Supplemental data

The E3 ubiquitin ligase TRAF6 intercedes starvation-induced skeletal muscle atrophy through multiple mechanisms

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The file contains:

Supplemental Figure Legends

Supplemental Figures S1-S7.

SUPPLEMENTAL FIGURES' LEGENDS

FIGURE S1. Role of TRAF6 in soleus muscle atrophy in response to starvation. 12-week-old $TRAF6^{f/f}$ and $TRAF6^{mko}$ mice were starved for 24h. **(A)** Representative photomicrographs of H&E-stained sections of soleus muscle sections of $TRAF6^{f/f}$ and $TRAF6^{mko}$ mice 24h after starvation. Scale bar: 20µm. **(B)** Average fiber cross-sectional area (CSA) in soleus muscle of control and 24h starved $TRAF6^{f/f}$ and $TRAF6^{mko}$ mice. N=4 in each group. Error bars represent SD. $^{*}p<0.05$, values significantly different from fasted $TRAF6^{f/f}$ mice.

FIGURE S2. Expression of ER stress responsive genes in $TRAF6^{+/+}$ and $TRAF6^{-/-}$ MEF in response to starvation or overexpression of ATF6. (A) Cultured $TRAF6^{+/+}$ and $TRAF6^{-/-}$ MEFs were incubated in serum-free medium for 24h followed measurement of mRNA levels of ER stress responsive genes ATF4, GADD34, GRP94, and CHOP by QRT-PCR. **(B)** Cultured $TRAF6^{+/+}$ and $TRAF6^{-/-}$ MEF were transduced with ATF6 adenovirus (Ad.ATF6) or control (Ad.Control) for 24h followed by measurement of mRNA levels of ER Chaperons GRP78 and GRP94. Error bars represent SD. $^{*}p<0.01$, values significantly different from serum-starved $TRAF6^{+/+}$ MEF. $^{\#}p<0.05$, values significantly different from unstarved $TRAF6^{+/+}$ MEF. $^{@}p<0.05$, values significantly different from Ad.ATF6-transduced $TRAF6^{+/+}$ MEF.

FIGURE S3. Effect of ER stressor on expression of UPS and ALS genes in C2C12 myotubes. C2C12 myoblasts were differentiated into myotubes and ER stress was induced by treatment with tunicamycin or thapsigargin for 18h. **(A)** QRT-PCR analyses showed that treatment with tunicamycin or thapsigargin significantly increased transcript levels of MAFBx, MuRF1, LC3B, and Beclin1 in myotubes. **(B).** Treatment with tunicamycin or thapsigargin significantly reduced mRNA levels of MHC4 in C2C12 myotubes. **(C)** Transcript levels of ER stress-related genes CHOP, GADD34 and ATF4 was increased upon treatment with tunicamycin or thapsigargin. Error bars represent SD. $^{*}p<0.05$, values significantly different from myotubes treated with vehicle alone.

FIGURE S4. Expression of autophagy-related genes is starved muscle is independent of TWEAK-Fn14 system. Three months old WT and TWEAK-KO mice were starved for 24 hrs and TA muscle was isolated for biochemical analyses. Starvation-induced increase in transcript levels of LC3B, Beclin1 and Atg12 did not show any significant difference between WT and TWEAK-KO mice. N=4 in each group. Error bars represent SD.

