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Maladaptative autophagy impairs adipose function in Congenital Generalized Lipodystrophy due to cavin-1 deficiency

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► **To cite this version:**

Laurence Salle-Teyssières, Martine Auclair, Faraj Terro, Mona Nemani, Solaf M Elsayed, et al.. Maladaptative autophagy impairs adipose function in Congenital Generalized Lipodystrophy due to cavin-1 deficiency. *Journal of Clinical Endocrinology and Metabolism*, 2016, 10.1210/jc.2016-1086 . hal-01318089

HAL Id: hal-01318089

<https://hal.sorbonne-universite.fr/hal-01318089>

Submitted on 19 May 2016

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1 **Maladaptative autophagy impairs adipose function**
2 **in Congenital Generalized Lipodystrophy due to cavin-1 deficiency**

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14 Québec Innovation Centre, Montréal, Canada (M.L.), Commissariat à l’Energie Atomique/ Institut de
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18
19 **Abbreviated title:** *PTRF* mutations and maladaptative autophagy

20 **Key terms:** *PTRF*, cavin-1, lipodystrophy, insulin resistance, autophagy, adipocyte differentiation

21 **Word count:** Main text, 3798 words; Figures, 5; Table, 1; References, 43

22
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24 médecine Pierre et Marie Curie, 27, rue Chaligny, 75012 Paris, France

25
26 **Funding sources:** This work was supported by grants from the French ‘Institut National de la Santé et
27 de la Recherche Médicale’ (Inserm), ‘Aide aux Jeunes Diabétiques’ and ‘Société Francophone du
28 Diabète’. Laurence Salle-Teyssières was the recipient of a master grant from the Limousin Regional

29 Health Agency and Mona Nemani received grants from ‘Région Ile-de-France’ and ‘Aide aux Jeunes
30 Diabétiques’.

31

32 **Disclosure statement:** The authors have nothing to disclose.

33

34 **Precis:**

35 Two new homozygous *PTRF* mutations associated with Congenital Generalized Lipodystrophy induce
36 cellular maladaptative autophagy resulting in insulin resistance and altered adipocyte differentiation.

37

38 **Abstract**

39

40 *Context:* Mutations in *PTRF* encoding cavin-1 are responsible for congenital generalized
41 lipodystrophy type 4 (CGL4) characterized by lipoatrophy, insulin resistance, dyslipidemia and
42 muscular dystrophy. Cavin-1 cooperates with caveolins to form the plasma membrane caveolae,
43 involved in cellular trafficking and signalling and in lipid turnover.

44 *Objective :* We sought to identify *PTRF* mutations in patients with CGL and to determine their impact
45 on insulin sensitivity, adipose differentiation and cellular autophagy.

46 *Design and patients :* We performed phenotyping studies and molecular screening of *PTRF* in two
47 unrelated families with CGL. Cellular studies were conducted in cultured skin fibroblasts from the two
48 probands and from control subjects, and in murine 3T3-F442A preadipocytes. Knockdown of cavin-1
49 or ATG5 was obtained by siRNA-mediated silencing.

50 *Results:* We identified two new *PTRF* homozygous mutations (p.Asp59Val or p.Gln157Hisfs*52) in
51 four patients with CGL4 presenting with generalized lipoatrophy and associated metabolic
52 abnormalities. In probands' fibroblasts, cavin-1 expression was undetectable and caveolin-1 and -2
53 barely expressed. Ultrastructural analysis revealed a loss of membrane caveolae and the presence of
54 numerous cytoplasmic autophagosomes. Patients' cells also showed increased autophagic flux and
55 blunted insulin signaling. These results were reproduced by *PTRF* knockdown in control fibroblasts
56 and in 3T3-F442A preadipocytes. Cavin-1 deficiency also impaired 3T3-F442A adipocyte
57 differentiation. Suppression of autophagy by siRNA-mediated silencing of *ATG5* improved insulin
58 sensitivity and adipocyte differentiation.

59 *Conclusions:* This study showed that cavin-1 deficiency resulted in maladaptative autophagy which
60 contributed to insulin resistance and altered adipocyte differentiation. These new pathophysiological
61 mechanisms could open new therapeutic perspectives for adipose tissue diseases including CGL4.

62

63 INTRODUCTION

64

65 Congenital generalized lipodystrophies (CGL) represent a group of rare monogenic disorders
66 characterized by an overall defect in adipose tissue development associated with severe insulin
67 resistance, diabetes, dyslipidemia and liver steatosis. CGL is a heterogeneous genetic disease, but the
68 great majority of cases are due to biallelic mutations in either *BSCL2* encoding seipin, a protein
69 involved in adipogenesis and lipid droplet formation, or *AGPAT2* encoding the enzyme 1-acyl-
70 glycerol-3-phosphate acyltransferase- β involved in the biosynthesis of triglycerides and
71 glycerophospholipids (1). A few cases of CGL have been shown to result from mutations in *CAVI*,
72 encoding caveolin-1 (2,3) or from mutations in *PTRF*, encoding polymerase I and transcript release
73 factor, also known as cavin-1. *PTRF* mutations are responsible for the association of generalized
74 lipoatrophy and muscular dystrophy, categorized as congenital generalized lipodystrophy type 4
75 (CGL4) (4-10).

76 Both cavin-1 and caveolin-1 are required for the formation of caveolae, which are specialized
77 omega-shaped microdomains of the plasma membrane involved in endocytosis, signal transduction,
78 and lipid transport and metabolism (11,12). Cavin-1 is a scaffold protein present in numerous cell
79 types, including myocytes, endothelial cells, fibroblasts and adipocytes, where caveolae are
80 particularly abundant. Cavin-1 has a critical importance for caveolae assembly (11), recruiting
81 caveolins into caveolae and preventing their degradation by the endolysosomal system (13). In
82 addition, cavin-1 colocalizes with caveolin-1 and -2, hormone-sensitive lipase and perilipin-1 at the
83 adipocyte lipid droplet surface, and contributes to the regulation of lipid droplet expandability (12,14-
84 18). Cavin-1 is also present in the cell nucleus, where it participates in transcription processes and
85 regulation of cellular senescence (19).

86 Few studies have investigated the cellular consequences of CGL4-linked cavin-1 mutations.
87 They were shown to result in a secondary deficiency of caveolins, which could play an important
88 pathophysiological role (2-4,7,8). Accordingly, cavin-1 knock-out in mice recapitulates the phenotype
89 observed in caveolin-1 deficient mice, including lack of cellular caveolae, lipodystrophy and insulin
90 resistance (20-22).

91 Macroautophagy – hereafter referred to as “autophagy” – is the main cellular pathway for
92 lysosomal degradation of altered proteins and organelles. Autophagy plays a crucial role in protein
93 quality control in a basal state, and contributes to cellular defense when activated in response to stress
94 conditions (adaptative autophagy) (23). Importantly, autophagy has also been shown to regulate lipid
95 metabolism, adipocyte differentiation and insulin sensitivity (24,25). Aberrant regulation of adipose
96 tissue autophagy has been reported in obesity and diabetes (26). Interestingly, caveolin-1 suppression
97 in mice has been shown to overactivate autophagy in stromal cells (22) and adipocytes (27). Caveolin-
98 1 was also demonstrated to regulate autophagy in lung and vascular endothelial cells (28,29).
99 However, the role of cavin-1 in the regulation of autophagy and associated metabolic functions
100 remains to be investigated.

101 In this study, we identified new *PTRF* mutations in two families with CGL and show that
102 subsequent loss of cavin-1 expression led to autophagy upregulation, resulting in insulin resistance
103 and altered adipocyte differentiation. These novel pathophysiological mechanisms could offer
104 innovative therapeutic perspectives for this severe orphan disease and other adipose tissue diseases
105 associated with maladaptative autophagy.

106

107 **SUBJECTS AND METHODS**

108

109 **Subjects**

110 Two unrelated patients issued from consanguineous families originating from Egypt and from
111 Switzerland, were referred to Saint-Antoine hospital (Paris, France) for CGL. Clinical, biological,
112 molecular, and cellular studies of the probands and their relatives were performed after full informed
113 consent according to legal procedures.

114

115 **Phenotype and genotype characterization**

116 Subjects underwent clinical evaluation and routine biological measurements, performed after an
117 overnight 12-h fast. Serum adiponectin and leptin levels were determined by ELISA (Quantikine,
118 R&D Systems, Oxford, UK). Genomic DNA was extracted from peripheral-blood leukocytes. The

119 entire coding region and splice junctions of genes involved in lipodystrophies (*i.e.* *BSCL2*, *AGPAT2*,
120 *CAVI*, *PTRF*, *LMNA*, *PPARG*, *AKT2*, *CIDEA*, *PLIN1*) were amplified by PCR with specific primers
121 in the two probands (primers sequences are available upon request). Purification of PCR products was
122 performed on Sephadex columns and sequencing used Big Dye Terminator chemistry (Applied
123 Biosystems). *PTRF* was also sequenced in the probands' family members and in 100 unrelated control
124 subjects.

125

126 **Cellular studies**

127 **Primary fibroblast cultures** were established from skin biopsies in the two probands and compared
128 to those from two healthy women aged 20 and 33 (30). Murine 3T3-F442A preadipocytes were
129 cultured and induced for adipocyte differentiation during 7 days as described (31).

130 For **mRNA silencing**, cells were incubated for 6h with 100 pmoles of control (scrambled) siRNA (sc-
131 37007), or a pool of 3-5 specific siRNA targeting murine or human *PTRF* (sc-76294 and sc-76293) or
132 *ATG5* (sc-41446 and sc-41445) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), in the
133 provided transfection reagent. Fibroblasts were studied 4 days after mRNA silencing. 3T3-F442A
134 preadipocytes were evaluated 3 days after mRNA silencing (D0, undifferentiated state), then 7 days
135 after adipocyte differentiation (D7).

