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Supplemental Information

Rbfox-Splicing Factors Maintain Skeletal Muscle Mass by Regulating Calpain3 and Proteostasis

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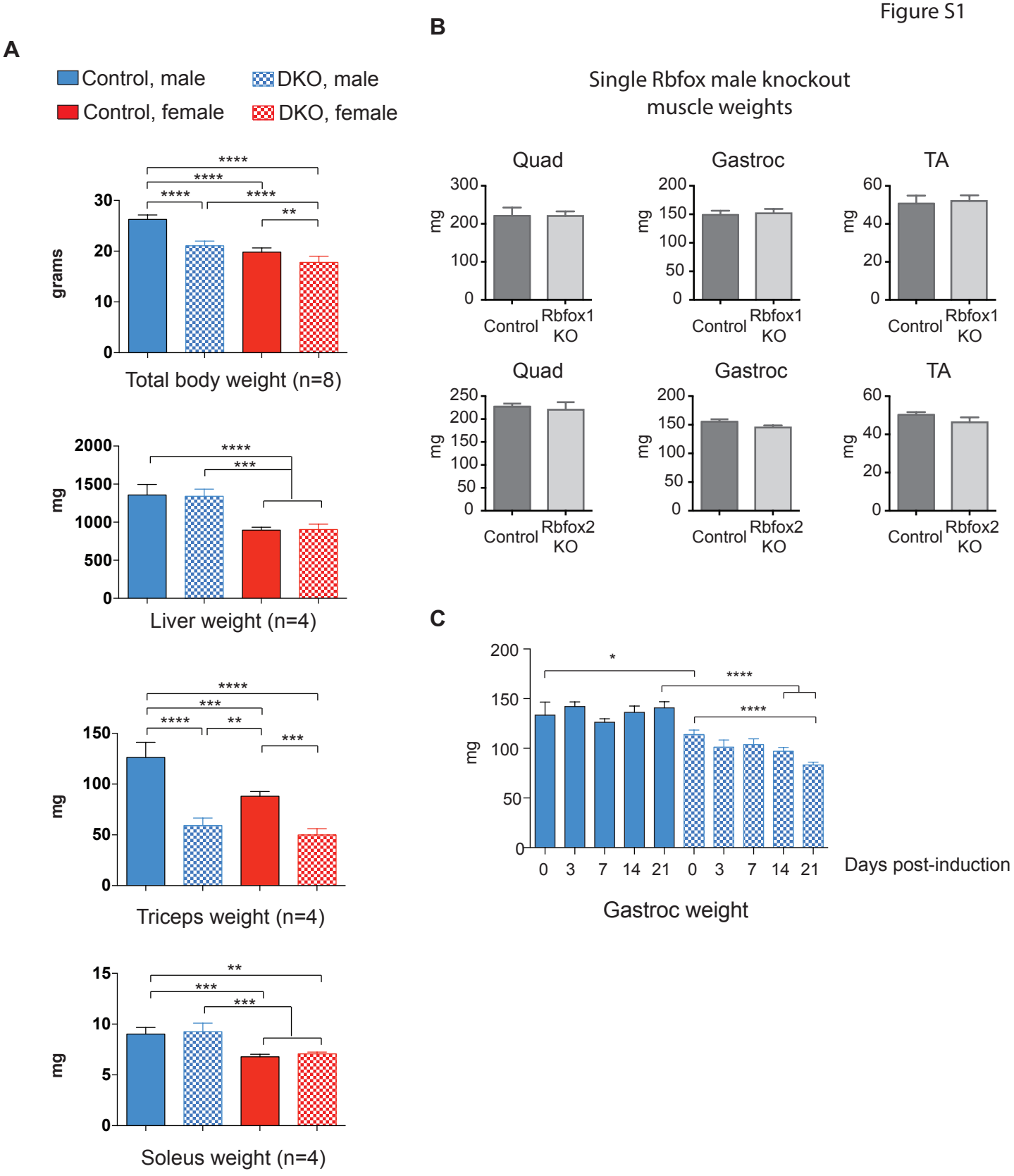


Figure S1. Deletion of Rbfox1 and Rbfox2 in adult skeletal muscle causes loss of muscle mass, related to Figure 1. A) Measurements of total body weight, and isolated liver, triceps and soleus muscle weights from control and DKO male and female mice (4-weeks after induction of Rbfox knock-out). B) Isolated muscle weights from control and single Rbfox1 (top) or Rbfox2 (bottom) knockout male mice. C) Isolated gastrocnemius muscle weight from uninduced and 3, 7, 14, and 21 days after starting doxycycline diet. N is ≥ 3 for all experiments in this figure. The columns and error bars in graphs indicate mean ± SD. p values as indicated by asterisk were calculated by ANOVA, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001.

