HDAC4 preserves skeletal muscle structure following long-term denervation by mediating distinct cellular responses

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Additional file

Figure S1 Denervation differentially affected HDAC4mKO and control mice. **a** Weight of HDAC4mKO and control (CTR) denervated TA muscles, over contralateral ones, expressed as the percentage, over time following denervation. Data are shown as mean ± SEM; n=4; two-way ANOVA (F=4.53; df 1; p=0.043) revealed an interaction between the genotypes and treatment (denervation); *p<0.05 by Tukey's HSD test. **b** Laminin staining of control (CTR) and HDAC4mKO TA muscles following one, two and four weeks of denervation. Scale bar=50 micron.

Figure S2 HDAC4mKO muscles did not show differences in dystophin glycoprotein complex. Representative images of immunofluorescence for dystophin or alpha-dystroglycan in HDAC4mKO and control TA muscles, following four weeks of denervation. Scale bar=50 micron.

Figure S3 Methylene blue and intermittent fasting efficiently activated UPS and autophagy in HDAC4mKO mice, respectively. **a** Proteasome activity in HDAC4mKO TA muscles, without (-) or

with MB treatment, two weeks following denervation. Data are shown as mean ± SEM; n=3-4. Two-way ANOVA (F=10; df 1; p=0.01) revealed an interaction between treatments (denervation and MB); *p<0.05; by Tukey's HSD test. **b** Western blot analyses for Myosin Heavy Chain (MHC) in contralateral and denervated HDAC4mKO muscles, two weeks following denervation, without (-) or with MB treatment. **c** Representative images of HDAC4mKO TA muscles co-electroporated with Ub-G76V-YFP (green) and dsRED (red) plasmids, without (-) or with MB treatment, and relative quantification. Scale bar=50 micron. n=3; between 45 and 300 $dsRED^+$ fibers were counted per each sample. Two-way ANOVA (F=8.56; df 1; p=0.02) revealed an interaction between treatments (denervation and MB); *p<0.05; by Tukey's HSD test. **d** Real-time PCR for autophagic markers in HDAC4mKO skeletal muscle, one week following denervation, in the absence (-) or IF. Data are shown as mean ± SEM; n=8. Two-way ANOVA revealed an effect of the treatment (IF) (F=29.3; df 1; p=0.0001 for Atg7, F=19.34; df 1; p=0.0003 for Gabarapl1, F=9.65; df 1; p=0.0045 for Atg5) and an interaction between untreated and treated denervated HDAC4mKO muscles; *p<0.05 by Tukey's HSD test. **e** Densitometric measurements of western blot analyses for LC3b and p62 proteins, one week following denervation, in the absence (-) or after IF treatment. Gapdh was used as loading control. Data are shown as mean ± SEM; n=3-4. Two-way ANOVA revealed an effect of the treatment (IF) (F=5.50; df 1; p=0.036 for LC3b, F=22.93; df 1; p=0.0006 for p62) and an interaction between untreated and treated denervated HDAC4mKO muscles; *p<0.05 by Tukey's HSD test.

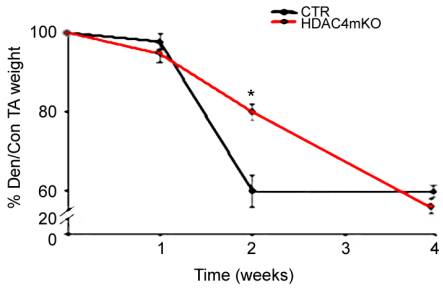
Figure S4 Effects of methylene blue and intermittent fasting on HDAC4mKO muscles. **a** Distribution analysis of HDAC4mKO fiber CSA, following 4 weeks of denervation, without or with MB treatment. Data are shown as mean ± SEM; n=4; two-way ANOVA (F=7.27; df 1; p=0.026 and F=7.27; df 1; p=0.026) revealed an interaction between treatments (denervation and MB) in 1000-1500 μm^2 and 2500-3000 μm^2 cross-sectional area classes, respectively; **p<0.01 by Tukey's HSD test. **b** Distribution analysis of HDAC4mKO fiber cross-sectional area, following 4 weeks of

denervation, without or with IF treatment. Data are shown as mean ± SEM; n=4; two-way ANOVA (F=10.2; df 1; p=0.01 for 1000-1500 μm^2 class and F=8.2; df 1; p=0.01 for 2500-3000 μm^2 class) revealed an interaction between treatments (denervation and IF); **p<0.01 by Tukey's HSD test. **c** Weight of HDAC4mKO muscles without (-) or after MB or IF treatment, over contralateral (-) ones. n=4-5; two-way ANOVA (F=103.5; df 1; p=0.001 for MB and F=40; df 1; p=0.0001 for IF) revealed an effect of denervation without interaction between treatments; *p<0.05 by Tukey's HSD test.