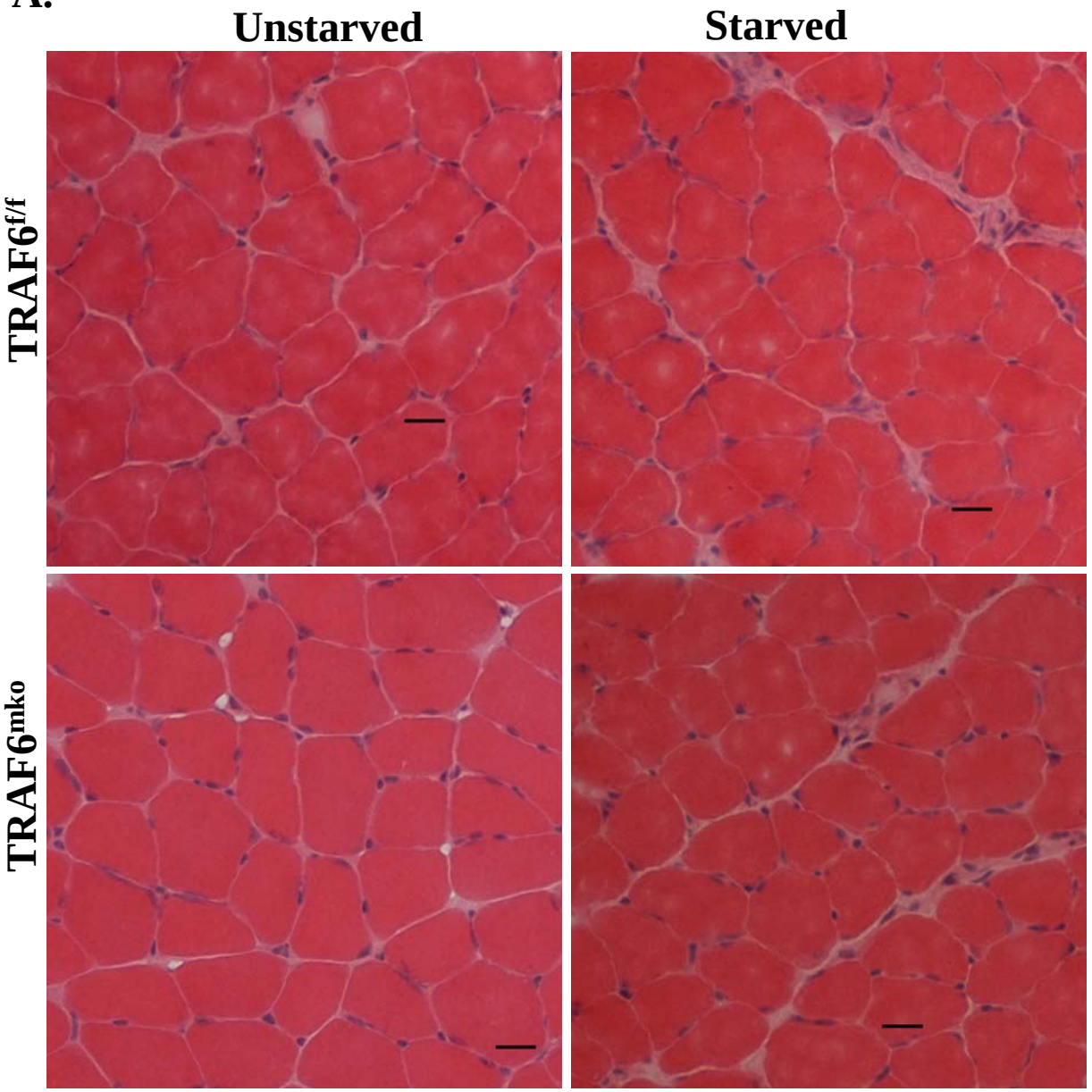
FIGURE S5. Ablation of TWEAK does not influence ER-stress genes in starved skeletal muscle of mice. QRT-PCR analyses demonstrate that there was no significant difference in transcript levels of ATF4, GADD34, CHOP, HERPS, PDI and GRP94 in TA muscle of WT and TWEAK-KO mice in response to starvation. N=4 in each group. Error bars represent SD.

FIGURE S6. Effect of overexpression of TRAF6C70A protein on starvation-induced myotube atrophy. C2C12 myotubes transfected with vector alone (i.e. pcDNA3) or TRAF6C70A plasmid were incubated in PBS. Representative phase contrasts pictures taken at different time point after addition of PBS are shown here. Scale bar: 20µm.

FIGURE S7. Quantification of myotube atrophy in TRAF6C70A transfected myotubes. Myotube diameter in vector or TRAF6C70A-expressing myotubes was measured after **(A)** 3h and **(B)** 6h of addition of PBS. $^{*}p<0.01$, values significantly different from 3h starved myotubes transfected with pcDNA3 alone. $^{\#}p<0.05$, values significantly different from 6h starved myotubes transfected with pcDNA3 alone.

FIGURE S1

A.


Unstarved
Starved
TRAF6f/f
TRAF6mko

B.