136 **Western blot analyses** were performed on whole cell extracts using specific antibodies listed in
137 **Supplemental Table 1**. Protein detection and semi-quantitative analysis of western blots *versus* beta-
138 actin were performed using ChemiGenius2 Bio-imaging system (Syngene, Cambridge, UK).

139 **Autophagy** is characterized by the formation of double-membrane organelles known as
140 autophagosomes, which surround targeted cytoplasmic materials. The trafficking of autophagosomes
141 and then their fusion with lysosomes leads to the degradation of the sequestered material (32). During
142 this process, the cytosolic protein LC3-I is modified to a lipidated form, LC3-II, which associates to
143 autophagosome membranes and is then submitted to the autophagic flux. We monitored autophagy as
144 previously described, in the presence or not of the autophagic flux blocker bafilomycin A1 (B1793,
145 Sigma-Aldrich) (100 nM for 4h), which inhibits the late phase of autophagy by preventing the fusion
146 between autophagosomes and lysosomes (33). We measured the amount of p62/sequestosome-1, an

147 autophagy substrate degraded in lysosomes. We also evaluated the rate of conversion of LC3-I to
148 LC3-II (LC3-II-to-LC3-I ratio), which correlates with autophagosome formation, and the levels of
149 LC3-II, which is also degraded in lysosomes.

150 To study **insulin signaling**, cells maintained for 24h in a serum-free medium were incubated or not
151 with 50 nmol/L human insulin (#I9278, Sigma-Aldrich) for 8min. We evaluated the total protein
152 expression of insulin receptor and of the signalling intermediates extracellular signal-regulated kinase
153 (ERK) 1/2 and Akt as well as their phosphorylated activated forms (**Supplemental Table 1**).

154 **Adipocyte conversion** of 3T3-F442A cells was assessed by the expression of adipocyte-specific
155 proteins and by Oil red O-staining of intracellular neutral lipid stores, which was quantified at 520 nm
156 after solubilization in 10% SDS and normalization to the amount of total proteins.

157 For **mRNA expression studies**, total RNA was isolated from cultured fibroblasts using RNeasy kit
158 (Qiagen, Courtaboeuf, France). cDNA was synthesized using random hexamers and AMV-RT
159 (Promega, Madison, WI, USA) for 60min at 42°C. Real-time PCR was performed with specific
160 primers (available upon request) using the FastStart SYBR Green I mix and the LightCycler detection
161 system (Roche Diagnostics, Meylan, France). TATA box binding protein (TBP) was used for
162 normalization. The relative expression of genes was calculated by the LightCycler Relquant program
163 using comparative C_t method.

164 **Immunofluorescence studies** were performed on fibroblasts grown on glass coverslips, after fixation
165 in methanol at -20°C. DAPI (4',6'-di-amidine-2-phenylindole dihydrochloride) was used for nuclear
166 staining. Cells were visualized and images were acquired using Leica SP2 confocal microscope and
167 software.

168 **Ultrastructural analysis by electronic microscopy** was performed on cultured fibroblasts fixed in
169 2.5% glutaraldehyde at 4°C. Cells were rinsed in PBS, post-fixed in 1% osmium tetroxide, dehydrated
170 using graded alcohol series then embedded in epoxy resin. Semi-fine sections (0.5 μ m) were stained
171 with toluidine blue. Ultrathin sections (60 nm) were contrasted with uranyl acetate and lead citrate and
172 examined using a JEOL 1010 electron microscope (JEOL, Tokyo, Japan) with an OSIS mega View III
173 camera.

174 Quantitative results, presented as mean \pm **SD**, were statistically analyzed by **non-parametric Mann-**

175 **Whitney test** using PRISM software (GraphPad Software, Inc, CA, USA). *P* values <0.05 were
176 considered as significant.

177

178 **RESULTS**

179

180 **Phenotype studies**

181 **Proband-1** was a 32-year-old woman, born from consanguineous parents (first-cousins) of Sicilian
182 origin. She presented with paucity of fat and muscular hypertrophy since birth and was diagnosed with
183 generalized lipodystrophy and diabetes at age 12. Despite high doses of insulin (up to 2U/kg/day)
184 associated with pioglitazone (30 mg/day), chronic hyperglycemia persisted (HbA1c: 8.8%) and was
185 complicated by proliferative retinopathy. Acute pancreatitis linked to major hypertriglyceridemia
186 occurred at age 16. The patient had also mild mental retardation diagnosed during early childhood and
187 hypergonadotrophic hypogonadism, with primary amenorrhea and limited breast development (Tanner
188 stage 3). Her height was 158 cm, her weight 48 kg (BMI: 19.2 kg/m²). Muscular strength was normal
189 but she complained of cramps. *Acanthosis nigricans* was present on neck and axillar folds. Abdominal
190 ultrasonography showed hepatomegaly, and liver steatosis was diagnosed at age 23 on histology.
191 Cardiac examination (including echography and electrocardiogram) was normal. Biological
192 measurements revealed high creatine kinase levels, high triglycerides and low HDL-cholesterol levels,
193 low leptin and adiponectin levels and mild renal insufficiency. Her 60 year-old mother (BMI 21.9,
194 normal physical examination) had type 2 diabetes since age 46. Her 62 year-old father and 35 year-old
195 sister were described as asymptomatic, without lipodystrophy, muscular signs, diabetes, or
196 dyslipidemia. They refused medical examination and molecular analyses.

197

198 **Proband-2** was a 13-year-old girl at examination. She was born from Egyptian consanguineous
199 parents (first-cousins), with a low birth weight (2000g) at term after an uneventful pregnancy. She was
200 diagnosed with generalized lipodystrophy at age 1, and mild mental retardation at age 10. Her height
201 was 151 cm, her weight 40 kg (BMI: 17.5 kg/m², Z-score: -0.47). Spontaneous menarche occurred at
202 age 15 after a normal pubertal development. In addition to typical generalized lipodystrophy, she showed

203 generalized muscular hypertrophy, pseudoacromegaloid features (enlarged hands and feet, prominent
204 eyebrows' arches), axillar and cervical *acanthosis nigricans*, and liver hypertrophy. Cardiac
205 examination was normal. Biological investigations revealed normal fasting glucose with high insulin
206 levels, slightly elevated liver enzymes, very low levels of leptin and adiponectin, and very high serum
207 creatine kinase, although she did not complain of any muscular symptoms. One of her four sisters died
208 at 2.5 months from respiratory distress. Her two youngest sisters, 2 and 6 years-old, were described
209 with congenital generalized lipodystrophy and muscle weakness, associated with achalasia in the older
210 one. Her 36 year-old mother, 41 year-old father, and 11 year-old sister were asymptomatic and their
211 physical examination was normal.

212

213 The main clinical and biological characteristics of the probands and affected relatives are summarized
214 in **Table 1**.

215

216 **Molecular studies**

217 *PTRF* sequencing in proband-1 revealed a homozygous c.176A>T transversion in exon 1, predicting a
218 p.Asp59Val substitution in the highly conserved N-terminal leucine-rich domain of cavin-1 (**Fig. 1**).
219 Her mother was heterozygous for the mutation. A homozygous transversion of the last nucleotide of
220 exon 1 (*PTRF* c.471G>C), predicting a Gln-to-His substitution at codon 157, was detected in proband-
221 2 and her two lipodystrophic sisters. Direct sequencing of cDNA derived from proband-2' cultured
222 skin fibroblasts showed that this mutation results in a splicing defect. The frameshift insertion of 143
223 nucleotides from intron 1, followed by a premature codon stop in exon 2, predicted the synthesis of a
224 mutated truncated protein (*PTRF* p.Gln157Hisfs*52) (**Fig. 1**). Proband-2' asymptomatic parents and
225 sister were heterozygous for the mutation. The family trees, sequence analyses and alignments are
226 shown in **Fig. 1**. The two *PTRF* mutations were absent in 100 unrelated control subjects (50 of
227 Caucasian and 50 of Egyptian origin) and in the 1000 Genome Project Database. The *PTRF*
228 p.Asp59Val mutation was reported in the Exome Aggregation Consortium (ExAC) Database with an
229 allelic frequency of 0.00001658, in the heterozygous but not the homozygous state (2 alleles among
230 120646). This mutation was not submitted to ClinVar. It was predicted to be damaging by Polyphen-2,

231 SIFT and MutationTaster. The truncating mutation *PTRF* p.Gln157Hisfs*52 was not reported in
232 ExAC. No pathological alteration was found in other lipodystrophy-related genes in the two probands
233 (i.e. *BSCL2*, *AGPAT2*, *CAVI*, *PTRF*, *LMNA*, *PPARG*, *AKT2*, *CIDEA*, *PLIN1*).

234

235 Cell studies

236 ***PTRF*-mutated fibroblasts from the two probands showed decreased number of caveolae and**
237 **increased autophagic flux**

238 Cavin-1 protein was not detectable in fibroblasts from the two probands, as shown by western blotting
239 and immunofluorescence microscopy (**Fig. 2A,B**). Cavin-1 mRNA expression was strikingly
240 decreased in fibroblasts from proband-2 but was not modified in proband-1 as compared to control
241 cells (data not shown), suggesting post-translational modifications leading to protein degradation in
242 this case. Protein levels of caveolin-1 and caveolin-2 were decreased (**Fig. 2A**). Electronic microscopy
243 showed, respectively, a decreased number and an absence of plasma membrane caveolae in fibroblasts
244 from proband-1 and -2 as compared to control cells (**Fig. 2C**). In addition, an increased number of
245 autophagic vacuoles was observed in fibroblasts from the probands. Some of these vacuoles, which
246 displayed multimembranous structures, were identified as autophagosomes (**Fig. 2D**).

