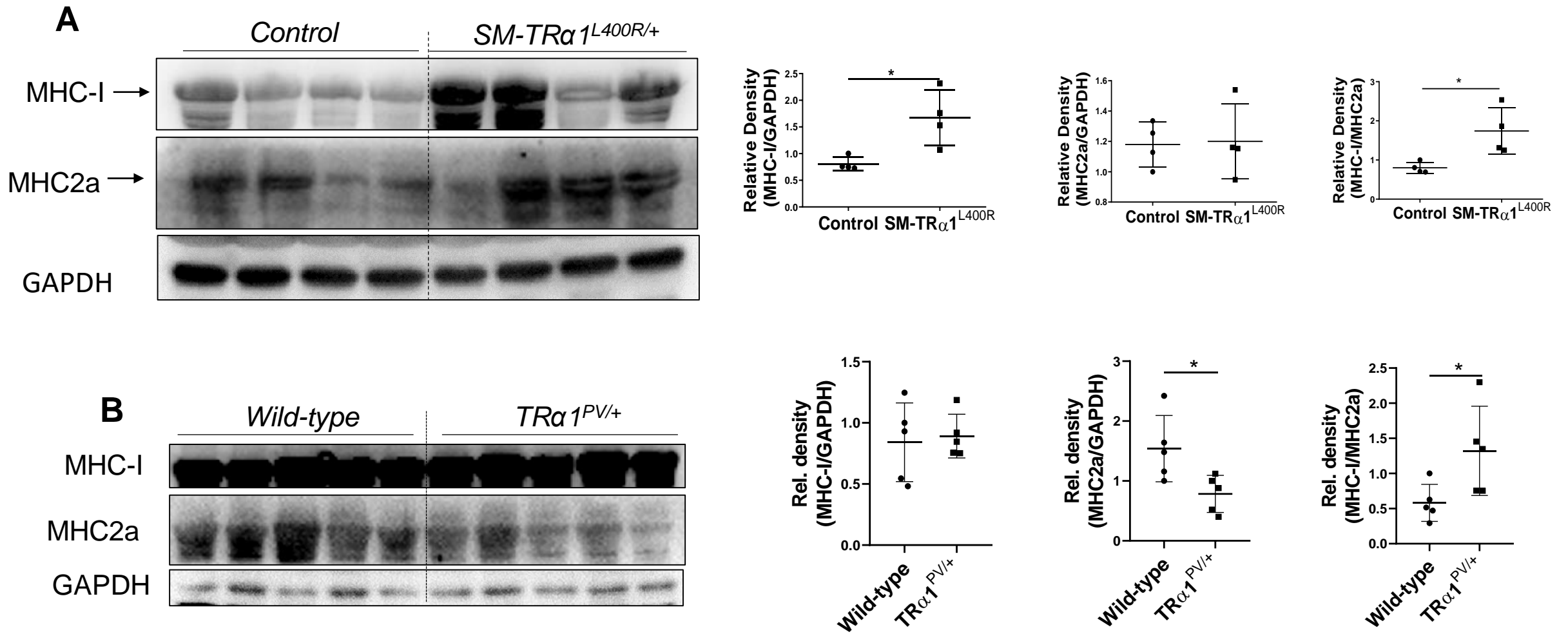
**Supplementary Figure 3.**  $TR\alpha 1^{PV}$  expression decreased protein level of Drp1 but not parkin. (A-C) Immunoblot (A) and densitometric analysis (B-C) showing protein level of Drp1 and parkin in gastrocnemius muscle from *wild-type* (n=5) and  $TR\alpha 1^{PV/+}$  (n=5) mice. Data are shown as mean $\pm$ SD.



**Supplementary Figure 4.** T<sub>3</sub> injection didn't affect Drp1 in gastrocnemius muscle from wildtype C57BL/6. Immunoblot and densitometric analysis of Drp1 in gastrocnemius muscle from control or T<sub>3</sub> injected wildtype male C57BL/6 mice. T<sub>3</sub> was given through intraperitoneal injection at a dose of 20 µg/100g body weight for 10 consecutive days.



