The LIM-Only Protein FHL2 is involved in autophagy to regulate the development of skeletal muscle cell

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Abstract

Four and a half LIM domain protein 2 (FHL2) is a LIM domain protein expressed in muscle tissue whose deletion is causative of myopathies. Although FHL2 has a confirmed important role in muscle development, its autophagy-related function in muscle differentiation has not been fully determined. To explore the role of FHL2 in autophagy-related muscle regulation, FHL2-silenced and -overexpressing C2C12 mouse cells were examined. Immunofluorescence and co-immunoprecipitation assay findings showed that FHL2 silencing reduced LC3-II protein expression and the amount of LC3 that co-immunoprecipitated with FHL2, indicating that FHL2 interacts with LC3-II in the formation of autophagosomes. Moreover, the expression of muscle development marker genes such as MyoD1 and MyoG was lower in FHL2-silenced C2C12 cells but not in FHL2-overexpressing C2C12 cells. Electron microscopy analysis revealed large empty autophagosomes in FHL2-silenced myoblasts, while flow cytometry suggested that FHL2 silencing made cells more vulnerable to staurosporine-induced cell death. In conclusion, we propose that FHL2 interacts with LC3-II in autophagosome formation to regulate the development of muscle cells.

Key words: FHL2, LIM-domain, autophagy, cell development, C2C12 cells

42 **Introduction**

43 Four and a half Lim domain protein 2 (FHL2) belongs to the FHL
44 protein family which contains five members: FHL1, FHL2, FHL3, FHL4,
45 and ACT [1]. Despite their high level of conservation among species,
46 their expression levels differ from each other between tissues. FHL1,
47 FHL2, and FHL3 are mainly expressed in skeletal and heart muscle [2],
48 whereas FHL4 and ACT are highly expressed in the testis [3]. FHL2 has
49 been shown to have a dual function in interacting with the cytoplasmic
50 domain of several integrin chains [4], and also as a transcriptional
51 coactivator of the androgen receptor [5]. Although FHL2 plays an
52 important role in muscle development and its deletion was reported to
53 lead to the development of myopathies [2, 6-10], the details of its
54 function in skeletal muscle development are unclear.

55 Autophagy is the major intracellular degradation system by which
56 cytoplasmic materials are delivered to and degraded in the lysosome,
57 which also serves as a dynamic recycling system that produces new
58 building blocks and energy for cellular renovation and homeostasis [11,
59 12]. Recently, the relationship between autophagy and muscle cell
60 development has been shown to play an important role in muscle mass
61 maintenance and integrity [13, 14]. As the main proteolytic system that
62 controls protein degradation in skeletal muscle cells, the autophagy
63 lysosome is activated in a number of catabolic disease states that lead to

64 muscle loss and weakness [15, 16]. Excessive activation of autophagy
65 aggravates muscle wasting by removing some cytoplasm, proteins, and
66 organelles; conversely, the inhibition or alteration of autophagy can
67 contribute to myofiber degeneration and weakness in muscle disorders
68 [17]. Additionally, autophagy protects against apoptosis during myoblast
69 differentiation [18].

70 Recently, a relationship between FHL2 and autophagy was identified,
71 with involvement of the FHL2-activated nuclear factor-κB pathway
72 reported in particulate matter 2.5-induced autophagy in mouse aortic
73 endothelial cells [19]. Muscle Lim protein (MLP)/CSRP3 was reported to
74 interact with microtubule-associated protein 1 light chain 3 (LC3) to
75 regulate the differentiation of myoblasts and facilitate autophagy [20].
76 Moreover, Sabatelli et al found that the aggresome–autophagy pathway
77 was involved in the pathophysiological mechanism underlying the muscle
78 pathology of the C150R mutation in the second LIM domain of *FHL1*
79 [21]. Because FHL2 contains the LIM domain, similar to FHL1 and
80 MLP/CSRP3, it is reasonable to speculate that FHL2 is involved in
81 autophagy to regulate the development of skeletal muscle. The present
82 study examined this hypothesis.

83 **Materials and Methods**

84 **Cell cultures**

85 The mouse C2C12 myoblast cell line (Fuheng Cell Center, Shanghai,

86 China) was maintained in growth medium composed of Dulbecco's
87 Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), and 1%
88 Antibiotic-Antimycotic (ABAM) at 37 °C under 5% CO_2. Differentiation
89 into myotubes was activated by replacing the growth medium with
90 differentiation medium composed of DMEM, 2% horse serum, and 1%
91 ABAM.

92 **FHL2 silencing and overexpression**

93 C2C12 cells were cultivated in 6-well plates and transfected with
94 siRNAs (sense: 5'-GCAAGGACUUGUCCUACAATT-3', antisense:
95 5'-UUGUAGGACAAGUCCUUGCTT-3'; Sangon Biotech, Shanghai,
96 China) when grown to a density of approximate 70% in plates. In contrast,
97 control cells were transfected with negative siRNA with same other
98 condition. The transfection reagent was Lipofectamine 3000 (Invitrogen,
99 Carlsbad, CA, USA). The knockdown efficiency was assessed by
100 quantitative RT-PCR of FHL2 mRNA and western blot assay of FHL2
101 protein.

102 C2C12 cells were transfected with a plasmid pcDNA3.1 which was
103 produced by cloning FHL2 cDNA into the pcDNA3.1 expression vector
104 (Sangon Biotech, Shanghai, China). The transfection reagent
105 Lipofectamine 3000 (Invitrogen) was mixed with optim-mem. Plasmid
106 with optim-mem was mixed with Lip3000. Next, they were mixed all
107 together at room temperature for 10 min. Finally, the mixture was added

108 into C2C12 6-well plates at 37 ℃ under 5% CO_2 for 48h.

109 **RNA extraction, cDNA synthesis and RT-PCR**

110 Total RNA was isolated using TRIzol (TAKARA, Dalian, China)
111 reagent according to the manufacturers' instruction. RNA was reverse
112 transcribed by TAKARA PrimeScript^{TM} RT reagent kit (TAKARA)
113 according to the manufacturers' instruction. Quantitative RT-PCR assay
114 was performed essentially as previously described [22].Primer are used in
115 Table 1.

116 **Western blot assay**

117 The cells were collected from the cultures, placed in the RIPA lysis
118 buffer on ice (BestBio, Shanghai, China). The whole proteins were
119 subjected to 10% sodium dodecyl sulfate polyacrylamide gel
120 electrophoresis (SDS-PAGE) and then transferred to polyvinylidene
121 fluoride membranes (PVDF; Millipore Corporation, Billerica, MA, USA).
122 The PVDF membrane was incubated with 5% defatted milk powder at
123 room temperature for 1 h, then incubation with the following specific
124 primary antibodies at 4℃ overnight: anti-FHL2 (Abcam, Cambridge,
125 MA, USA), anti-MYOD1 (Abcam), anti-MyoG (Abcam), anti-MYH3
126 (Abcam), anti-ATG5 (Cell Signaling, Danvers, MA, USA), anti-ATG7
127 (Cell Signaling), and anti-β-Actin (Abcam). The secondary antibodies
128 HRP-labeled mouse and rabbit IgG (Cell Signaling) were added at room
129 temperature for 1h. Following each step, the membranes were washed

130 five times with PBS-T for 3 min. The proteins were visualized by
131 enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway,
132 NJ, USA) with a Kodak imager (Eastman Kodak, Rochester, NY, USA).
133 Quantification of protein blots was performed using the Quantity One
134 1-D software (version 4.4.0) (Bio-Rad, Hercules, CA, USA) on images
135 acquired from an EU-88 image scanner (GE Healthcare, King of Prussia,
136 PA, USA).