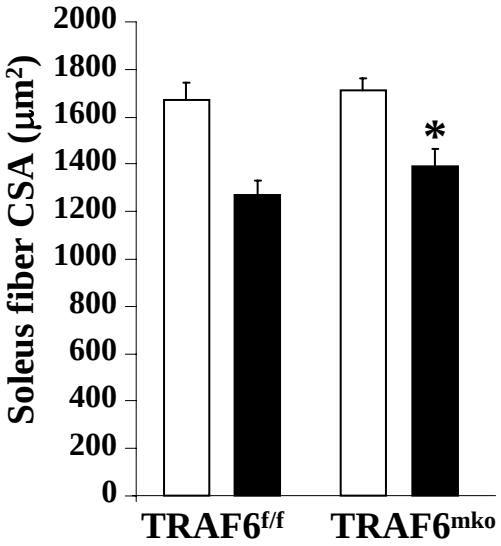

Soleus fiber CSA (μm2)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
TRAF6f/f
TRAF6mko

FIGURE S2

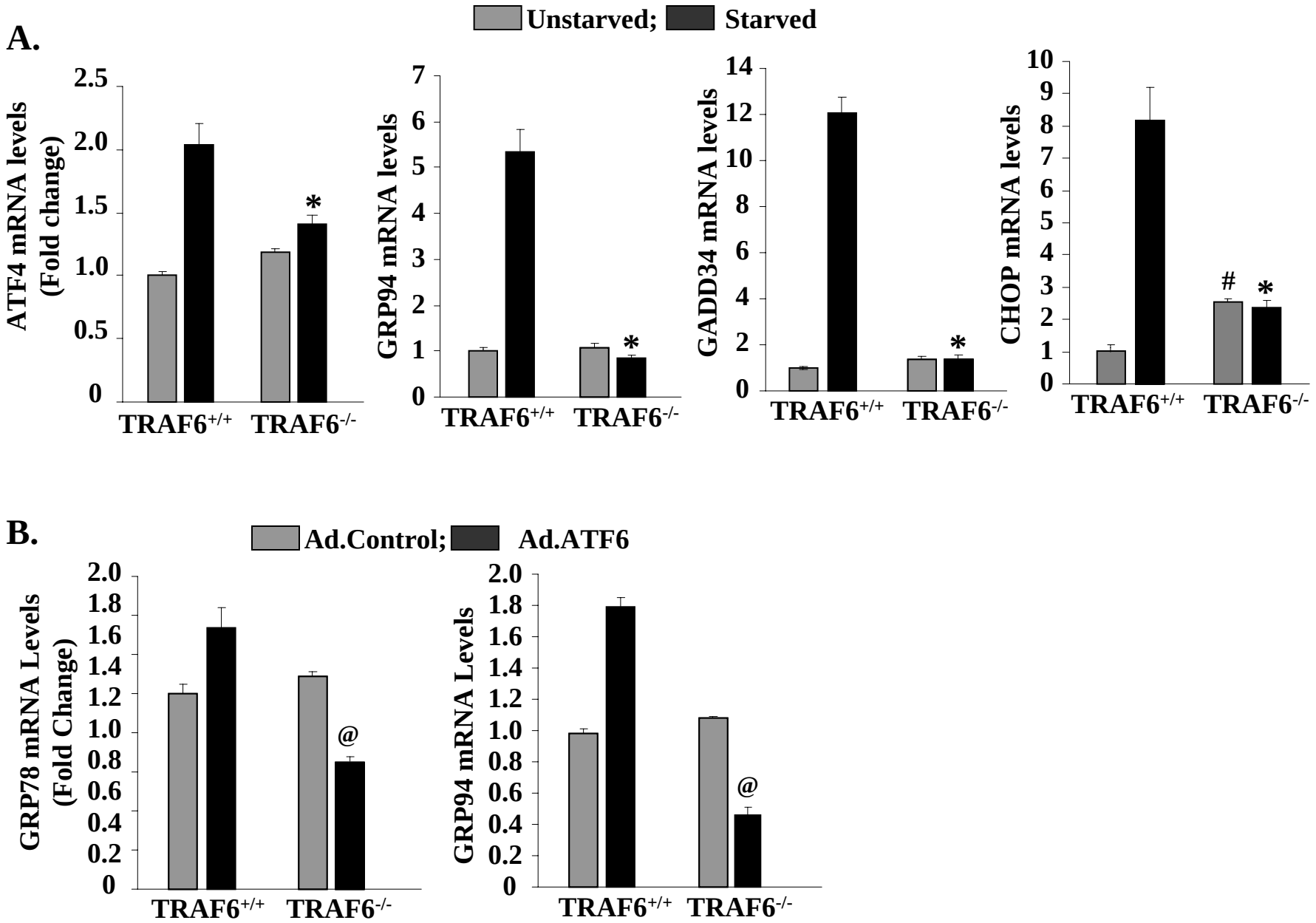

A.
Unstarved; Starved
ATF4 mRNA levels (Fold change)
2.5
2.0
1.5
1.0
0.5
0
*
TRAF6+/+
TRAF6-/-
GRP94 mRNA levels
7
6
5
4
3
2
1
0
*
TRAF6+/+
TRAF6-/-
GADD34 mRNA levels
14
12
10
8
6
4
2
0
*
TRAF6+/+
TRAF6-/-
CHOP mRNA levels
10
9
8
7
6
5
4
3
2
1
0
#
*
TRAF6+/+
TRAF6-/-
B.
Ad.Control; Ad.ATF6
GRP78 mRNA Levels (Fold Change)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
@
TRAF6+/+
TRAF6-/-
GRP94 mRNA Levels
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
@
TRAF6+/+
TRAF6-/-