247 To further characterize intracellular autophagy in *PTRF*-mutated vs control fibroblasts, we evaluated
248 the conversion from soluble microtubules-associated light chain 3 protein (LC3-I) to vacuolar
249 membrane-associated LC3-II, which correlates with autophagosome formation (34).
250 Immunofluorescence detection of LC3 showed an increase in the number of LC3-positive bright
251 puncta, depicting LC3-II associated with autophagosomes, in cells from probands as compared to
252 controls (**Fig. 2B**). Western blot revealed increased LC3-II levels and LC3-II-to-LC3-I ratios, showing
253 that the number of autophagosomes was increased, in patients' versus controls' fibroblasts (**Fig. 2E**).
254 The selective autophagy substrate p62, which is degraded by lysosomes when autophagy is activated,
255 was decreased in patients' versus control cells, in favor of an enhanced autophagic flux (**Fig. 2E**). As
256 expected, blockade of the autophagic flux by bafilomycin A1, which prevents the fusion between
257 autophagosomes, endosomes and lysosomes (33), increased LC3-II-to-LC3-I ratio and inhibited

258 substrate degradation as reflected by increased p62 levels, in both control and mutated fibroblasts
259 **(Fig. 2F).**

260 Taken together, these results show that patients' fibroblasts with cavin-1 deficiency displayed
261 decreased caveolae formation and increased autophagic flux.

262

263 ***PTRF*-mutated fibroblasts from the two probands displayed cellular insulin resistance, and**
264 **siRNA-mediated *PTRF* knockdown in control fibroblasts recapitulated increased autophagy and**
265 **insulin resistance**

266 We evaluated insulin-stimulated activation of the insulin receptor and the insulin signaling
267 intermediates protein kinase B (PKB/Akt) and extracellular-regulated kinases (ERK1/2) in fibroblasts
268 from the two patients and from control subjects. Insulin-induced activation of the signaling proteins
269 was blunted in probands' fibroblasts as compared to controls **(Fig. 3A).**

270 To determine the involvement of cavin-1 deficiency in increased autophagy flux and insulin
271 resistance, we performed siRNA-mediated silencing of *PTRF* in control fibroblasts. Efficient *PTRF*
272 knockdown also led to a drastic reduction in caveolin-1 and -2 proteins **(Fig. 3B)**, and activated the
273 autophagic flux, as shown by an increase in LC3-II level and LC3-II-to-LC3-I ratio and a decrease in
274 p62 level **(Fig. 3C)**. In addition, *PTRF* knockdown reduced insulin-activated phosphorylation of Akt
275 and ERK1/2 **(Fig. 3D)**.

276 These results demonstrated that cavin-1 deficiency activated autophagy and reduced insulin response
277 in human fibroblasts.

278

279 ***PTRF* silencing in 3T3-F442A preadipocytes activated autophagy, induced insulin resistance and**
280 **impaired adipocyte differentiation**

281 We then studied the effects of *PTRF* mRNA silencing in murine 3T3-F442A preadipocytes. Cells
282 were evaluated 3 days after siRNA transfection, in the undifferentiated state (D0), and 7 days after
283 induction of differentiation (D7). As observed in fibroblasts, *PTRF* silencing was associated with a
284 decrease in caveolin-1 and caveolin-2 proteins, both at D0 (data not shown) and D7 of differentiation
285 **(Fig. 4A)**. *PTRF* knockdown also activated autophagy, as shown by increased LC3-II-to-LC3-I ratio

286 and decreased p62 protein amount (**Fig. 4A**). In addition, insulin signaling, studied at D7 of
287 differentiation, was blunted in cavin-1 deficient 3T3-F442A cells. *PTRF* silencing decreased insulin-
288 induced phosphorylation of insulin receptor β -subunit, Akt and ERK1/2, indicating cellular insulin
289 resistance (**Fig. 4B**). Beside, *PTRF* knockdown significantly decreased the total amount of insulin
290 receptor β -subunit, which also represents a marker of mature adipocytes (1.27 ± 0.14 vs 0.66 ± 0.05
291 arbitrary units (**mean \pm SD**) in control and cavin-1 deficient 3T3-F442A cells, respectively, $p=0.05$).
292 In accordance, adipocyte differentiation of 3T3-F442A cells, assessed by increased protein expression
293 of key adipocyte transcription factors (C/EBP- α , PPAR γ , SREBP-1c) and of the mature adipocyte
294 marker fatty acid synthase (FAS) from D0 to D7, was impaired by *PTRF* knockdown (**Fig. 4C**).
295 Finally, *PTRF* mRNA silencing decreased the ability of differentiating 3T3-F442A cells to store
296 lipids, as indicated by decreased Oil red O-staining at D7 of differentiation (**Fig. 4D**).
297 Therefore, in 3T3-F442A cells, cavin-1 knockdown was responsible for activation of autophagy,
298 insulin resistance and impaired adipocyte differentiation.

299

300 **In *PTRF*-mutated fibroblasts from the two probands, suppression of autophagy by *ATG5*** 301 **knockdown partially reversed cellular insulin resistance**

302 To further investigate the role of increased autophagy in *PTRF* knockdown-induced insulin resistance,
303 we blocked the mRNA expression of *ATG5*, a key component of the molecular core machinery of
304 autophagy (33), in fibroblasts from probands and controls. As expected, efficient *ATG5* silencing
305 reversed the overactivation of autophagic processes associated with cavin-1 deficiency, as shown by a
306 decreased LC3-II-to-LC3-I ratio and an increased p62 amount in knocked-down versus untreated
307 probands' cells (**Supplemental Fig. 1A**). **We also observed that reducing autophagy in patients'**
308 **fibroblasts significantly increased the amount of p.Asp59Val mutated cavin-1 from proband 1, though**
309 **it still remained lower than in control cells (Supplemental Fig. 1A).** This suggests that overactivation
310 **of autophagy partially contributed to the posttranslational degradation of this cavin-1 mutant.** *ATG5*
311 silencing also increased insulin-induced phosphorylation of Akt and ERK1/2 in patients' fibroblasts
312 (**Fig. 5A**). Importantly, *ATG5* knockdown in control fibroblasts did not significantly alter the LC3-II-

313 to-LC3-I ratio and p62 level (**Supplemental Fig. 1A**) and the insulin-mediated activation of Akt and
314 ERK1/2 (**Fig. 5A**).

315

316 **In 3T3-F442A cells, suppression of autophagy by *ATG5* siRNA-mediated silencing reversed**
317 ***PTRF* knockdown-induced insulin resistance and altered adipocyte differentiation**

318 We then studied the effects of *ATG5* mRNA silencing in murine 3T3-F442A preadipocytes subjected
319 or not to *PTRF* knockdown prior to induction of differentiation. As expected, *PTRF* knockdown-
320 induced activation of autophagy was partially reversed by concomitant *ATG5* silencing, the increased
321 LC3-II-to-LC3-I ratio and decreased p62 levels being partly rescued at D7 of differentiation
322 (**Supplemental Fig. 1B** and data not shown). As observed in patients' fibroblasts, *ATG5* knockdown
323 in 3T3-F442A cells partly reversed the insulin resistance resulting from cavin-1 deficiency, as
324 assessed by increased insulin-mediated phosphorylation of Akt and ERK1/2. Moreover, *ATG5* mRNA
325 silencing increased both total protein level and insulin-mediated tyrosine phosphorylation of the
326 insulin receptor β -subunit in *PTRF* knockdown cells (**Supplemental Fig. 1C**).

327 In addition, suppression of autophagy by *ATG5* silencing prevented the decrease in *C/EBP α* , *PPAR γ* ,
328 *SREBP-1c* and *FAS* proteins induced by cavin-1 deficiency in differentiating 3T3-F442A cells (**Fig.**
329 **5B**). In accordance, it also improved the lipid storage capacity of cavin-1 deficient 3T3-F442A cells,
330 evaluated by Oil red O-staining at D7 of differentiation (**Fig. 5B**).

331 All together, these results showed that activation of autophagy induced by cavin-1 deficiency
332 contributed to insulin resistance and impaired adipogenesis.

333

334 **DISCUSSION**

335

336 CGL are rare diseases of diverse molecular origin which are all characterized by the
337 association of adipose tissue deficiency with metabolic complications usually observed in obese
338 subjects (insulin resistance, hypertriglyceridemia and liver steatosis, among others), pointing to the
339 critical role of adipose tissue in metabolic health. Thus, defective lipid storage in adipose tissue, due to
340 disruption of adipogenesis and/or lipid droplet formation induced by *AGPAT2* or *BSCL2* mutations,

341 the main molecular alterations responsible for CGL, were shown to result in insulin resistance and
342 associated metabolic dysfunction (1). In the present study, we assessed the cellular consequences of
343 *PTRF*/cavin-1 deficiency, responsible for rare cases of CGL. We show that the lack of cavin-1 protein
344 expression resulted in maladaptative autophagy, which triggered insulin resistance and altered
345 adipocyte differentiation. These results add new insights into the complex relationships between
346 adipose tissue and whole body metabolism.