137 **Microscopy**

138 Cellular morphology was evaluated in proliferating myoblasts and
139 differentiated myotubes by phase-contrast microscopy without
140 preliminary fixation. Pictures were produced using the Olympus IX73
141 inverted microscope (OLYMPUS, Tokyo, Japan) and the Hamamatsu
142 C11440 digital camera (HAMAMATSU, Shizuoka, Japan).

143 **Transmission electron microscopy**

144 C2C12 myoblasts or myotubes were detached from the plates using
145 a manual scraper, washed with PBS for a while. Then the cells were
146 suspended and fixed overnight at 4 °C in 2% glutaraldehyde with 1%
147 tannic acid in 0.1M sodium cacodylate, pH 7.3. The cells were rinsed
148 three times in the sodium cacodylate buffer and incubated in 2% osmium
149 tetroxide in the same buffer for 2 h at room temperature. Afterwards, the
150 cells were rinsed three times in distilled water and exposed to 1% uranyl
151 acetate in water for 15 min at room temperature and twice in distilled

152 water. Next, the cells were spun down into 3% agarose at 45 °C and
153 cooled to form blocks. The agarose blocks were dehydrated in graded
154 steps of acetone and embedded in Spurr's low-viscosity media. Following
155 polymerization overnight at 65 °C, 80-nm sections were cut on a
156 Reichert-Jung Ultracut E ultramicrotome and picked up on copper grids.
157 The grids were post-stained in uranyl acetate and bismuth subnitrate. The
158 sections were observed on a Philips CM-10 TEM (HT7700) and
159 micrographs were recorded on a Kodak 4489 sheet film (Eastman
160 Kodak).

161 **Immunofluorescence assay**

162 Dry the cell climbing slides slightly and mark the objective area with
163 liquid blocker pen for 20 min at room temperature, where add 50-100 μl
164 of permeabilize working solution. Then eliminate obvious liquid, mark
165 the objective tissue with liquid blocker pen. Cover objective area with 3%
166 BSA at room temperature for 30 min and incubate cells with primary
167 antibody overnight at 4 °C, placed in a wet box. Cell climbing slides were
168 covered with secondary antibody labelled with HRP at room temperature
169 for 50 min. Then DAPI solution was added for incubation at room
170 temperature for 10 min, kept in dark place. Put the slides on a glass
171 microscope slide and then mount with resin mounting medium. Finally,
172 Microscopy detection and images collection were performed by
173 Fluorescent Microscopy (OLYMPUS).

174 **Co-immunoprecipitation assay**

175 Protein concentration was determined using the BCA Protein
176 Quantitation Kit (BestBio). 1mg of lysate was mixed with lysis buffer
177 including phosphatase inhibitor to a volume of 1ml. Then Lysates were
178 precleared with 5 μg of appropriate control IgG (Santa Cruz
179 Biotechnology) and 20 μl of protein A/G plus-agarose (Santa Cruz
180 Biotechnology) for 1 h rotation at 4 °C. Lysates were centrifuged (500 ×
181 g for 5 min at 4 °C) and 5 μg of FHL2 (Abcam) or LC3-II antibody
182 (Abcam) or corresponding IgG was added to the precleared lysates and
183 kept on ice for ~ 4 h. After incubation, 30 μl of protein A/G plus-agarose
184 was added to each tube and kept on a rotator overnight at 4 °C. Lysates
185 were then centrifuged (500 × g for 5 min at 4 °C). The pellet fractions
186 were washed four times with PBS-PI and then resuspended in 20 μl of
187 loading buffer. Samples were electrophoresed on a 12% SDS-PAGE gel
188 and immunoblotted with the appropriate antibody.

189 **Flow cytometry assay**

190 Apoptosis was induced by treating cells with 2uM Staurosporine
191 (STS) (Selleckchem, Houston, TX, USA) in 12-well plates. Then cells
192 were collected and digested by pancreatic enzyme to be cell suspension.
193 Then the cells were washed twice with cold PBS and then resuspended
194 cells in 1×binding buffer (BD Pharmingen, Santiago, CA, USA) at a
195 concentration of 1×106 cells/mL. One hundred microliters of the solution

196 were transferred to a 5-mL culture tube, and then 5 μL of Annexin
197 V-FITC (BD Pharmingen) and 5 μL of PI (BD Pharmingen) were added.
198 The cells were gently vortexed and incubated for 15 min at room
199 temperature (25 °C) in the dark. Four hundred microliters of 1×binding
200 buffer was added to each tube and analyzed by flow cytometry (BD
201 FACSCalibur, BD Pharmingen) within 1 h.

202 **Statistical analysis**

203 All statistical analyses were performed using SPSS 17.0 (SPSS Inc.,
204 Chicago, IL, USA). Data are presented as least squares means ± standard
205 error of the mean (SEM), and values were considered statistically
206 different at $P<0.05$.

207 **Results**

208 **The role of FHL2 in skeletal muscle differentiation**

209 To explore the potential role of FHL2 in skeletal muscle
210 differentiation, we performed a knockdown assay in C2C12 myoblasts
211 derived from mouse satellite cells. C2C12 cells transfected with FHL2
212 siRNA or scrambled siRNA were induced to differentiate, and *FHL2*
213 mRNA expression was shown to be reduced significantly after
214 knockdown in both myoblasts and myotubes compared with controls (Fig.
215 1A). Western blot analysis revealed a decrease in FHL2 protein in
216 FHL2-silenced cells compared with control cells (Fig. 1B). Next,
217 morphological differences between negative control and FHL2

218 siRNA-transfected groups were compared during C2C12 differentiation
219 into myotubes. The FHL2-silenced group showed reduced myotube
220 formation (Fig. 1C), and the expression of myogenic marker genes
221 *MyoD1*, *MYH3*, and *MyoG* was significantly reduced in FHL2-silenced
222 cells compared with controls (Fig. 1D–F). Moreover, western blotting
223 revealed that MYH3 and MyoG protein levels were reduced after FHL2
224 silencing (Fig. 1G). These results suggest that FHL2 siRNA was effective
225 and that FHL2 plays an important role in muscle differentiation by
226 regulating myogenesis-related genes. The overexpression of FHL2 in
227 C2C12 myoblasts and myotubes (Fig. 2A and B) had no significant effect
228 on *MyoG*, *MyoD1*, or *MYH3* expression levels (Fig. 2C–E).

229 **FHL2 regulated autophagy in skeletal muscle cells**

230 To determine whether FHL2 silencing in skeletal muscle influenced
231 the induction of autophagy, the expression of autophagy genes *ATG5* and
232 *ATG7* was measured and shown to be significantly reduced in myoblasts
233 and myotubes from FHL2-silenced cells compared with control cells (Fig.
234 3A). Next, LC3 protein level changes were examined to monitor
235 autophagy induction, and the ratio of LC3-II to LC3-I protein was found
236 to be reduced in FHL2-silenced myoblasts and myotubes compared with
237 controls (Fig. 3B). Interestingly, the starvation of FHL2-silenced
238 myoblasts and myotubes, which should have been able to activate
239 autophagy, did not induce the accumulation of LC3-II (Fig. 3B).

240 Moreover, FHL2 overexpression did not significantly influence the
241 expression of *ATG5* or *ATG7* (Fig. 3C).

242 To further confirm our findings, we used TEM to observe the
243 ultrastructure of myoblasts. As shown in Fig. 4, both negative control and
244 FHL2-overexpressing myoblasts were observed to contain normal
245 autophagosomes whereas large empty autophagosomes were present in
246 FHL2-silenced myoblasts, which was indicative of impaired autophagy.

247 **FHL2–LC3-II co-localization**

248 Immunofluorescence and co-immunoprecipitation assays were next
249 performed to determine whether FHL2 co-localized with the
250 autophagosomal marker LC3 during myoblast differentiation.
251 Immunofluorescence analysis showed similar FHL2 and LC3 protein
252 distribution in myoblasts (Fig. 5A), while FHL2 silencing decreased both
253 FHL2 and LC3II expression which was indicative of their co-expression
254 (Fig. 5A). The co-immunoprecipitation assay revealed co-localization of
255 FHL2 and LC3 proteins in C2C12 cells during myoblast differentiation,
256 and reduced co-immunoprecipitation in FHL2-silenced myoblasts
257 compared with control cells (Fig. 5B). These results support a role for
258 FHL2 in the formation of autophagosomes by binding LC3 in an
259 intracellular complex.