**Supplementary Figure 5.** The effect of mutant TR $\alpha$ 1 expression on protein level of CPT1 $\beta$ . (A) Immunoblot and densitometric analysis showing protein level of CPT1 $\beta$  in gastrocnemius muscle from *Control* (n=4) and *SM-TR $\alpha$ 1<sup>L400R/+</sup>* (n=4) mice. (B) Immunoblot and densitometric analysis showing protein levels of CPT1 $\beta$  in gastrocnemius muscle from *wild-type* (n=5) and *TR $\alpha$ 1<sup>PV/+</sup>* (n=5) mice. Data are shown as mean $\pm$ SD.



**Supplementary Figure 6.** Protein levels of MHC-I and MHC2a in gastrocnemius muscle. (A) Immunoblot and densitometric analysis showing protein levels of MHC-I and MHC2a in gastrocnemius muscle from *Control* (n=4) and *SM-TR $\alpha$ 1<sup>L400R/+</sup>* (n=4) mice (A) or wild-type (n=5) and *TR $\alpha$ 1<sup>PV/+</sup>* (n=5) mice (B). Data are shown as mean $\pm$ SD.



**Supplementary table 1. Antibody list**

Antibody	Company	Catalogue number	Antibody	Company	Catalogue number
LC3B	Cell Signaling Technology	2775	cathepsin B	Santa Cruz Biotechnology	sc-13985
p62		5114	cathepsin D		sc-6486
AMPK		5831	$\beta$ -actin		sc-47778
p-AMPK (Thr172)		2535	p-ATGL (Ser406)	Abcam	ab135093
p-P70S6K (Thr389)		9205	ATGL		ab99532
P70S6K		9202	ERRa		07-662
4EBP1		9452	CPT1 $\beta$		ab134988
p-4EBP1 (Thr37/46)		2855	TFAM		ab131607
Tomm20		42406	PINK1		ab23707
COXIV		4850	TFEB		ab2636
Drp1		8570	p-TFEB (Ser142)	Millipore	ABE1971
parkin		4211	PGC1a	Sigma Chemical Co	SAB2500781
LAMP1		9091	LAMP2	Thermo Fisher Scientific	PA1-655
GAPDH		5174			
VDAC1		4661			

**Supplementary Table 2.** Pathway analysis of differentially expressed genes in the gastrocnemius muscle from *SM-TRα1<sup>L400R/+</sup>* VS. *Control* mice

<i>SM-TRα1<sup>L400R/+</sup></i> vs. <i>Control</i>	Pathway ID	description	Adjusted p-value	Gene list
<b>Down-regulated</b>	GO:0046461	neutral lipid catabolic process	0.00074	Apob Ces1d Cps1 Apoa2 Apoc3 Apoa1
	GO:0032870	cellular response to hormone stimulus	0.001445	Aldob Rhoq Fgb Apob Acvr1 Nr6a1 Fyn Eif5a Nr3c1 Arg1 Rxrg Egr1 Junb Fos Cpeb2 Myo5a Uri1 Mef2c Plcb1 Cacna1h Jak2 Tsc1 Akt3 Cps1
	GO:0032368	regulation of lipid transport	0.012703	Acsl4 Mapk9 Apoa2 Abca7 Pla2g6 Apoa1 Apoc3 Atp8a1
	GO:0006639	acylglycerol metabolic process	0.001501	Apoa1 G6pc Agpat1 Apoc3 Cps1 Slc27a5 Acsl4 Apoa2 Apob Ces1d
	GO:0032409	regulation of transporter activity	0.013862	Myo5a Apoa2 Cbarp Camk2d Plcb1 Pla2g6 Mef2c Gpd1 Neto2 Kmt2a Cacng7 Wnk2
<b>Up-regulated</b>	GO:0005581	collagen	8.58E-08	Col4a2 Col5a2 Col11a2 Col11a1 Col1a1 Col5a1 Col5a3 Col15a1 Col6a1 Col6a3 Col12a1 Col3a1 Col4a1 Col14a1 Pcolce Col19a1 Col6a2 Col1a2
	GO:0071560	cellular response to transforming growth factor beta stimulus	2.31E-07	Nrep Peg10 Aspn Ankrd1 Smad5 Col1a1 Fbn1 Axin1 Smad2 Fn1
	GO:0046332	SMAD binding	1.33E-05	Ankrd1 Flna Acvr1 Mef2a Pml Smad7 Col5a2 Zeb2 Col3a1 Tcf12 Rnf111 Col1a2 Smad2 Axin1
	GO:0014888	striated muscle adaptation	1.20E-06	Gtf2ird1 Tead1 Tnni1 Tnnc1 Kdm4a Atp2a2 Ppargc1a Myh7 Cacna1s Gsn Tnnt1
	GO:0060415	muscle tissue morphogenesis	1.85E-05	Rbpj Tnnc1 Ankrd1 Tnni1 Col11a1 Rxra Myh7 Smad7 Tbx1 Myl2 Myl3 Col3a1