347 We identified two new homozygous *PTRF* mutations, predicting a point mutation or a
348 truncation in cavin-1 (p.Asp59Val or p.Gln157Hisfs*52 alterations), in two families with CGL4,
349 characterized by generalized lipodystrophy, insulin resistance and/or diabetes, and high levels of creatine
350 kinase with or without symptomatic muscular dystrophy. Both mutations led to a completely abolished
351 cavin-1 protein expression in patients' cells.

352 Although its physiological roles have not been completely deciphered, cavin-1 is recognized
353 as a scaffold protein interacting with caveolin-1 both at the level of plasma caveolae and at the surface
354 of adipocyte lipid droplet (11,14). Caveolin-1, involved in rare cases of CGL3, was shown to regulate
355 the cellular process of autophagy (22, 27-29). Our results reveal, for the first time, that cavin-1 also
356 contributes to the regulation of autophagy.

357 We first confirmed, in fibroblasts from the two probands, that cavin-1 deficiency impaired
358 caveolae formation and expression of caveolins (8,20). More importantly, we showed that
359 patients' cells displayed intrinsic proximal insulin signaling defects, with alterations of insulin-
360 mediated activation of the insulin receptor and its downstream molecular targets, together with an
361 increased autophagic flux. Recapitulation of these defects by cavin-1 knockdown, both in fibroblasts
362 and in differentiating adipocytes, was consistent with the assumption that these alterations directly
363 resulted from the lack of cavin-1. Interestingly, absence of caveolae, by leading to accumulation of
364 glycosphingolipids into lysosomes, was shown to increase autophagy (12). In addition, insulin
365 resistance and increased autophagy were previously described in caveolin-1 null mice (27,35). In
366 accordance with these studies, we found that cavin-1 knockdown induced a decreased expression of
367 the insulin receptor in adipocytes, which was rescued upon autophagy inhibition. Moreover, our
368 results revealed that lack of cavin-1 also resulted in a global defect of insulin signaling pathways.

369 Interestingly, a decreased level of cavin-1 induced by hypoxia was recently associated with impaired
370 insulin signaling in adipocytes (36).

371 Importantly, our results show that cavin-1 deficiency impaired adipocyte differentiation and
372 that inhibition of autophagy by *ATG5* knockdown rescued, at least partially, altered adipocyte
373 differentiation and cellular insulin resistance. Taken together, our results strongly suggest that the
374 metabolic defects induced by cavin-1 deficiency were mediated by a constitutive upregulation of
375 autophagic flux. ~~Cavin-1 could thus act as a physiological negative regulator of
376 autophagy. Consistently, autophagy which is known to regulate adipocyte differentiation and insulin
377 sensitivity (24,25), has also been shown to contribute to lipid droplet breakdown (37). Interestingly,
378 dysregulation of autophagy in adipose tissue could play an important role in the pathophysiology of
379 obesity and diabetes (26).~~

380 Autophagy is known to regulate adipocyte differentiation, adipose brown/white remodeling
381 and insulin sensitivity (24,25). Suppression of autophagy by systemic *Atg5* or adipose-specific *Atg7*
382 knock-out in mice was previously shown to impair adipogenesis and induce lipodystrophy (24,37,38).
383 However, increased autophagy due to the absence of cavin-1 (this study) or to caveolin-1 knock-out in
384 mice (27) is also associated with impaired adipogenesis and lipodystrophy. In addition, autophagy was
385 shown to be decreased (39), increased (40), or dysregulated (41) in adipose tissue from obese patients.
386 This suggests that a finely regulated adaptative activation of autophagy is required for a proper
387 homeostasis of adipose tissue. In accordance with our results, *IGF1R* mutations responsible for a
388 subtype of SHORT syndrome with lipodystrophy and decreased insulin-induced Akt activation, were
389 recently shown to activate autophagy (42). ~~This suggests that~~ Maladaptative autophagy could ~~thus~~
390 contribute to lipodystrophy and insulin resistance in several pathogenic situations.

391 Although further studies are needed to decipher the precise underlying mechanisms linking
392 cavin-1 deficiency and increased autophagy, our results show that maladaptative autophagy, triggering
393 altered adipocyte differentiation and insulin resistance, could contribute to the pathophysiology of
394 lipodystrophy and the associated metabolic dysfunctions in CGL4. Our study add further evidence for
395 the role of lipid droplets, their coated proteins and/or caveolae proteins in dynamic cellular functions,

396 including autophagy (43), and could open new therapeutic options in the field of genetic
397 lipodystrophies and other adipose tissue diseases associated with maladaptive autophagy.

398

399 **ACKNOWLEDGEMENTS**

400

401 We thank the patients who participated in these studies, Drs Jean-Philippe Bastard and Soraya Fellahi
402 from AP-HP, Department of Biochemistry, Tenon Hospital, Paris, France for leptin and adiponectin
403 measurements, Marie-Christine Verpont, from Institut Fédératif de Recherche 65, Paris, France, for
404 her expertise in electronic microscopy and Emilie Capel from Inserm U938, Paris, France, for
405 technical assistance.

406

407 **FIGURE LEGENDS**

408

409 **Figure 1. Proband-1 (A) and proband-2 (B) families and PTRF/cavin-1 molecular alterations.**

410 Patients with generalized lipodystrophy are depicted with black symbols and probands with arrows.
411 *PTRF* sequences in control and probands are shown, with the consequences of each mutation on
412 mRNA transcription and translation. Alignment of *PTRF/cavin-1* aminoacid sequences includes the
413 N-terminal leucine zipper domain from various species, with the conserved Asp59 residue in blue. In
414 proband-2, *PTRF* sequencing revealed a homozygous c.471G>C transversion, affecting the last
415 nucleotide of exon 1. Direct sequencing of cDNA derived from cultured skin fibroblasts showed that
416 this mutation results in a splicing defect. The frameshift insertion of 143 nucleotides from intron 1,
417 followed by a premature codon stop in exon 2, predicted the synthesis of a mutated truncated protein
418 (p.Gln157Hisfs*52).

419

420 **Figure 2. Fibroblasts from patients with CGL4 showed lack of cavin-1 protein, decreased** 421 **amount of plasma membrane caveolae and increased autophagic flux**

422 **A-** Protein expression of cavin-1 and its partners caveolin-1 and -2 were determined by western blot in
423 control (Ctrl) and probands' fibroblasts (P1 and P2) as described in Methods. Beta-actin was used as an

424 index of the cellular protein level. A representative western blot (for each protein) is shown, with
425 semi-quantitative analyses of western blots from experiments performed in triplicate (expressed as
426 means \pm SD). *: $p < 0.05$ as compared to control cells. **B-** Representative photographs of
427 immunofluorescence microscopy. Cavin-1 was revealed by red staining (absent in
428 probands' fibroblasts), and LC3 by green signals, either diffuse (cytosolic form of LC3-I, control
429 cells), or punctuated (LC3-II form associated with autophagosomes, probands' cells). Cell nuclei are
430 stained in blue with DAPI. Scale bar: 10 μ m. **C, D-** Fibroblasts were examined using electron
431 microscopy. The number of plasma membrane caveolae (C, arrows) was decreased in fibroblasts from
432 proband-1 and absent in fibroblasts from proband-2. The number of autophagosomes (D, arrows) was
433 increased in probands' cells (inset: magnification showing the characteristic double membrane of an
434 autophagosome). Scale bar: 1 μ m. **E, F-** Representative western blots showing the protein expression
435 of LC3-I and LC3-II isoforms and of p62, which is degraded in lysosomes when autophagy is
436 activated. Semi-quantitative analyses of western blots (expressed as means \pm SD) show that LC3-II-to-
437 LC3-I ratio were increased in probands' fibroblasts as compared to control cells whereas p62 was
438 decreased, showing activation of autophagy. (E) *: $p < 0.05$ as compared to control cells. (F) Increased
439 autophagic flux in patients' cells was confirmed using bafilomycin, an inhibitor of the late phase of
440 autophagy, which increased the LC3-II-to-LC3-I ratio and increased p62 in control and patients' cells.

441

442 **Figure 3. PTRF-mutated probands' fibroblasts showed cellular insulin resistance, and PTRF**
443 **knockdown in control fibroblasts recapitulated increased cellular autophagy and insulin**
444 **resistance**

445 **A-** Insulin signaling activation was assessed in control (Ctrl) and probands' fibroblasts (P1 and P2).
446 Representative western blots are shown, with quantification expressed as fold-stimulation by insulin of
447 the activated/phosphorylated-to-total protein ratios (means \pm SD). *: $p < 0.05$ as compared to control
448 cells. IR β : insulin receptor β -subunit, pTyr IR β : phosphotyrosine-IR β , pAkt: phospho-Ser473-Akt,
449 pERK1/2: phospho-Tyr204-ERK1/2. **B-** Control fibroblasts were transfected or not with scrambled
450 (scr) or PTRF-specific siRNA as indicated and assessed for protein expression of cavin-1, caveolin-1

451 and caveolin-2 as described in Methods. A representative western blot (performed in triplicate) is
452 shown, with quantifications of the protein levels normalized to β -actin, expressed as means \pm SD. *:
453 $p < 0.05$ as compared to non-transfected cells. **C-** Western blot detection of LC3 protein isoforms LC3-
454 I and LC3-II, p62 and β -actin (loading control), in control fibroblasts transfected or not with
455 scrambled or PTRF-specific siRNA as indicated. Semi-quantitative analyses of LC3-II-to-LC3-I ratios
456 and p62 levels normalized to β -actin are expressed as means \pm SD. *: $p < 0.05$ as compared to non-
457 transfected cells. **D-** Insulin signaling activation was assessed as in (A) in control fibroblasts
458 transfected or not with scrambled or PTRF-specific siRNA as indicated.