260 **FHL2 silencing promoted apoptosis in both myoblasts and myotubes**

261 Flow cytometry was next used to measure cell death after treatment

262 of C2C12 cells with 2 μM STS for 12 h. Cell death was observed in 2.74%
263 and 3.51% of wild-type (WT) myoblasts and myotubes, respectively,
264 compared with 6.30% and 10.77%, respectively, in si-FHL2 groups (Fig.
265 6B and D). This indicated that FHL2 silencing enhanced apoptosis in
266 both myoblasts and myotubes. Moreover, a decreased resistance to STS
267 was detected in FHL2-silenced myoblasts and myotubes (Fig. 6A–D).
268 Western blotting of PARP and caspase-3 proteins under different
269 conditions revealed the increased accumulation of PARP in myoblasts
270 following FHL2 silencing (Fig. 6E). Caspase-3 protein expression was
271 not detected in WT cells, but was observed in si-FHL2 myotubes,
272 suggesting an important role for FHL2 in protecting myotubes from
273 apoptosis (Fig. 6E). We also found that myotubes were more resistant to
274 STS-induced apoptosis than myoblasts (Fig. 6E).

275 **Discussion**

276 Although previous studies suggested that FHL2 is involved with
277 autophagy and may be associated with LC3 protein [19, 23], its
278 autophagy-related role in muscle differentiation had not been fully
279 determined. In this study, we explored the mechanism of FHL2 in muscle
280 differentiation through autophagy induction.

281 We initially confirmed that FHL2 played a role in muscle
282 development by measuring the gene and protein expression of
283 muscle-related MyoD1, MyH3, and MyoG after FHL2 silencing and

overexpression. FHL2 expression was significantly lower after siRNA transfection, indicating the efficiency of siRNA. Expression of the muscle-related genes was decreased in FHL2-silenced myoblasts or myotubes, and their protein expression was slightly reduced. However, no reduction in their expression was detected in FHL2-overexpressing cells. Those results confirm that FHL2 play a part in muscle development.

Previous studies detected LC3 protein accumulation during muscle cell development by western blotting [24, 25]. We investigated the relationship between FHL2 and autophagy using two methods: first, measuring the expression of genes essential for autophagy such as *ATG5* and *ATG7* [26] following FHL2 knockdown, and second, measuring the correlation between FHL2 and LC3II. Knockdown, but not the overexpression, of FHL2 significantly decreased the expression of *ATG5* and *ATG7* in both myoblasts and myotubes. Additionally, the autophagy level as indicated by the ratio of LC3-II to LC3-I protein [27] was reduced in FHL2-silenced myoblasts and myotubes. These results indicate a role for FHL2 in autophagy induction.

It is reasonable to assume that the function of FHL2 in muscle development is associated with autophagy because similar effects on muscle-related genes and autophagy markers were detected following FHL2 silencing or overexpression. Moreover, the starvation that activated autophagy in control cells did not induce LC3-II accumulation in either

306 FHL2-silenced myoblasts or myotubes, indicative of a function for FHL2
307 in autophagy induction. Further TEM analysis showed that FHL2
308 knockdown caused organelle abnormalities and impaired autophagy in
309 C2C12 cells, but not in control or FHL2-overexpressing C2C12 cells. The
310 persistent activation of catabolic pathways in muscle causes atrophy and
311 weakness, and autophagy is required to maintain muscle mass [17, 28].
312 Therefore, our results suggest that FHL2 has a role in myotube formation
313 by activating cell autophagy to maintain cellular homeostasis.

314 MLP/CSRP3 containing a LIM domain has been reported to
315 participate in muscle differentiation and autophagosome formation by
316 interacting with LC3-II [20]. FHL2 also contains four and a half LIM
317 domains that act as a protein–protein binding interface involved in muscle
318 differentiation [29]. Considering the potential of FHL2 to bind LC3-II, its
319 correlation with LC3-II at the protein level in C2C12 cells, and its likely
320 role in the assembly of extracellular membranes [30], we hypothesized
321 that FHL2 participates in autophagosome formation by interacting with
322 LC3-II. We therefore next explored their localization in C2C12 cells. The
323 protein distribution of FHL2 in myoblasts was shown to resemble that of
324 LC3-II, and immunoprecipitation revealed the co-localization of LC3-II
325 and FHL2. More importantly, FHL2 silencing simultaneously reduced
326 both FHL2 and LC3 protein expression. These findings provided
327 evidence of a role for FHL2 in muscle development by interacting with

328 LC3-II and participating in the formation of autophagosomes.

329 Insufficient autophagy contributes to the accumulation of waste
330 material inside the cell and the induction of apoptosis. However,
331 autophagy can also protect cells from apoptosis [31]. Study has revealed
332 autophagy protects myoblasts against apoptosis [32]. In a previous study,
333 the specific autophagy inhibitor 3MA [33] was used to treat C2C12 cells,
334 resulting in increased *CASP3* activity which is a marker of increased
335 apoptosis [18]. If FHL2 functions in the induction of autophagy,
336 FHL2-silenced C2C12 cells would be expected to show more apoptosis.
337 Indeed, we observed increased STS-induced death of both FHL2-silenced
338 myoblasts and myotubes compared with controls. These results suggested
339 that FHL2 knockdown reduces autophagy, indicating that it regulates
340 muscle development by controlling autophagy and thereby FHL2 may
341 regulate muscle development through autophagy against apoptosis.
342 However, exactly how autophagy prevents apoptosis requires additional
343 study. Furthermore, to validate the results of flow cytometry, the protein
344 levels of cleaved caspase-3 and PARP-1, markers of apoptosis [34, 35],
345 were measured in WT and STS-treated groups by western blotting.
346 Increased cleavage of PARP-1 and caspase-3 was observed in
347 FHL2-silenced cells relative to controls, and increased PARP-1 cleavage
348 was also detected during the differentiation of FHL2-silenced cells. These
349 results reflect increased apoptosis in the absence of FHL2, likely caused

350 by repressed autophagy.

351 In conclusion, our findings suggest that FHL2 interacts with LC3-II
352 protein to regulate muscle development through autophagy. As a result,
353 the deletion of FHL2 inhibited muscle development and damaged
354 autophagosomes. However, further studies of mechanism of autophagy in
355 muscle cells are needed to understand in detail how FHL2 regulates
356 autophagy and its contribution to muscle development or myopathies.