FIGURE S3

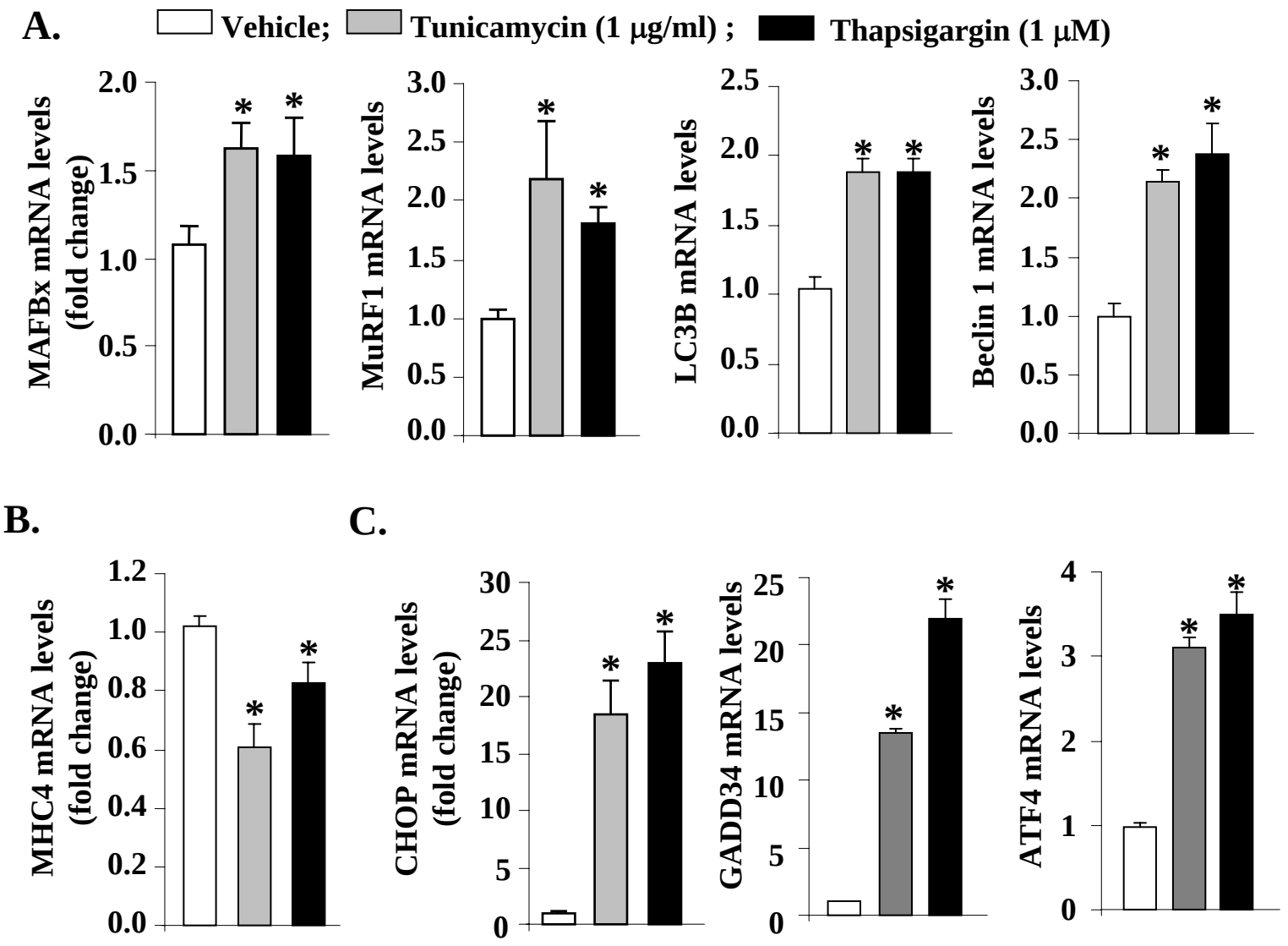

A.
Vehicle;
Tunicamycin (1 μg/ml) ;
Thapsigargin (1 μM)
MAFBx mRNA levels (fold change)
MuRF1 mRNA levels
LC3B mRNA levels
Beclin 1 mRNA levels
B.
MHC4 mRNA levels (fold change)
C.
CHOP mRNA levels (fold change)
GADD34 mRNA levels
ATF4 mRNA levels