459

460 **Figure 4. PTRF mRNA silencing in 3T3-F442A preadipocytes activated autophagy, induced**
461 **insulin resistance and impaired adipocyte differentiation**

462 3T3-F442A cells were untransfected (none) or submitted to scrambled (scr) or PTRF-specific mRNA
463 silencing as indicated. **A-** Representative western blots showing the effects of PTRF silencing on
464 protein expression of cavin-1, caveolin-1 and caveolin-2, and on the conversion of LC3-I to LC3-II
465 and the p62 protein amount, at D7 of adipocyte differentiation in 3T3-F442A cells. Quantifications of
466 the protein levels normalized to β -actin are expressed as means \pm SD. *: $p < 0.05$ as compared to non-
467 transfected cells. **B-** Insulin signaling activation was assessed at D7 by evaluating insulin-induced
468 phosphorylation of insulin receptor β -subunit, Akt and ERK1/2, normalized to the total corresponding
469 protein content. IR β : insulin receptor β -subunit, pTyr IR β : phosphotyrosine-IR β , pAkt: phospho-
470 Ser473-Akt, pERK1/2: phospho-Tyr204-ERK1/2. **C-** Adipocyte differentiation of 3T3-F442A cells
471 was evaluated by the protein expression of adipogenic factors (C/EBP α , PPAR γ , SREBP-1c) and of
472 the marker of mature adipocyte fatty acid synthase (FAS) at D0 and D7. A representative western blot
473 and the quantifications of the proteins are shown, as described in (A) (means \pm SD, *: $p < 0.05$ as
474 compared to non-transfected cells). **D-** Oil red O-staining of intracellular stored lipids at D7 of
475 differentiation was shown by fluorescence microscopy (cell nuclei are stained in blue with DAPI) and
476 quantified with normalization to the total protein content as described in Methods.

477

478 **Figure 5. Insulin resistance and impaired adipocyte differentiation induced by *PTRF***
479 **knockdown were partially rescued by *ATG5* siRNA-mediated silencing**

480 **A-** Representative western blots showing the effects of *ATG5* siRNA-mediated silencing on insulin-
481 mediated activation of Akt and ERK1/2 in control (Ctrl) and probands' fibroblasts (P1 and P2). Cells
482 were either untransfected or transfected with scrambled or *ATG5*-specific siRNA as indicated.
483 Quantification was expressed as fold-stimulation by insulin of the activated/phospho-to-total protein
484 ratios (means \pm SD). *: $p < 0.05$ as compared to non-transfected cells from the same subject.

485 pAkt: phospho-Ser473-Akt, pERK1/2: phospho-Tyr204-ERK1/2.

486 **B-** Adipocyte differentiation of 3T3-F442A cells either untransfected, or submitted to scrambled,
487 *PTRF* or to double *PTRF* and *ATG5* siRNA silencing, was evaluated at D7 by protein expression of
488 adipocyte markers and Oil red O-staining of intracellular stored lipids. Representative western blots
489 and phase-contrast microscopy images are shown. The quantifications of the proteins levels
490 normalized to β -actin are expressed as means \pm SD. Oil red O-staining was quantified with
491 normalization to the total protein content as described in Methods. Scale bar: 1 μ m. *: $p < 0.05$ as
492 compared to non-transfected cells.

493

494 **Supplemental Figure 1. Reversion of cavin-1 deficiency-mediated alterations by *ATG5***
495 **knockdown in patients' fibroblasts and *PTRF*-knocked down 3T3-F442A cells**

496 **A-** Autophagic flux was evaluated by LC3-II-to-LC3-I ratio and p62 levels in control and
497 patients' cells, either untransfected, or transfected with scrambled or *ATG5*-specific siRNA as
498 indicated. Quantifications of the protein levels normalized to β -actin are expressed as means \pm SD. *:
499 $p < 0.05$ as compared to non-transfected cells from the same subject. #: $p < 0.05$ as compared to non-
500 transfected cells control cells.

501 **B, C-** 3T3-F442A cells either untransfected, or submitted to scrambled, *PTRF* or to double *PTRF* and
502 *ATG5* siRNA silencing as indicated, were studied at D7 of differentiation. Autophagic flux (**B**) and
503 activation of insulin signaling (**C**) were evaluated.

504 Representative western blots are shown, with quantifications expressed as protein levels normalized to

505 β -actin or fold-stimulation by insulin of the activated-to-total protein ratios (means \pm SD).

506 *: $p < 0.05$ as compared to non-transfected cells. IR β : insulin receptor β -subunit, P-Tyr IR β :

507 phosphotyrosine-IR β , pAkt: phospho-Ser473-Akt, pERK1/2: phospho-Tyr204-ERK1/2.

508

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- 641
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Table 1**Characteristics of patients with *PTRF* homozygous mutations.**

	Proband-1	Proband-2	Sister 1 of Proband-2	Sister 2 of Proband-2	Reference ranges
<i>PTRF</i> homozygous mutation	p.D59V	p.Q157HfsX52	p.Q157HfsX52	p.Q157HfsX52	
Age at examination (years)	32	13	6	2	
Sex	Female	Female	Female	Female	
BMI (kg/m²) (Z-score in children)	19.2	17.5 (-0.47)	15.4 (0.13)	16.1 (-0.24)	
Lipodystrophy	Congenital Generalized	Congenital Generalized	Congenital Generalized	Congenital Generalized	
<i>Acanthosis nigricans</i>	Neck, axillae	Neck, axillae	-	No	
Mental retardation	Mild	Mild	No	No	
Muscular signs	Generalized muscular hypertrophy, cramps	Generalized muscular hypertrophy	Calf hypertrophy, generalized muscle weakness	Generalized muscle weakness	
Cardiac examination	Normal	Normal	Normal	Normal	
Creatine kinase (IU/L)	308	1054	618	1202	15-95
Fasting glucose (mmol/L)	9.5	4.5	4.2	4.3	4-5.6
Fasting insulin (mIU/L)	Insulin-treated	16.3	9.2	4.5	2-10
Triglyceride (mmol/L)	2.3	1.7	0.7	1.0	0.4-1.5
Total cholesterol (mmol/L)	4.3	3.4	3.5	3.7	4.1-6.2
HDL-cholesterol (mmol/L)	0.4	0.7	0.9	0.8	1.3-2.1
Leptin (µg/L)	3.2	0.2	0.2	0.7	4-20
Adiponectin (mg/L)	2.8	0.9	1.7	2.8	3.9-12.9
Liver enzymes (AST/ALT) (IU/L)	32/34	43/53	44/60	43/52	5-35 / 5-35
Liver examination	Hepatomegaly and steatosis (histology)	Hepatomegaly	Hepatomegaly	-	

BMI, body mass index; AST: aspartate aminotransferase; ALT: alanine aminotransferase