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363 **Competing Interests**

364 The authors have declared that no competing interest exists.

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Table 1. Primers used for quantitative real-time PCR

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
FHL2	TGCGTGCAGTGCAAAAAG	TGTGCACACAAAGCATTCCT
MyoD1	AGCACTACAGTGGCGACTCA	GGCCGCTGTAATCCATCA
MyoG	TACAGCGACCAACAGTACGC	TCTGCATTGTTTCCATCCTG
MYH3	CGGCTGCCTAAAGTGGAGAT	AGGCCTGTAGGCGCTCAA
ATG5	AGCAGCTCTGGATGGGACTGC	GCCGCTCCGTCGTGGTCTGA
ATG7	GCTCCTCATCACTTTTTGCCAACA	GGAGCCACCACATCATTGC
β-actin	CGTGAAAAGATGACCCAGATCA	CACAGCCTGGATGGCTACGT

Fig 1a

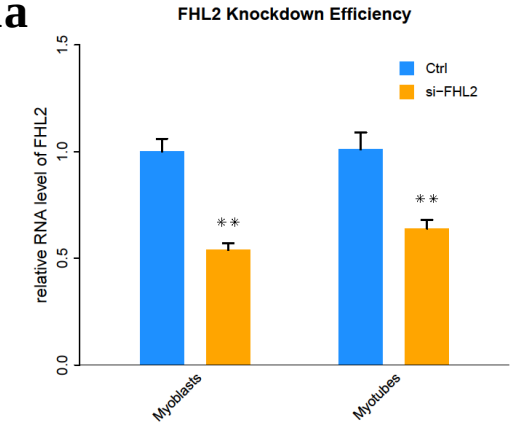


Fig 1b

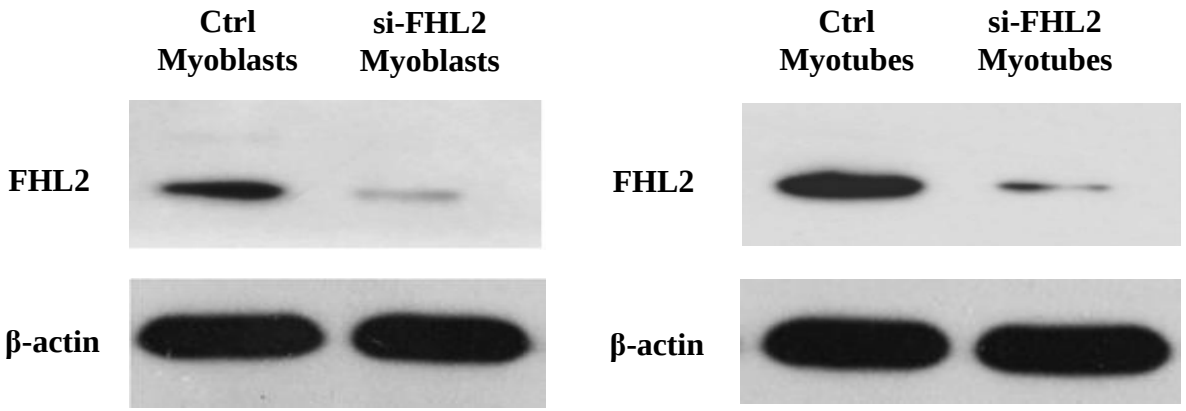
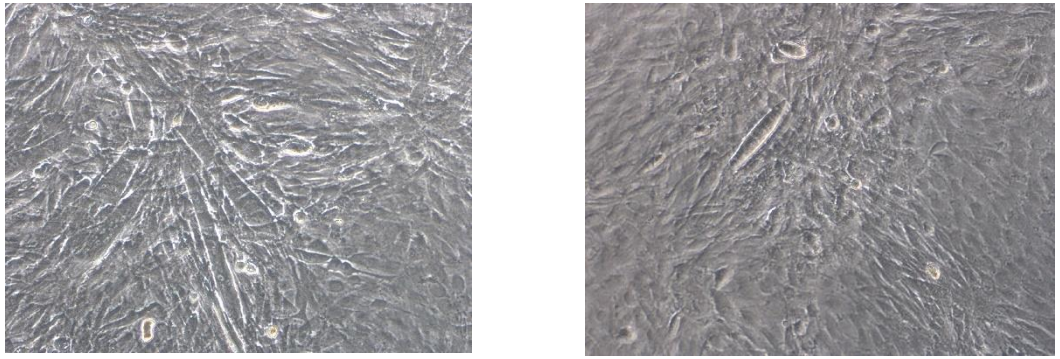