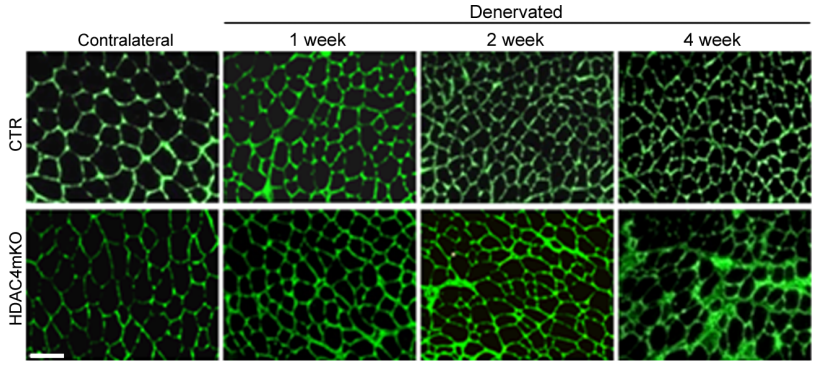
Figure S5 HDAC4mKO mice showed altered levels of Gp91phox upon denervation. Representative western blot analyses for Gp91phox in control (CTR) and HDAC4mKO muscle after **a** one, **b** two, and **c** four weeks following denervation. **d** Real-time PCR of antioxidant enzymes in control (CTR) and HDAC4mKO skeletal muscles, four week following denervation. Data are shown as mean ± SEM; n=4. Two-way ANOVA (F=7.97; df 1; p=0.018 for catalase) revealed an effect of denervation; *p<0.05 by Tukey's HSD test.

Figure S6 Trolox treatment efficiently reduces free radical levels in HDAC4mKO mice. **a** DHE staining of HDAC4mKO skeletal muscle following four weeks of denervation without (-) or after Trolox (TRX) treatment and relative quantification. Scale bar=50 micron. n=3; two-way ANOVA (F=209; df 1; p=0.0001) showed an interaction between treatments (denervation and Trolox); *p<0.05 by Tukey's HSD test. **b** Quantification of free radical levels (ROS and RNS) in HDAC4mKO skeletal muscle following four weeks of denervation without (-) or after Trolox treatment. n=3; two-way ANOVA (F=14.4; df 1; p=0.0053) revealed an effect of denervation.

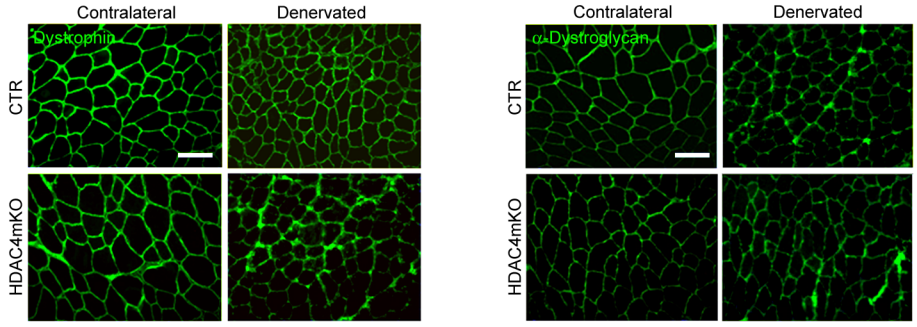
a


CTR
HDAC4mKO
100
80
60
20
0
% Den/Con TA weight
*
1
2
3
4
Time (weeks)