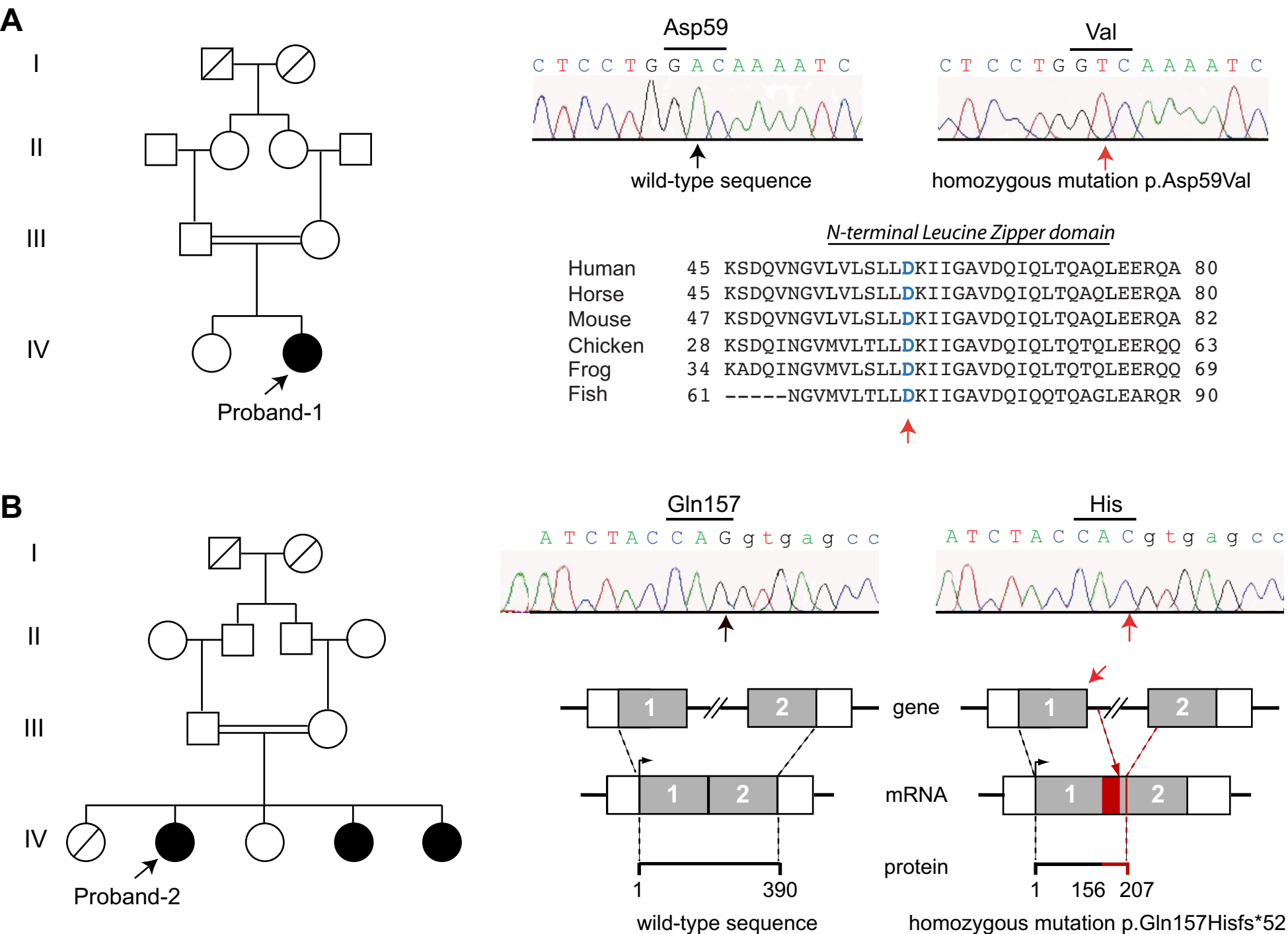


Figure 1

Human fibroblasts

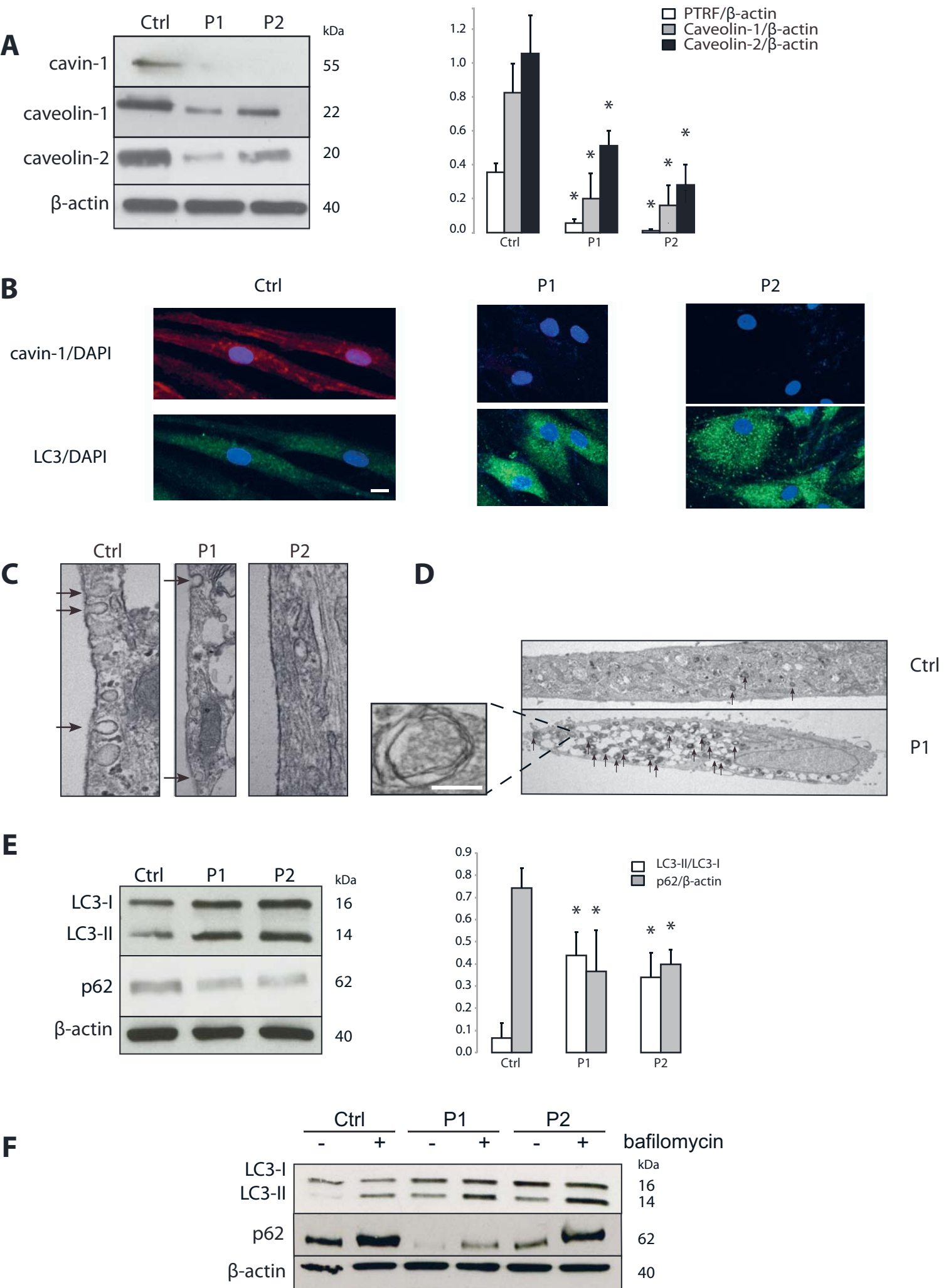
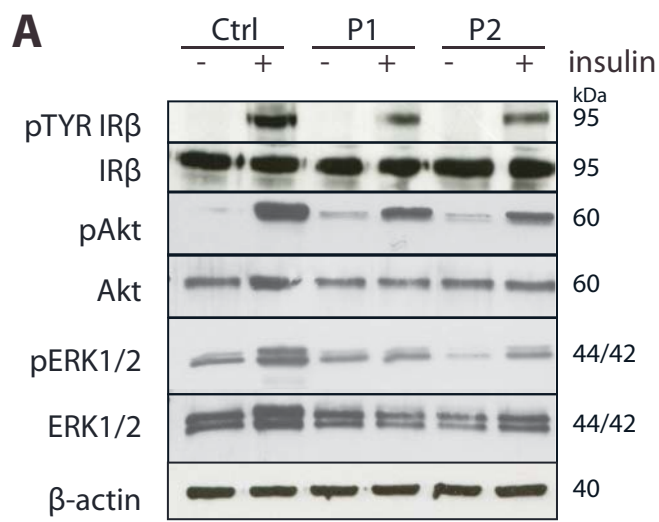