Fig 1c



control

si-FHL2

Fig 1g

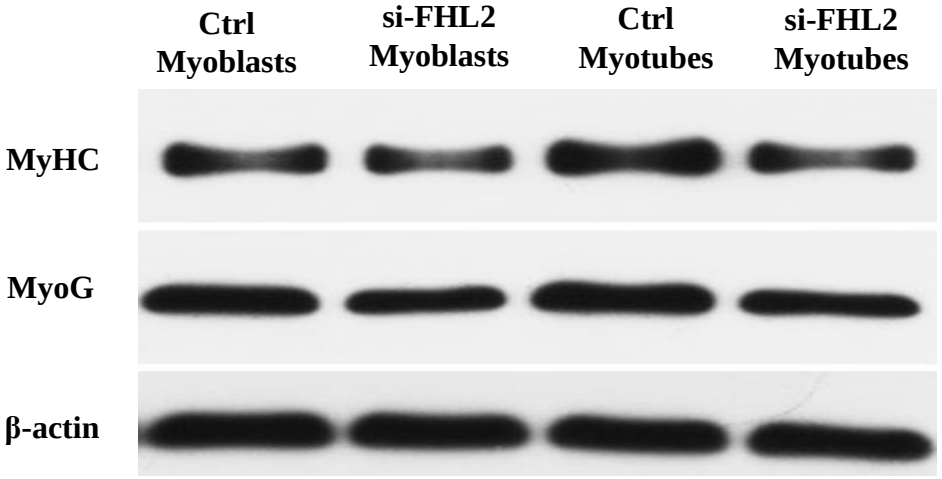


Fig 1 d-f

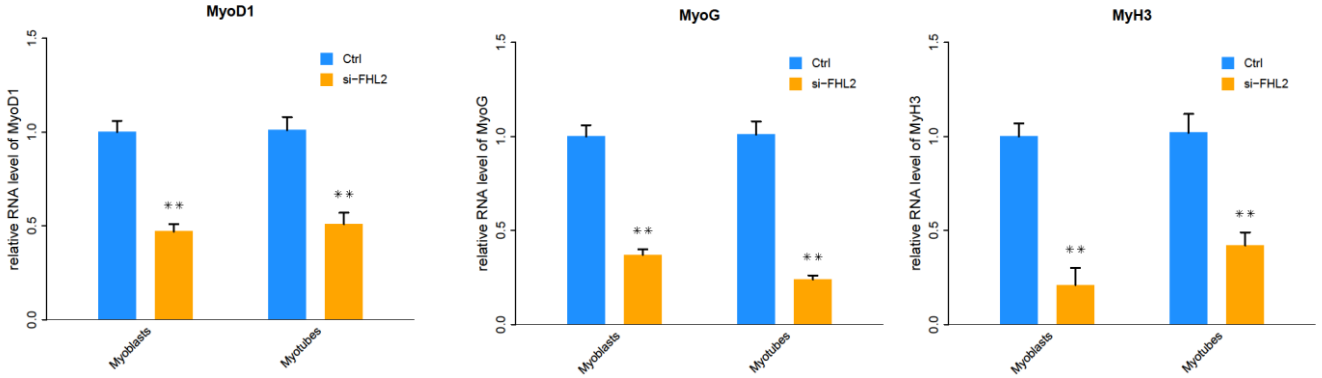


Fig 2a

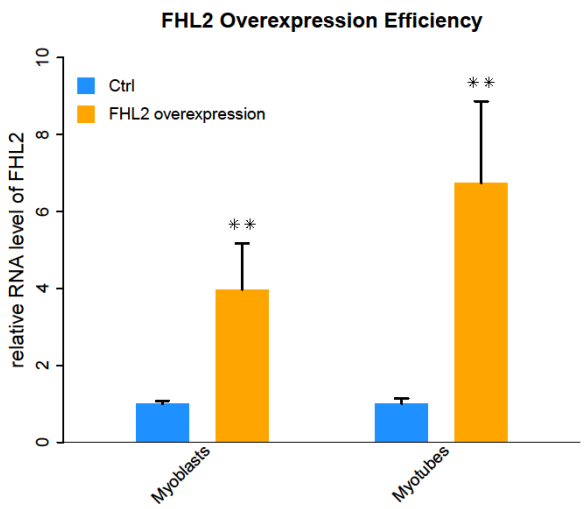


Fig 2b

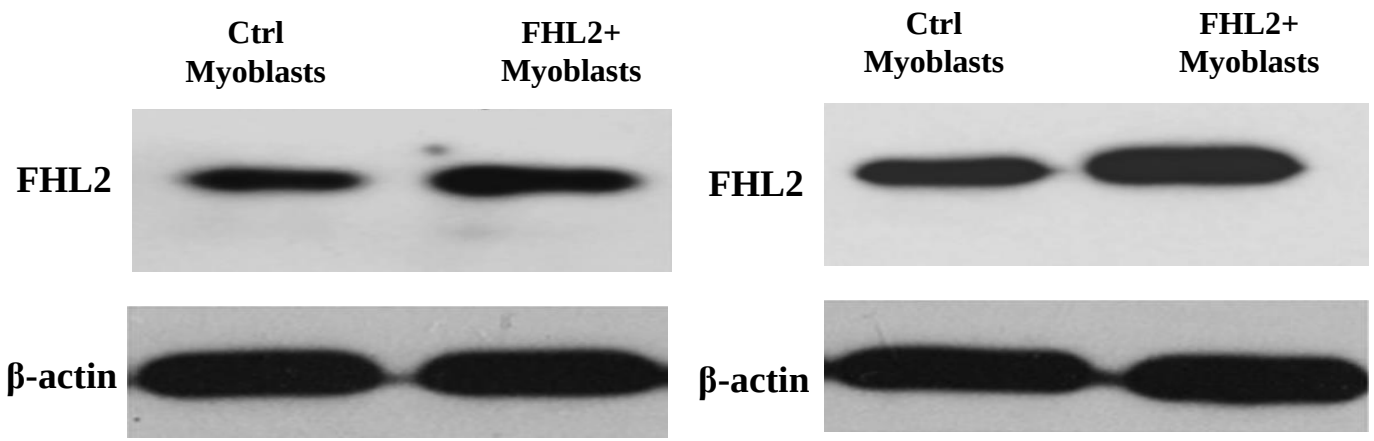


Fig 2 c-e