FIGURE S4

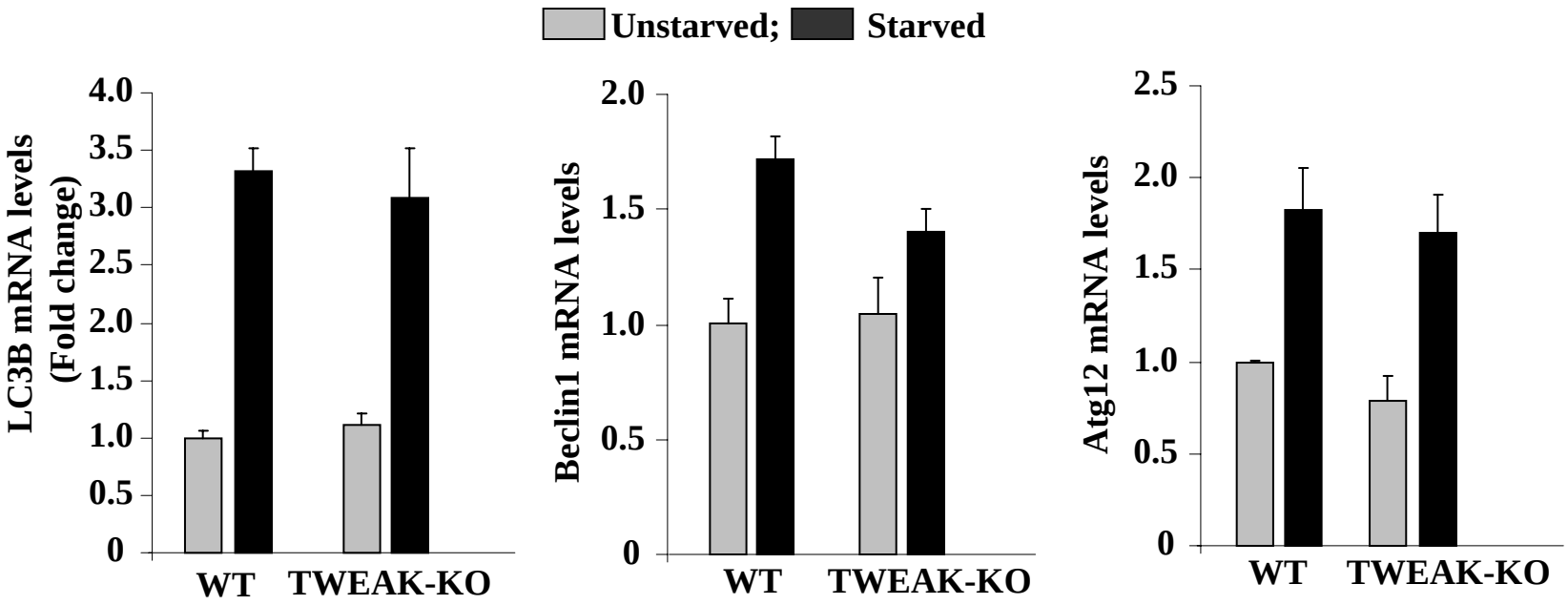

Unstarved;
Starved
LC3B mRNA levels (Fold change)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
WT
TWEAK-KO
Beclin1 mRNA levels
2.0
1.5
1.0
0.5
0
WT
TWEAK-KO
Atg12 mRNA levels
2.5
2.0
1.5
1.0
0.5
0
WT
TWEAK-KO