Figure 2



Human fibroblasts

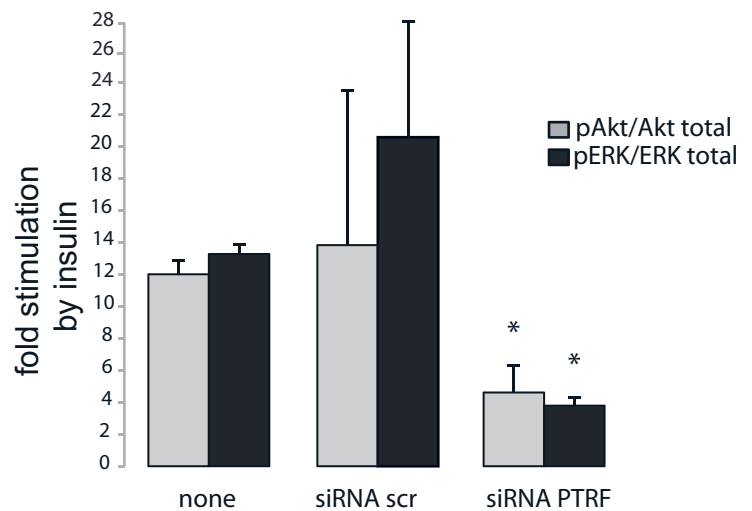
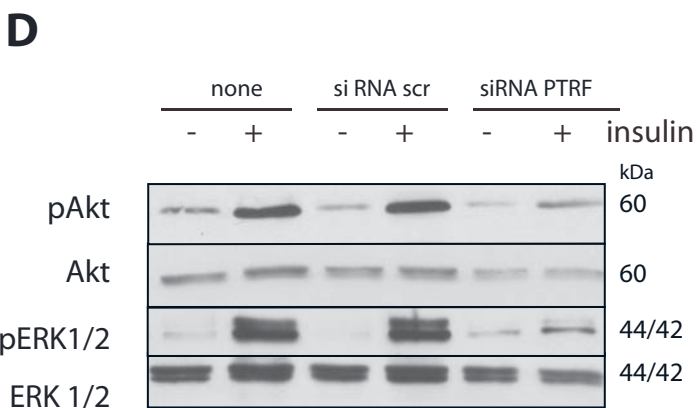
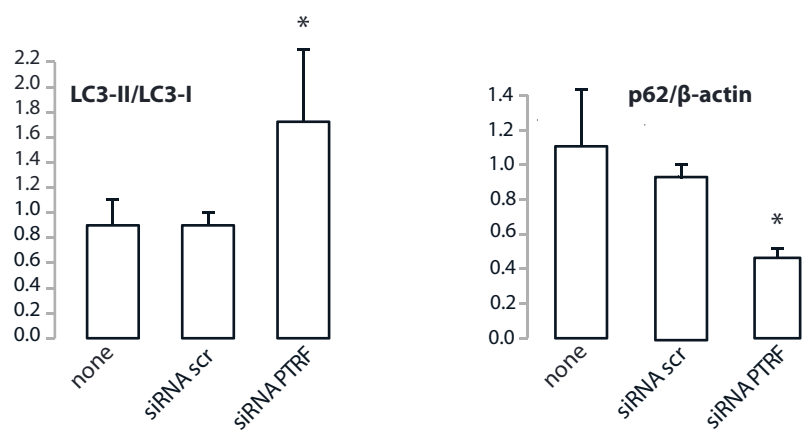
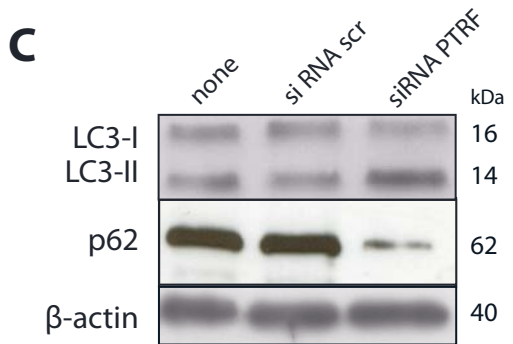
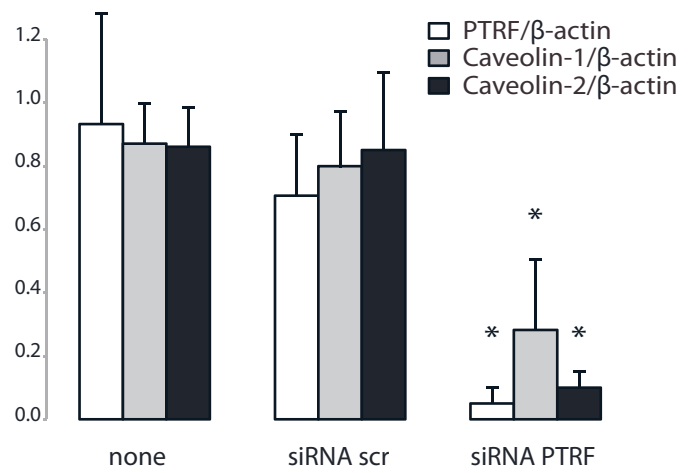
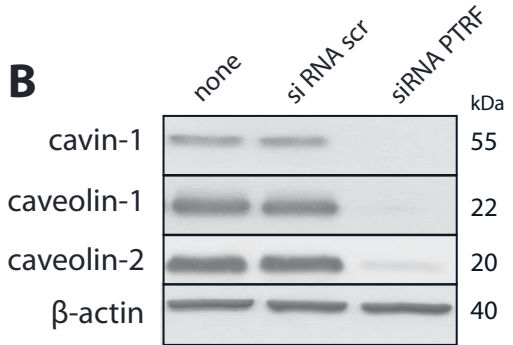
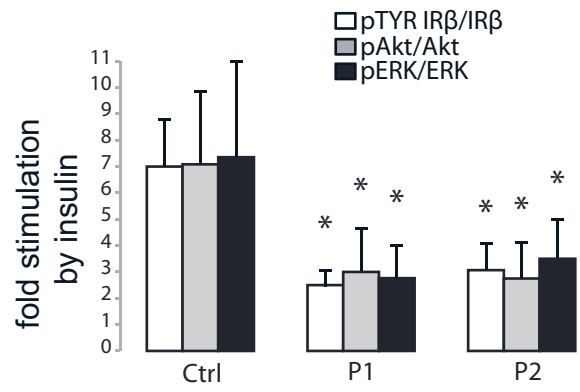
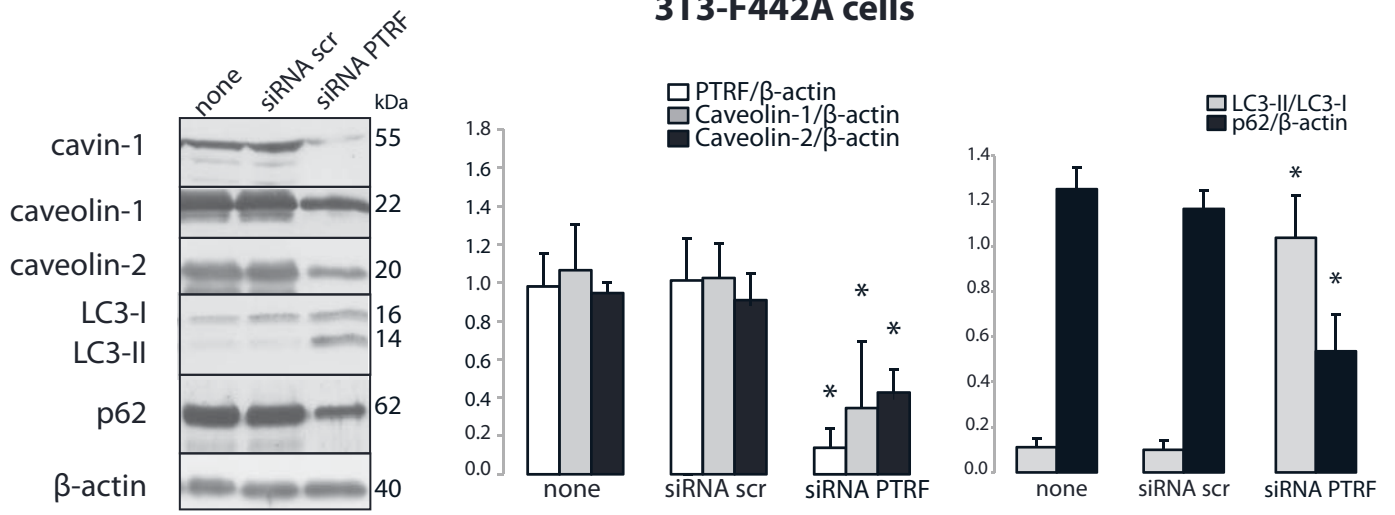