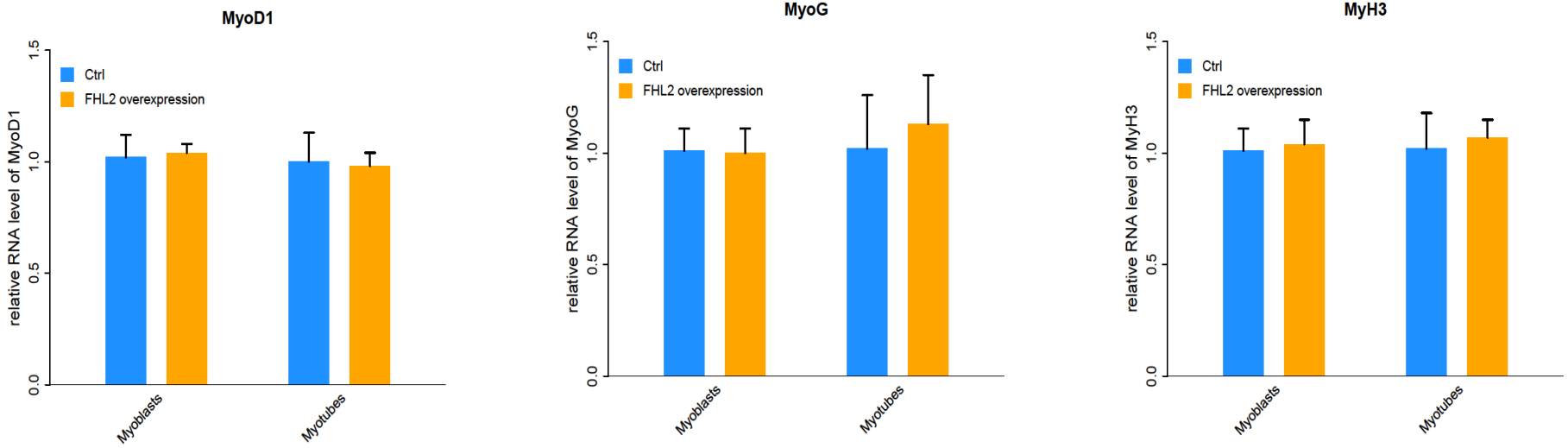


Fig 3a

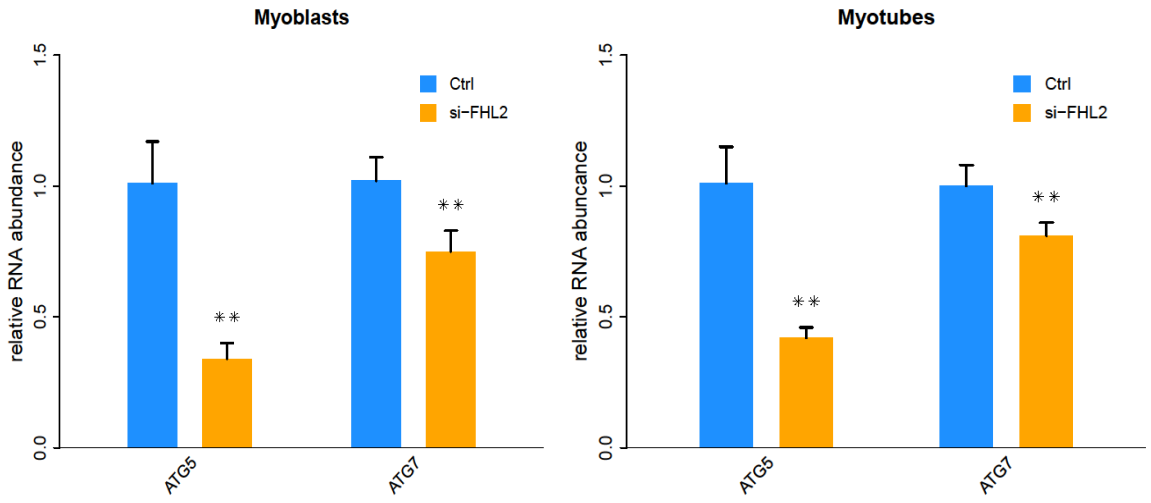


Fig 3b

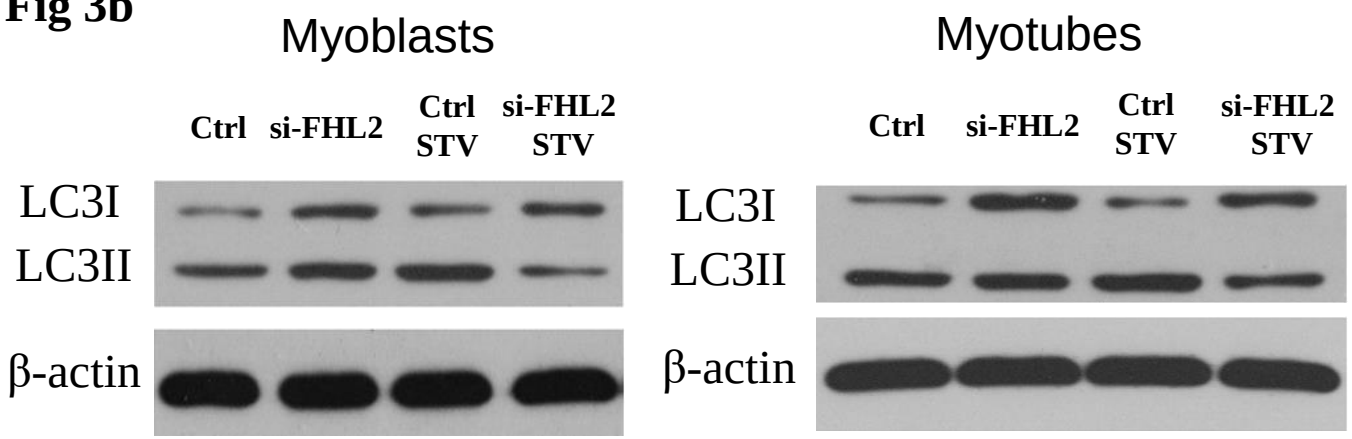


Fig 3c

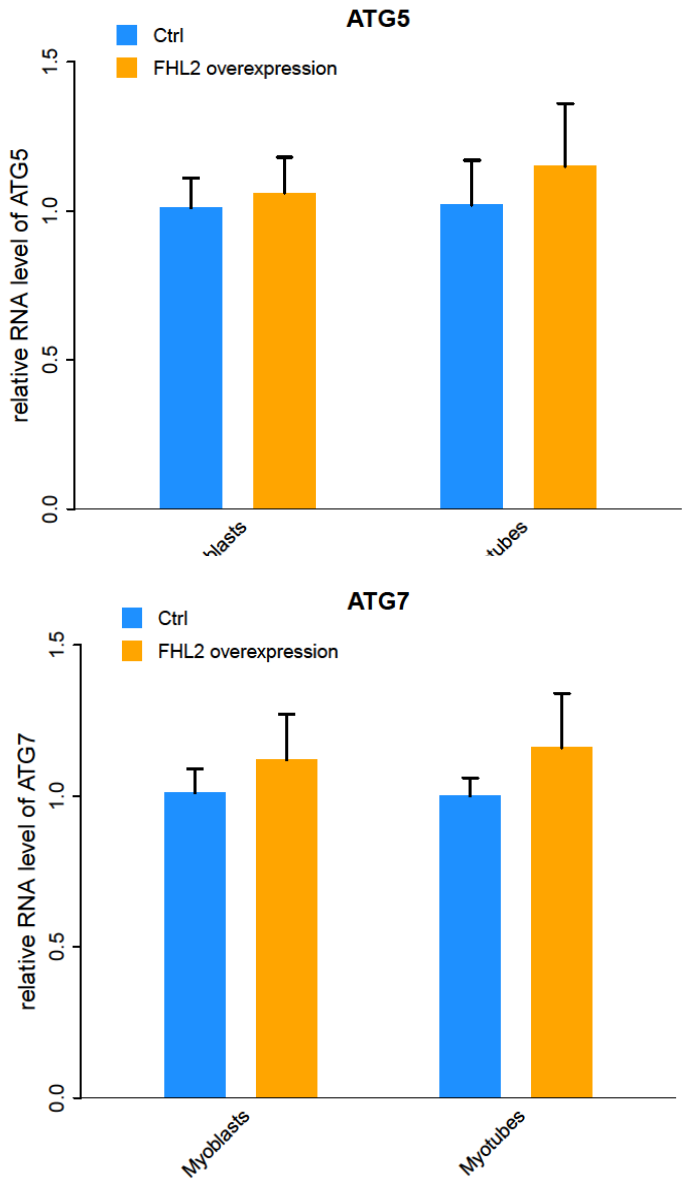
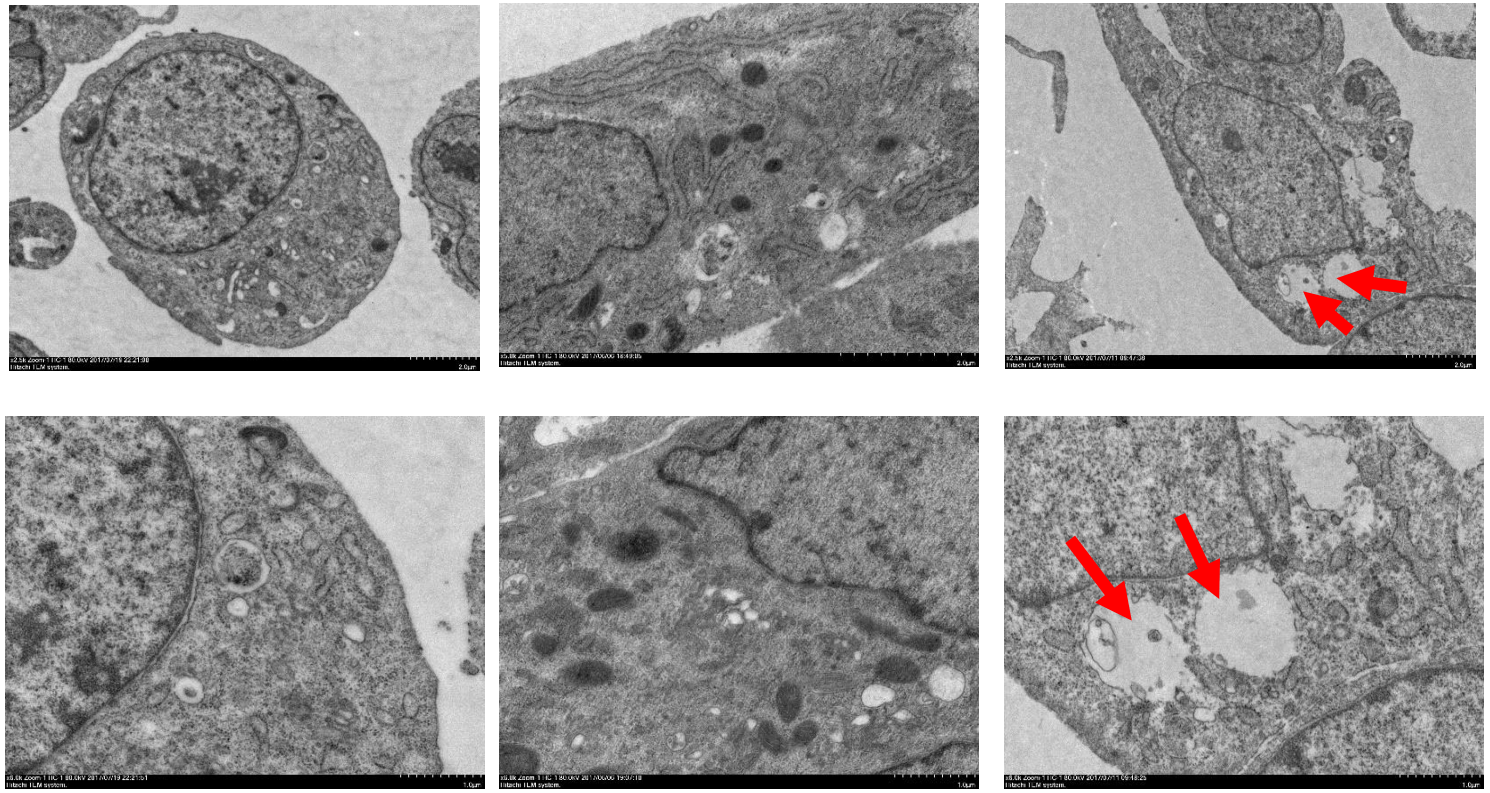