FIGURE S5

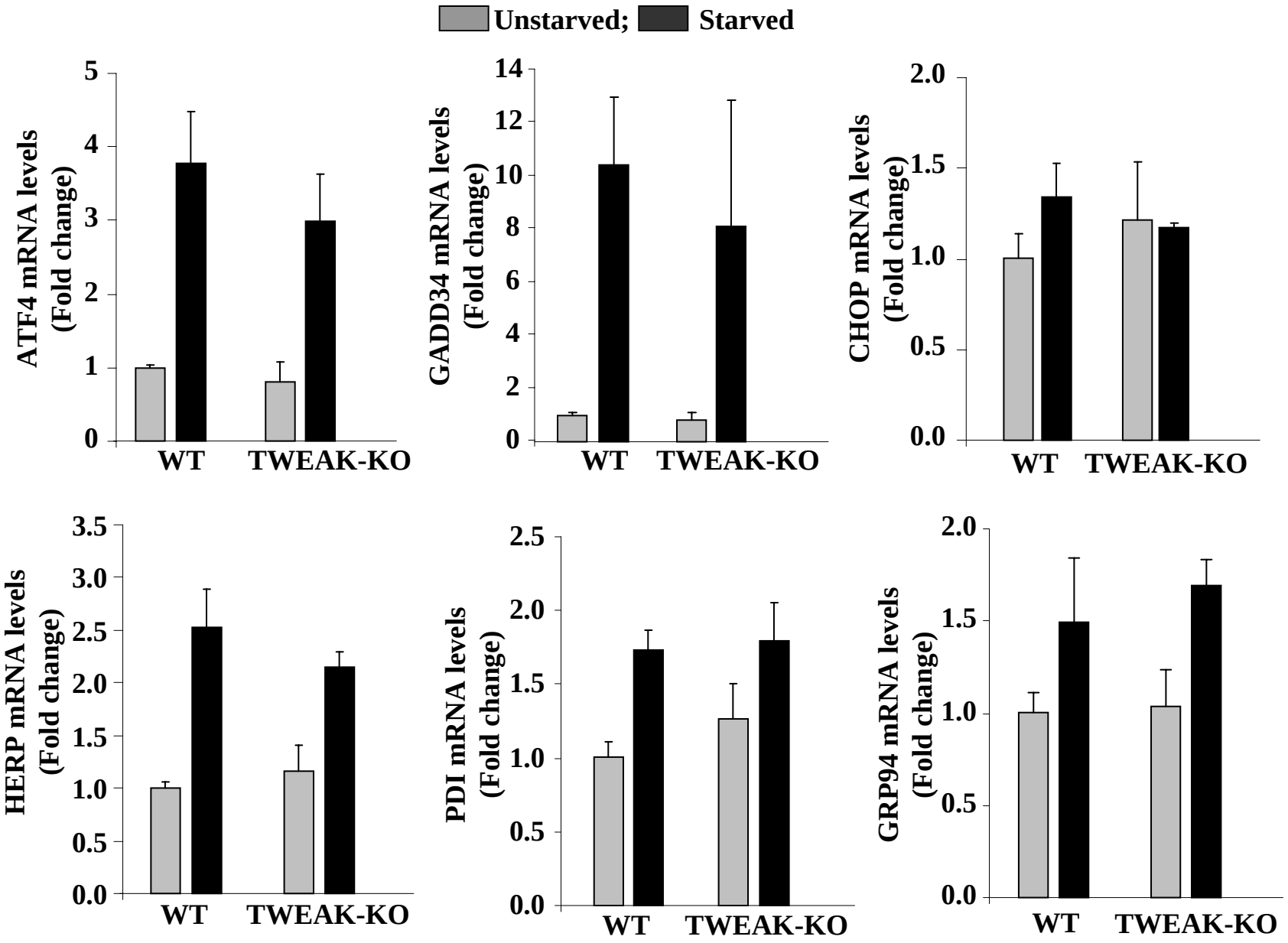

Unstarved; Starved
ATF4 mRNA levels (Fold change)
0
1
2
3
4
5
WT
TWEAK-KO
GADD34 mRNA levels (Fold change)
0
2
4
6
8
10
12
14
WT
TWEAK-KO
CHOP mRNA levels (Fold change)
0.0
0.5
1.0
1.5
2.0
WT
TWEAK-KO
HERP mRNA levels (Fold change)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
WT
TWEAK-KO
PDI mRNA levels (Fold change)
0.0
0.5
1.0
1.5
2.0
2.5
WT
TWEAK-KO
GRP94 mRNA levels (Fold change)
0.0
0.5
1.0
1.5
2.0
WT
TWEAK-KO