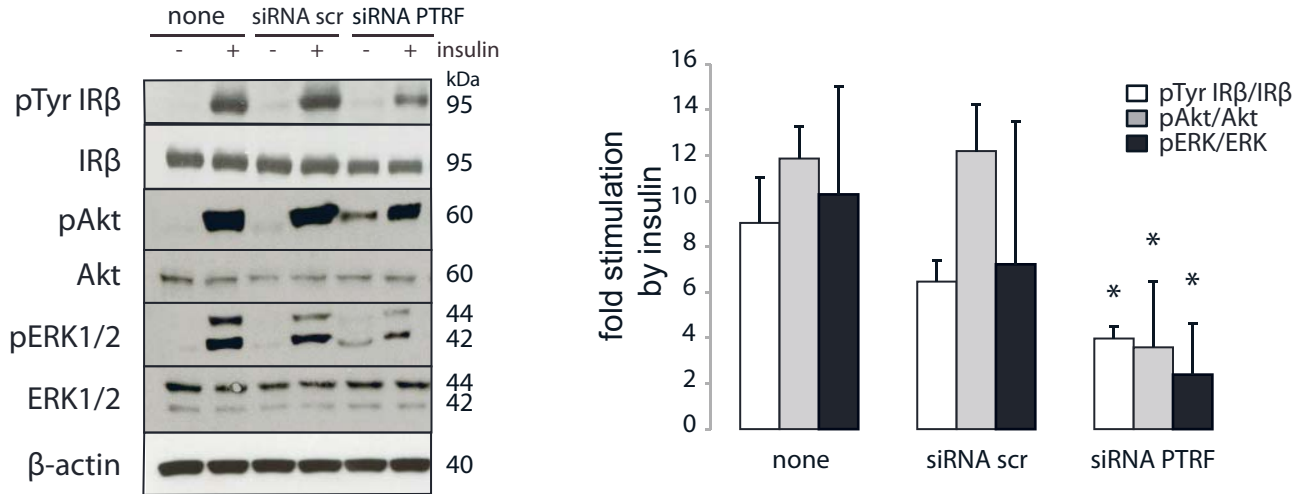
Figure 3

3T3-F442A cells

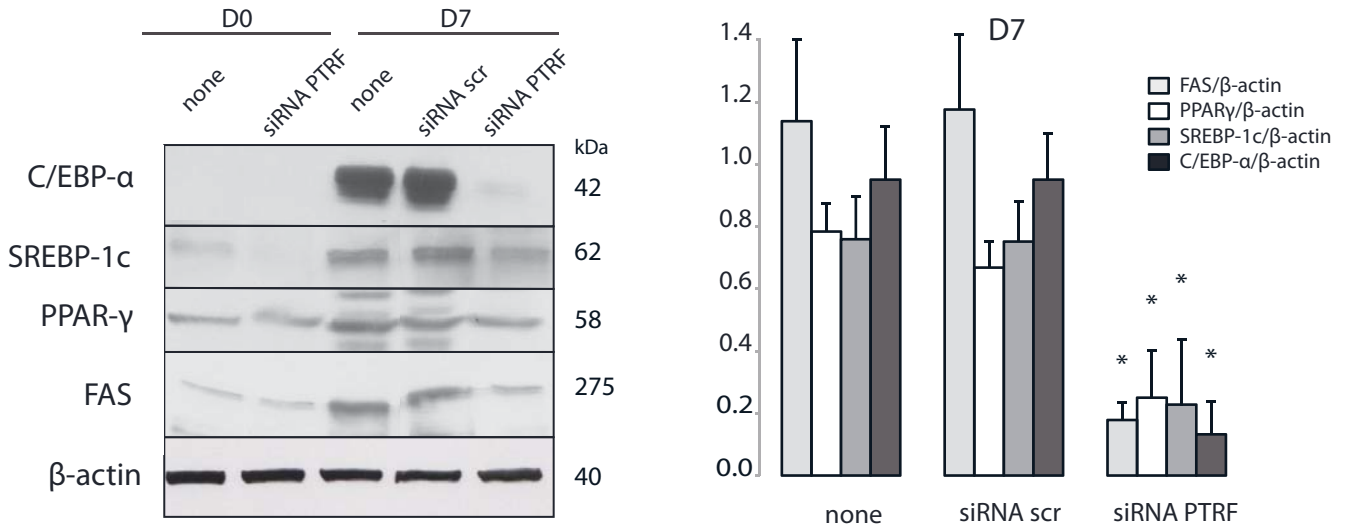
A



B



C



D

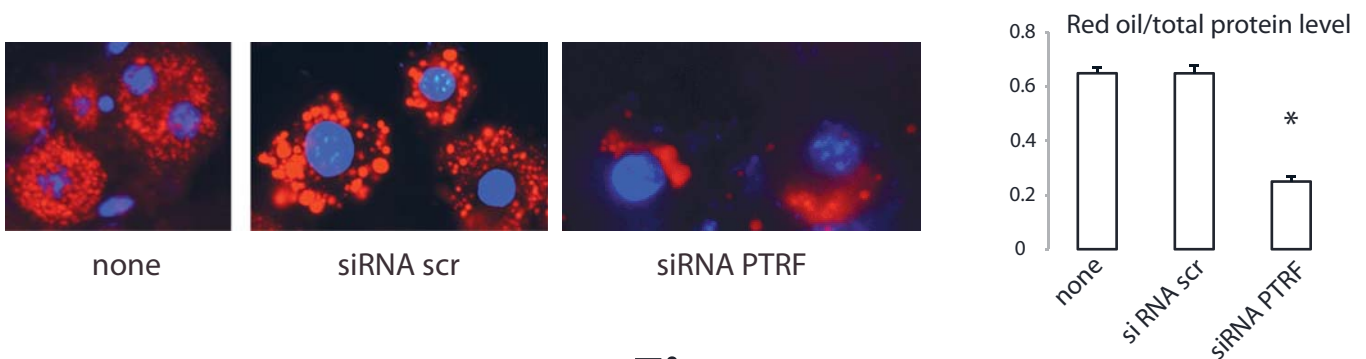
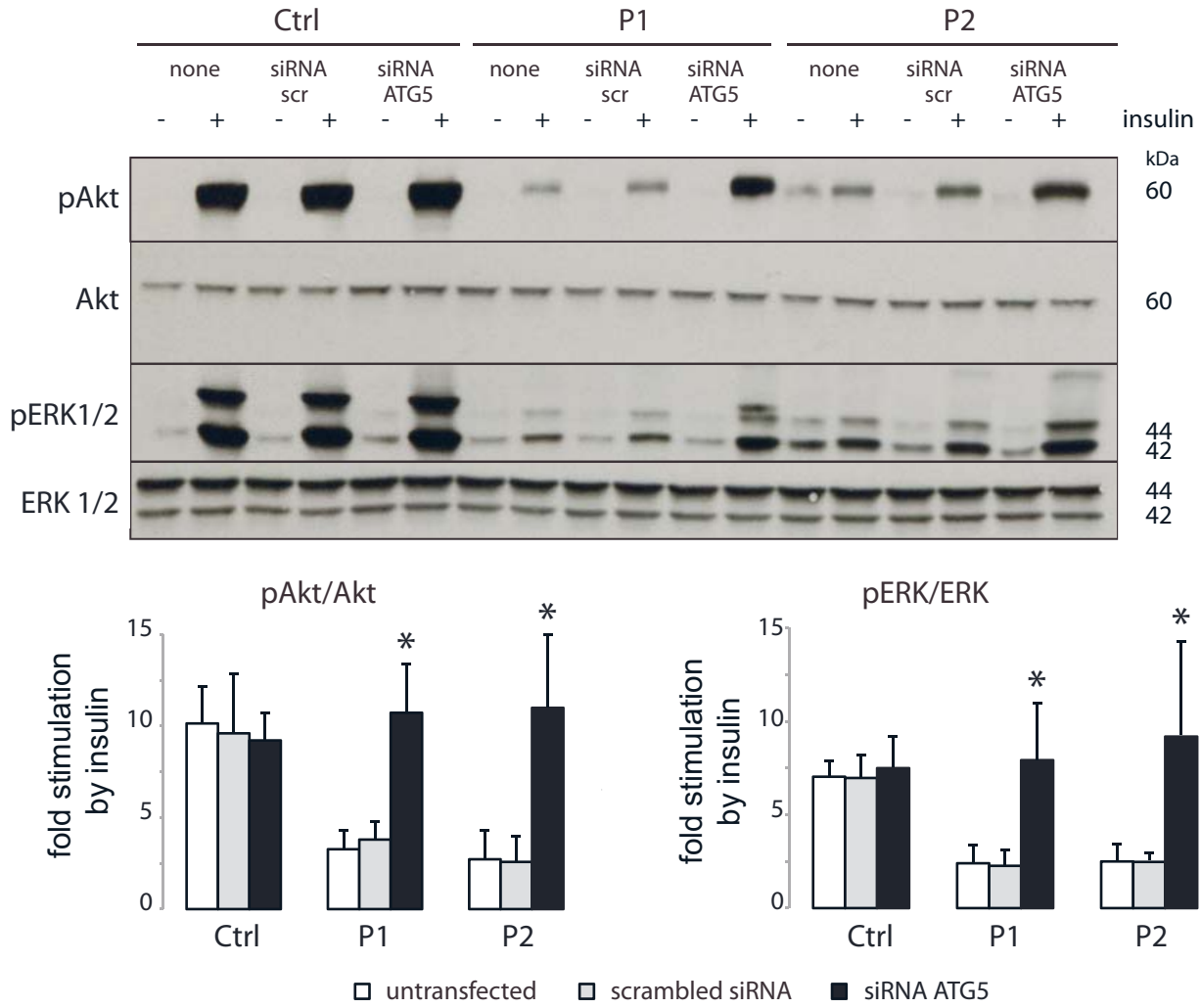


Figure 4

A Human fibroblasts



B 3T3-F442A cells at D7 of adipocyte differentiation

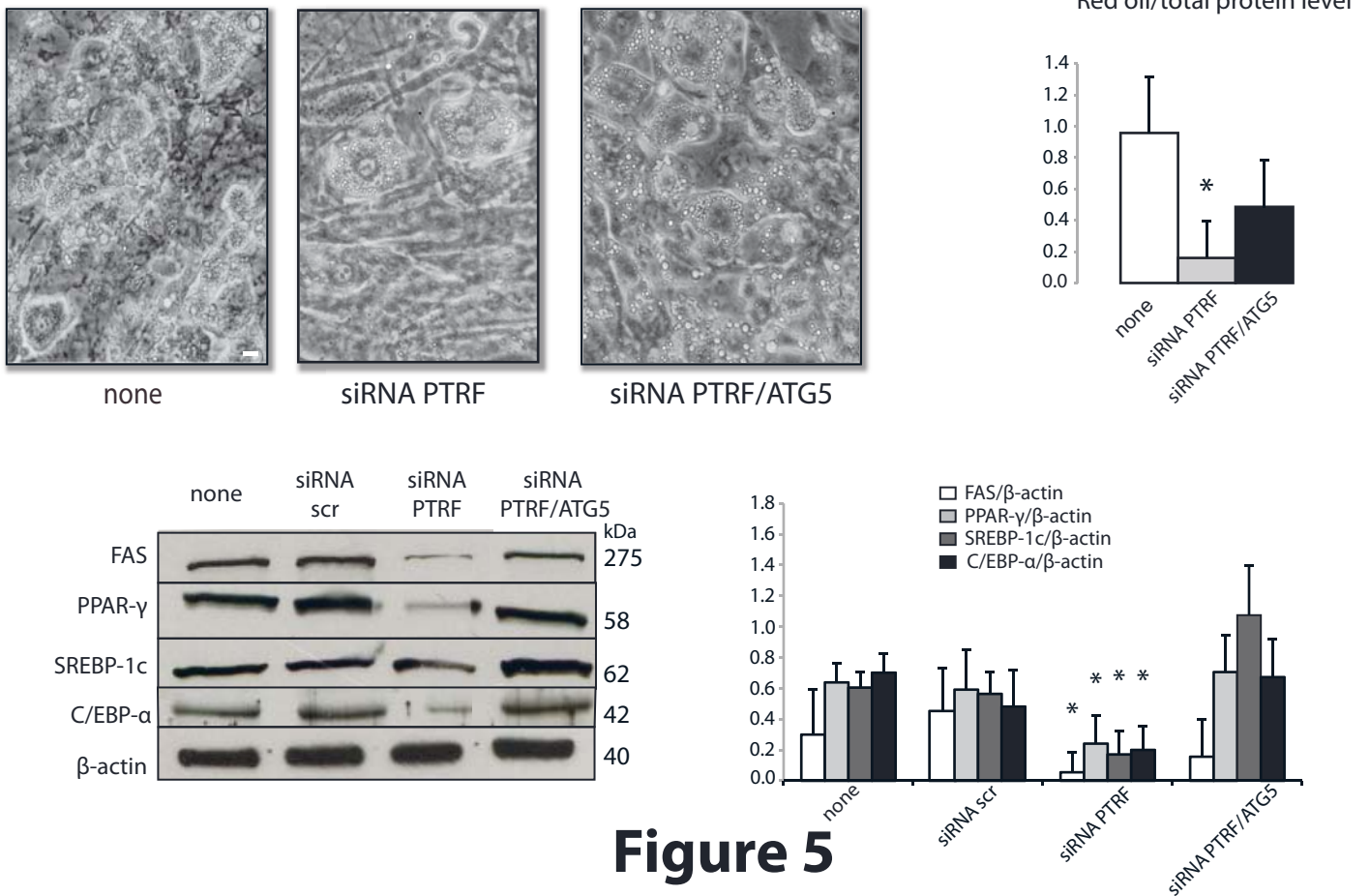


Figure 5