Fig 4

Fig 4a **Fig 4b** **Fig 4c**

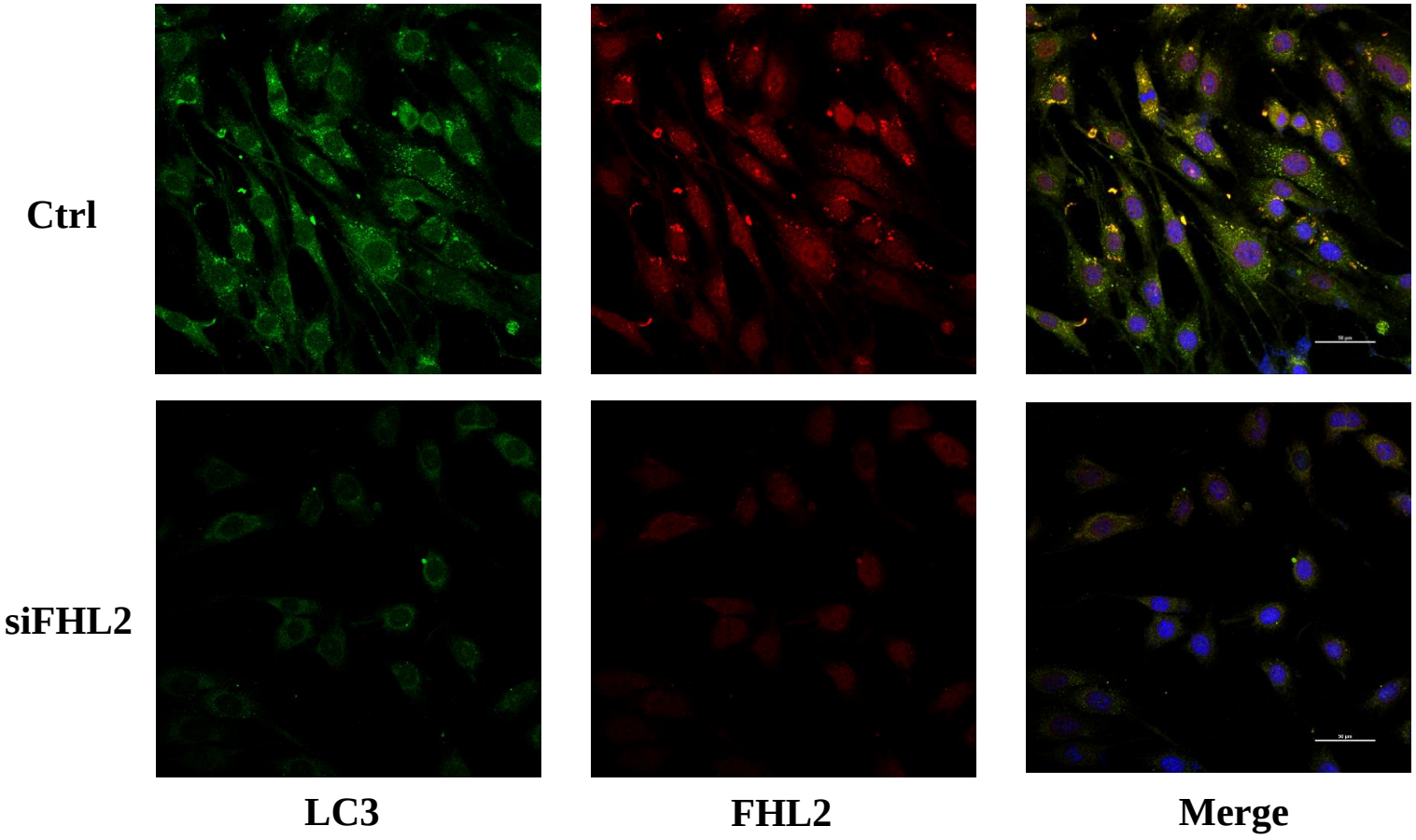
X2500

X6000



ctrl **FHL2 overexpression** **FHL2 knockdown**

Fig 5a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LC3
FHL2
Merge

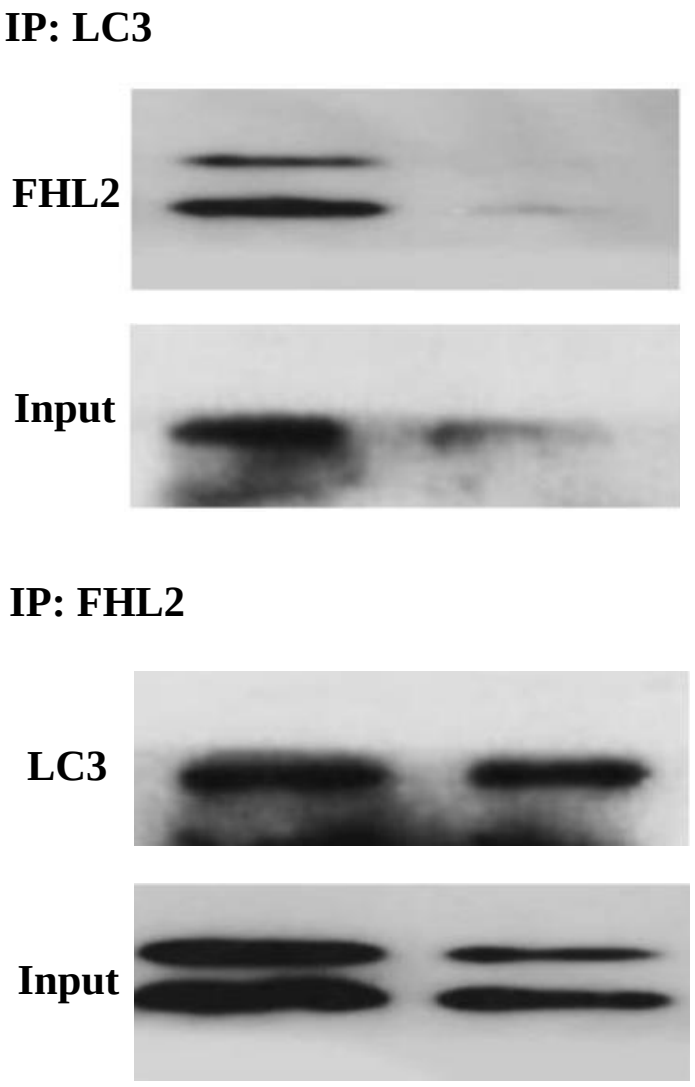

IP: LC3
FHL2
Input
IP: FHL2
LC3
Input

Fig 6a

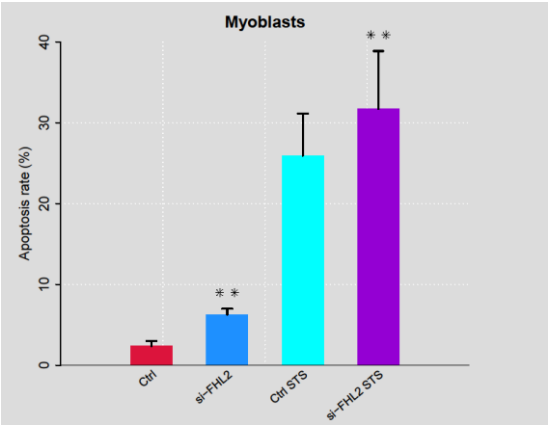


Fig 6c

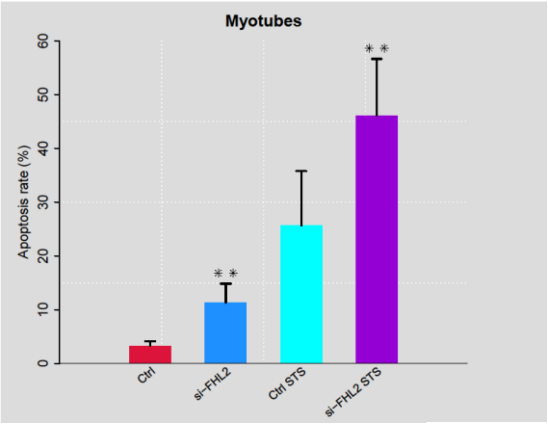