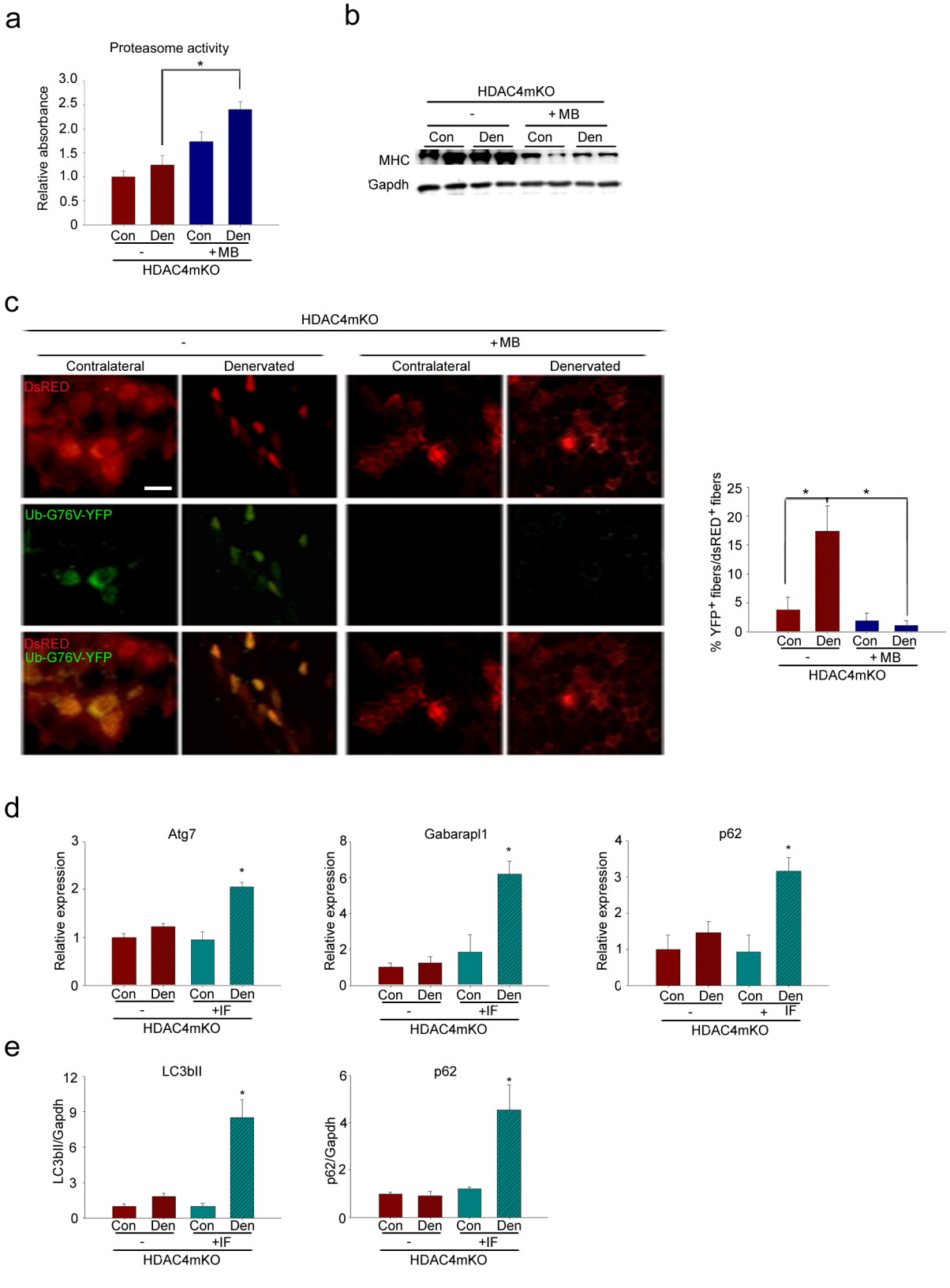
b


Denervated
Contralateral
1 week
2 week
4 week
CTR
HDAC4mKO

S2

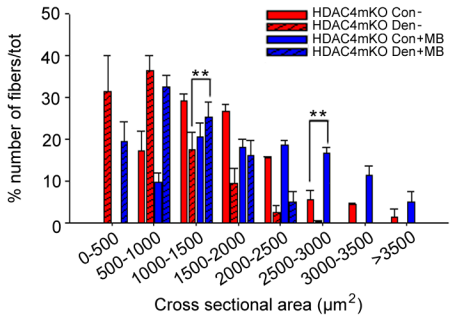

Contralateral
Denervated
Dystrophin
CTR
HDAC4mKO
Contralateral
Denervated
α-Dystroglycan
CTR
HDAC4mKO