FIGURE S6

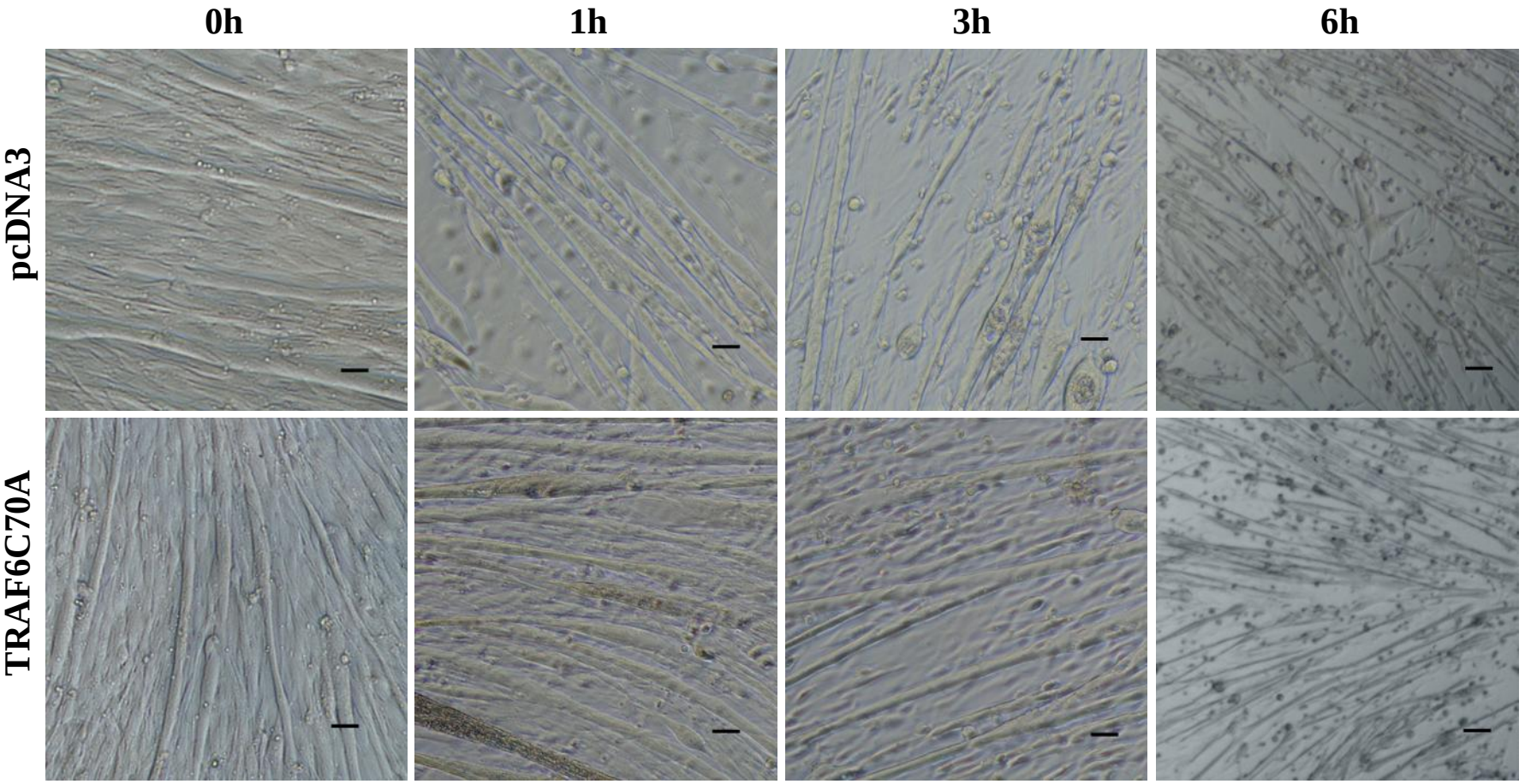
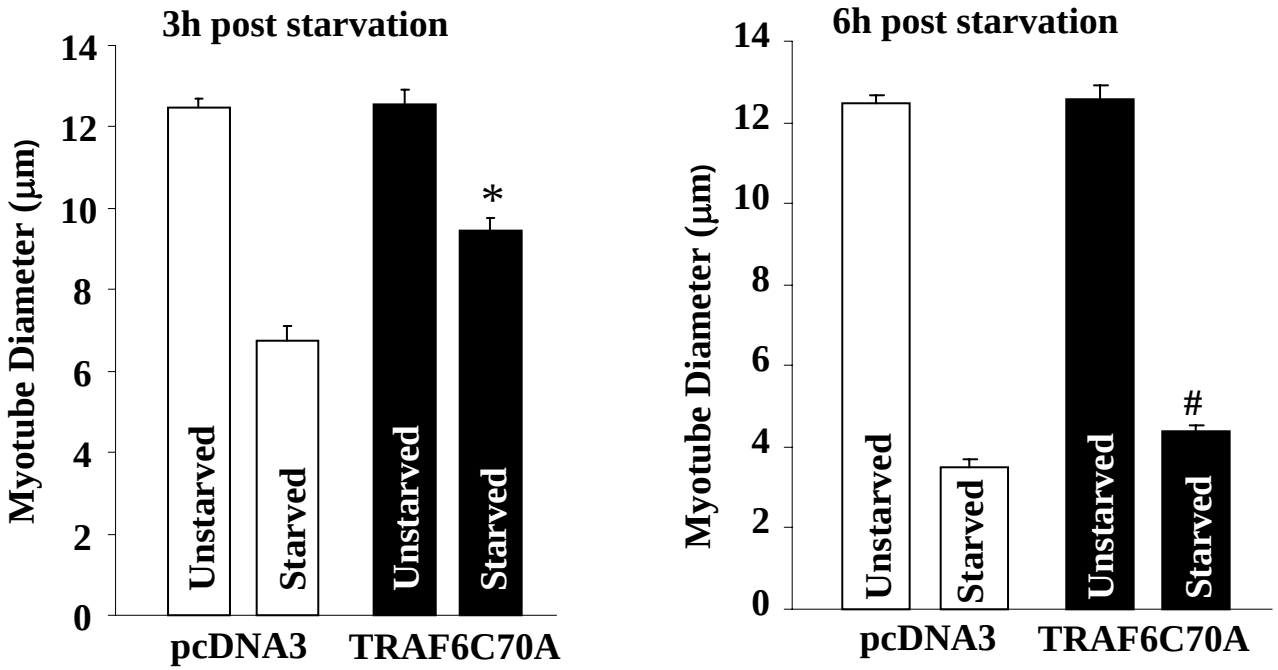


FIGURE S7


3h post starvation
Myotube Diameter (μm)
14
12
10
8
6
4
2
0
Unstarved
Starved
Unstarved
Starved
*
pcDNA3
TRAF6C70A
6h post starvation
Myotube Diameter (μm)
14
12
10
8
6
4
2
0
Unstarved
Starved
Unstarved
Starved
#
pcDNA3
TRAF6C70A