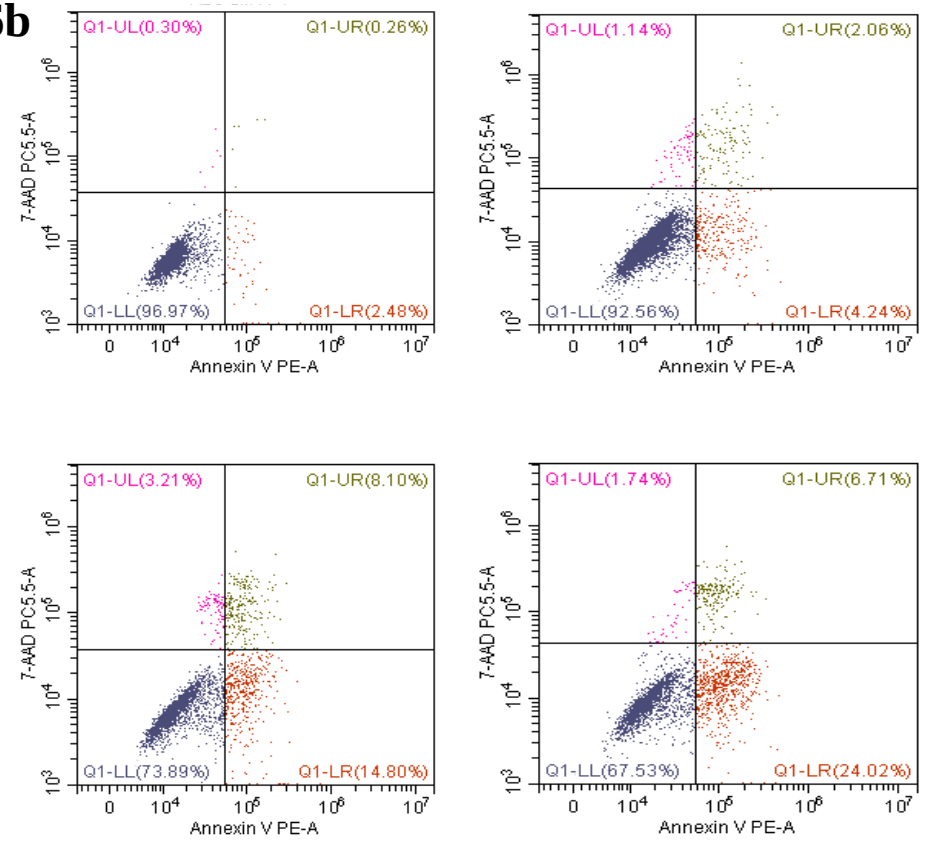
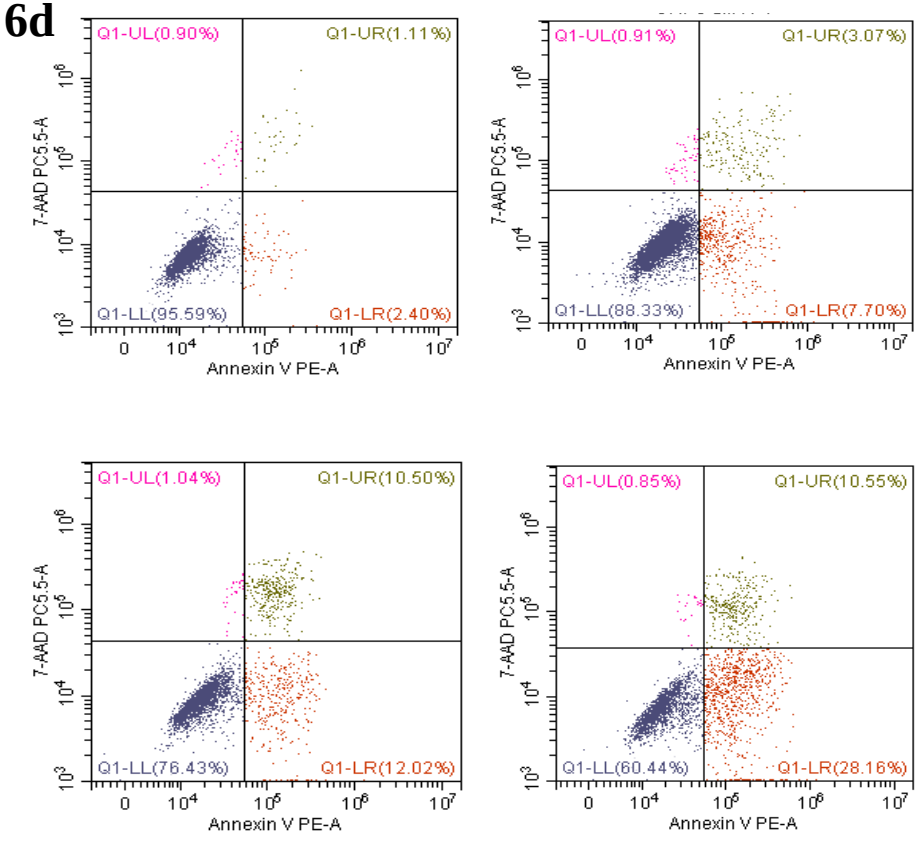


Fig 6b



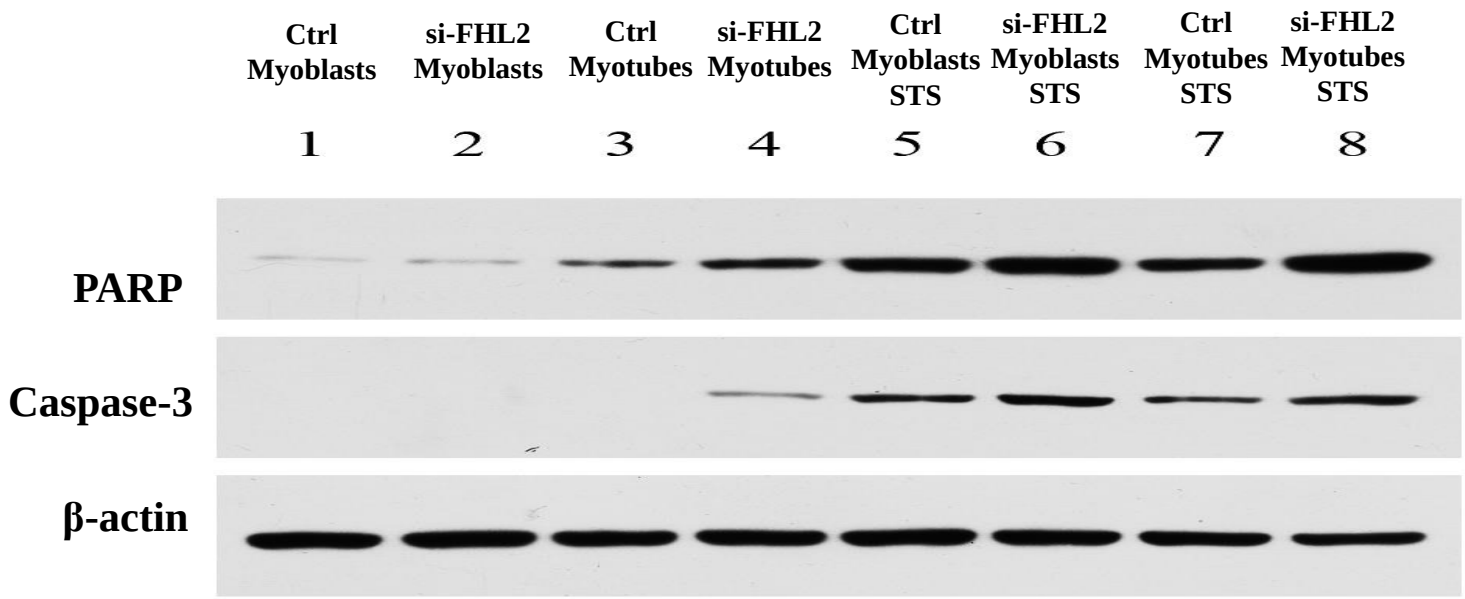
Ctrl + STS **SiRNA+ STS**

Fig 6d



Ctrl + STS **SiRNA+ STS**

Fig 6e


Ctrl Myoblasts
si-FHL2 Myoblasts
Ctrl Myotubes
si-FHL2 Myotubes
Ctrl Myoblasts STS
si-FHL2 Myoblasts STS
Ctrl Myotubes STS
si-FHL2 Myotubes STS
1
2
3
4
5
6
7
8
PARP
Caspase-3
β-actin