S3

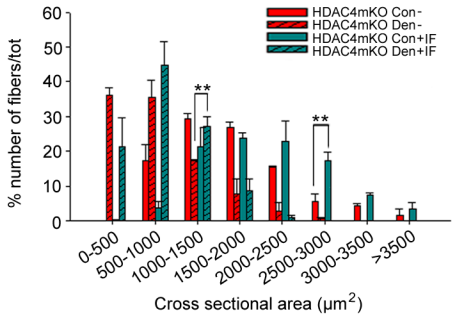

a
Proteasome activity
Relative absorbance
3.0
2.5
2.0
1.5
1.0
0.5
0
*
Con Den Con Den
-
+MB
HDAC4mKO
b
HDAC4mKO
-
+MB
Con Den Con Den
MHC
Gapdh
c
HDAC4mKO
-
+MB
Contralateral
Denervated
Contralateral
Denervated
DsRED
Ub-G76V-YFP
DsRED
Ub-G76V-YFP
% YFP+ fibers/dsRED+ fibers
25
20
15
10
5
0
* *
Con Den Con Den
-
+MB
HDAC4mKO
d
Atg7
Relative expression
3
2
1
0
*
Con Den Con Den
-
+IF
HDAC4mKO
Gabarapl1
Relative expression
8
6
4
2
0
*
Con Den Con Den
-
+IF
HDAC4mKO
p62
Relative expression
4
3
2
1
0
*
Con Den Con Den
-
+
IF
HDAC4mKO
e
LC3bII
LC3bII/Gapdh
12
9
6
3
0
*
Con Den Con Den
-
+IF
HDAC4mKO
p62
p62/Gapdh
6
4
2
0
*
Con Den Con Den
-
+IF
HDAC4mKO

S4

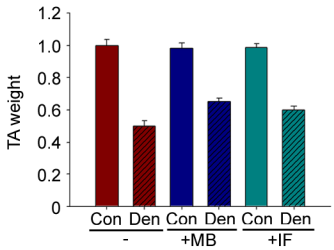
a


HDAC4mKO Con-
HDAC4mKO Den-
HDAC4mKO Con+MB
HDAC4mKO Den+MB
% number of fibers/tot
50
40
30
20
10
0
**
**
0-500
500-1000
1000-1500
1500-2000
2000-2500
2500-3000
3000-3500
>3500
Cross sectional area (μm2)