## Supplementary figure legends

**Supplementary Figure 1.** T<sub>3</sub> decreased p62 in gastrocnemius muscle. Immunoblot and densitometric analysis of p62 in gastrocnemius muscle from control or T<sub>3</sub> injected wildtype male C57BL/6 mice. T<sub>3</sub> was given through intraperitoneal injection at a dose of 20 µg/100g body weight for 10 consecutive days.

**Supplementary Figure 2.** TRα1<sup>PV</sup> expression increased phosphorylation of 4EBP1, and decreased phosphorylation of ATGL. (A-B) Immunoblot (A) and densitometric analysis (B) showing phosphorylation of 4EBP1 and 4EBP1 in gastrocnemius muscle from *wild-type* (n=5) and TRα1<sup>PV/+</sup> (n=5) mice. (C-D) Immunoblot (C) and densitometric analysis (D) showing phosphorylation ATGL in gastrocnemius muscle from *wild-type* (n=5) and TRα1<sup>PV/+</sup> (n=5) mice. Data are shown as mean±SD.

**Supplementary Figure 3.** TRα1<sup>PV</sup> expression decreased protein level of Drp1 but not parkin. (A-C) Immunoblot (A) and densitometric analysis (B-C) showing protein level of Drp1 and parkin in gastrocnemius muscle from *wild-type* (n=5) and TRα1<sup>PV/+</sup> (n=5) mice. Data are shown as mean±SD.

**Supplementary Figure 4.** T<sub>3</sub> did not affect Drp1 in gastrocnemius muscle. Immunoblot and densitometric analysis of Drp1 in gastrocnemius muscle from control or T<sub>3</sub> injected wildtype male C57BL/6 mice. T<sub>3</sub> was given through intraperitoneal injection at a dose of 20 µg/100g body weight for 10 consecutive days.

**Supplementary Figure 5.** The effect of mutant TRα1 expression on protein level of CPT1β. (A) Immunoblot and densitometric analysis showing protein level of CPT1β in gastrocnemius muscle from *Control* (n=4) and *SM-TRα1<sup>L400R/+</sup>* (n=4) mice. (B) Immunoblot and densitometric analysis showing protein levels of CPT1β in gastrocnemius muscle from *wild-type* (n=5) and TRα1<sup>PV/+</sup> (n=5) mice. Data are shown as mean±SD.

**Supplementary Figure 6.** Protein levels of MHC-I and MHC2a in gastrocnemius muscle. (A) Immunoblot and densitometric analysis showing protein levels of MHC-I and MHC2a in gastrocnemius muscle from *Control* (n=4) and *SM-TR $\alpha$ 1<sup>L400R/+</sup>* (n=4) mice (A) or wild-type (n=5) and *TR $\alpha$ 1<sup>PV/+</sup>* (n=5) mice (B). Data are shown as mean $\pm$ SD.