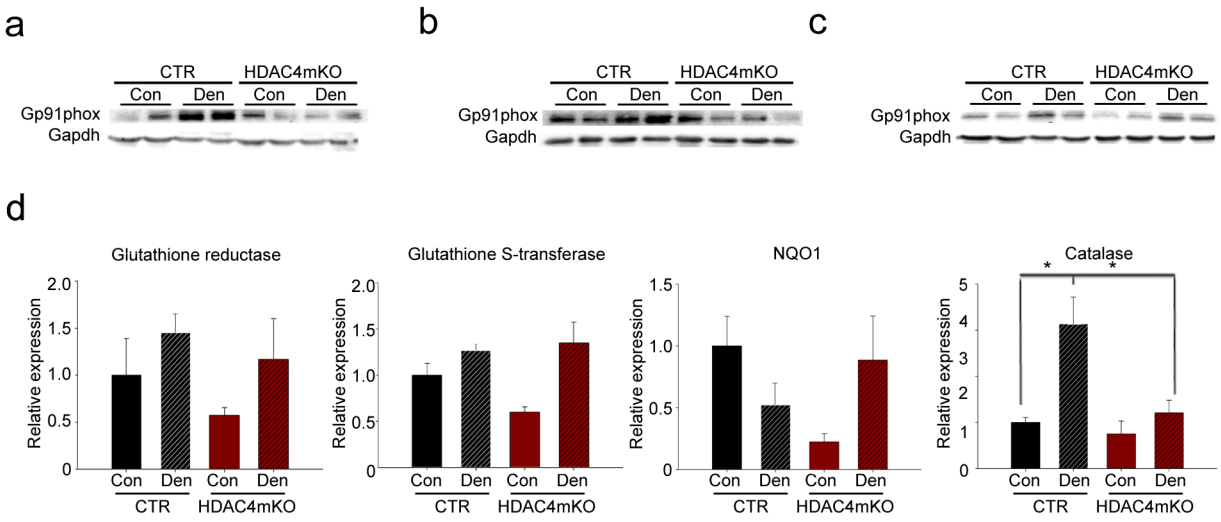
b


HDAC4mKO Con-
HDAC4mKO Den-
HDAC4mKO Con+IF
HDAC4mKO Den+IF
% number of fibers/tot
60
50
40
30
20
10
0
**
**
0-500
500-1000
1000-1500
1500-2000
2000-2500
2500-3000
3000-3500
>3500
Cross sectional area (μm2)

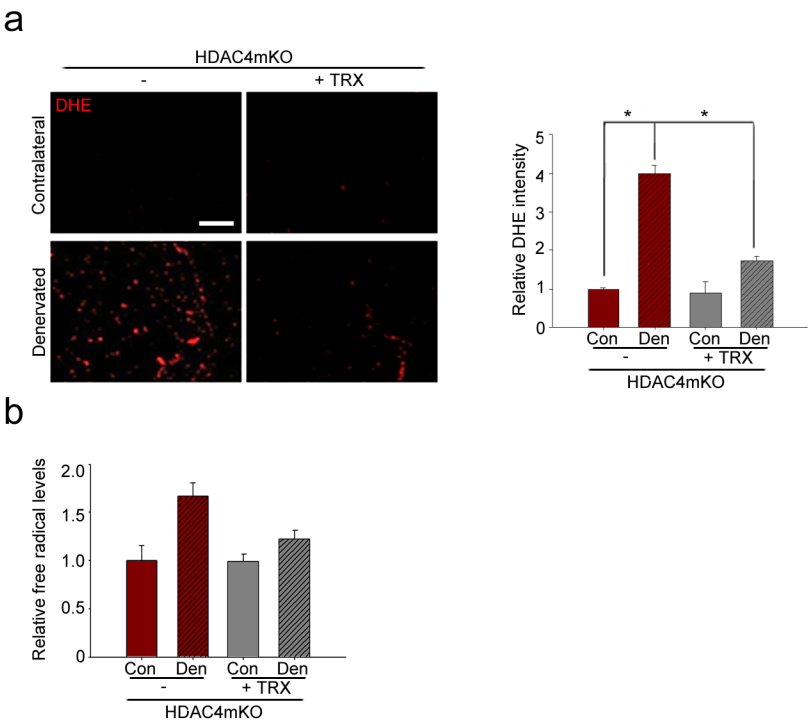
c


TA weight
1.2
1.0
0.8
0.6
0.4
0.2
0
Con Den Con Den Con Den
-
+MB
+IF

S5


a
CTR
HDAC4mKO
Con
Den
Con
Den
Gp91phox
Gapdh
b
CTR
HDAC4mKO
Con
Den
Con
Den
Gp91phox
Gapdh
c
CTR
HDAC4mKO
Con
Den
Con
Den
Gp91phox
Gapdh
d
Glutathione reductase
Relative expression
2.0
1.5
1.0
0.5
0
Con
Den
Con
Den
CTR
HDAC4mKO
Glutathione S-transferase
Relative expression
2.0
1.5
1.0
0.5
0
Con
Den
Con
Den
CTR
HDAC4mKO
NQO1
Relative expression
1.5
1.0
0.5
0
Con
Den
Con
Den
CTR
HDAC4mKO
Catalase
*
*
Relative expression
5
4
3
2
1
0
Con
Den
Con
Den
CTR
HDAC4mKO

S6


a
HDAC4mKO
-
+ TRX
DHE
Contralateral
Denervated
Relative DHE intensity
0
1
2
3
4
5
*
*
Con
Den
Con
Den
-
+ TRX
HDAC4mKO
b
Relative free radical levels
0
0.5
1.0
1.5
2.0
Con
Den
Con
Den
-
+ TRX
HDAC4mKO