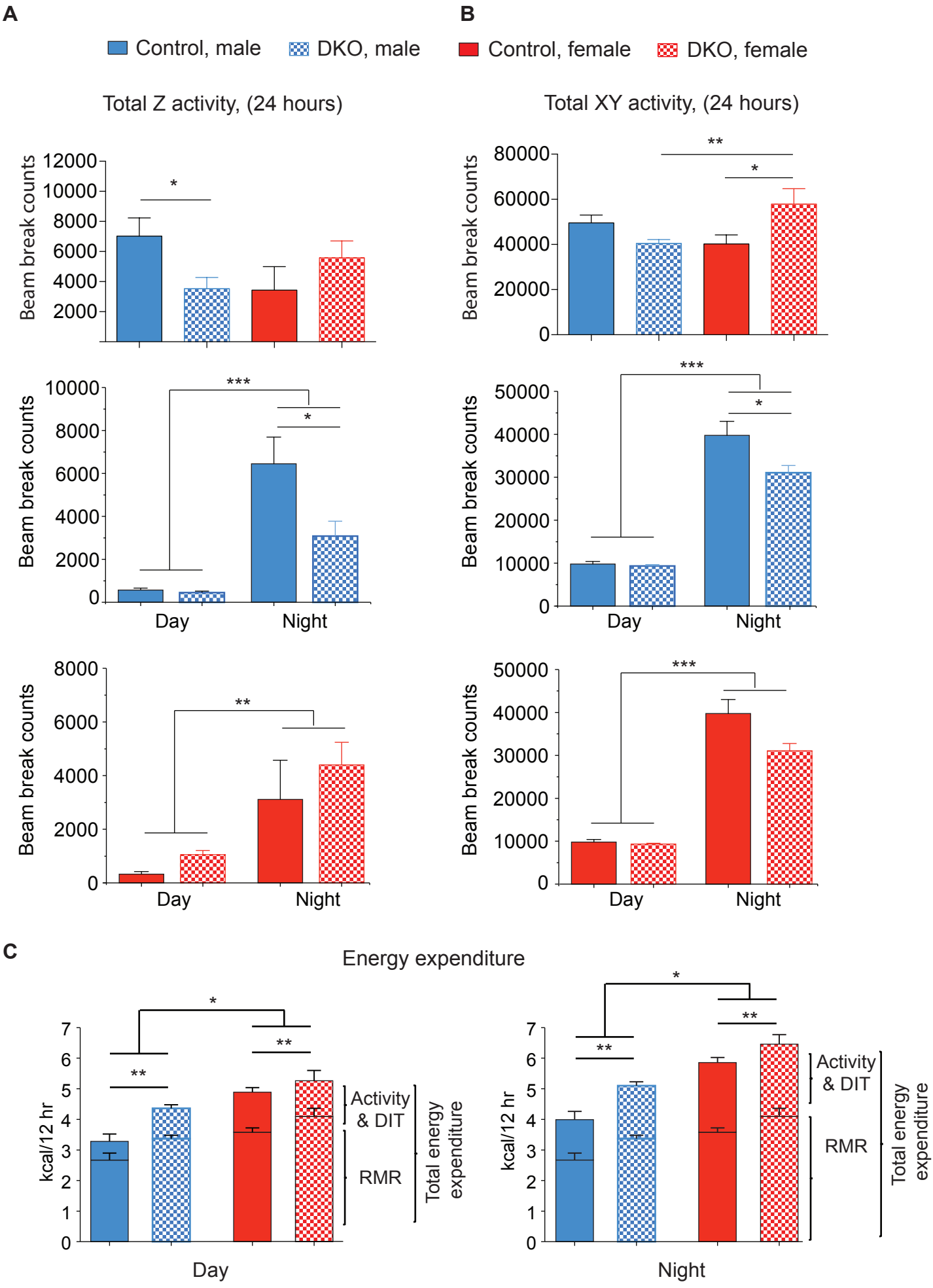

A
Control, male
DKO, male
Total Z activity, (24 hours)
Beam break counts
12000
10000
8000
6000
4000
2000
*
10000
8000
6000
4000
2000
0
***
*
Day
Night
8000
6000
4000
2000
0
**
Day
Night
B
Control, female
DKO, female
Total XY activity, (24 hours)
Beam break counts
80000
60000
40000
20000
0
**
*
50000
40000
30000
20000
10000
0
***
*
Day
Night
50000
40000
30000
20000
10000
0
***
Day
Night
C
Energy expenditure
kcal/12 hr
7
6
5
4
3
2
1
0
*
**
**
Activity & DIT
RMR
Total energy expenditure
Day
*
**
**
Activity & DIT
RMR
Total energy expenditure
Night

Figure S2. Activity and energy expenditure of control and Rbfox DKO male and female mice approximately 2 weeks after knockout induction, related to Figure 2. A-B) Average 24-hour rearing or Z-plane activity (A) and horizontal-plane activity (B) in male and female control and Rbfox DKO animals (n=5 for male, n≥3 for female). Activity data in A and B was divided into 12-hour day (rest period, 6AM to 6PM) and night (active period, 6PM to 6AM) for male (middle panels) and female (bottom panels). For the analysis of effects of time of day; we used a repeated measure ANOVA with time of day as the repeated measure for each mouse. C) Energy expenditure (adjusted for lean mass) from control and DKO mice for day (left panel) and night (right panel) are indicated (n=5 for male, n≥3 for female). Abbreviation: RMR - resting metabolic rate, DIT- diet induced thermogenesis. Activity and DIT is not significantly different between genotype or gender. The columns and error bars in graphs indicate means ± SE. p values as indicated by Asterisk were calculated by ANOVA, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Figure S3

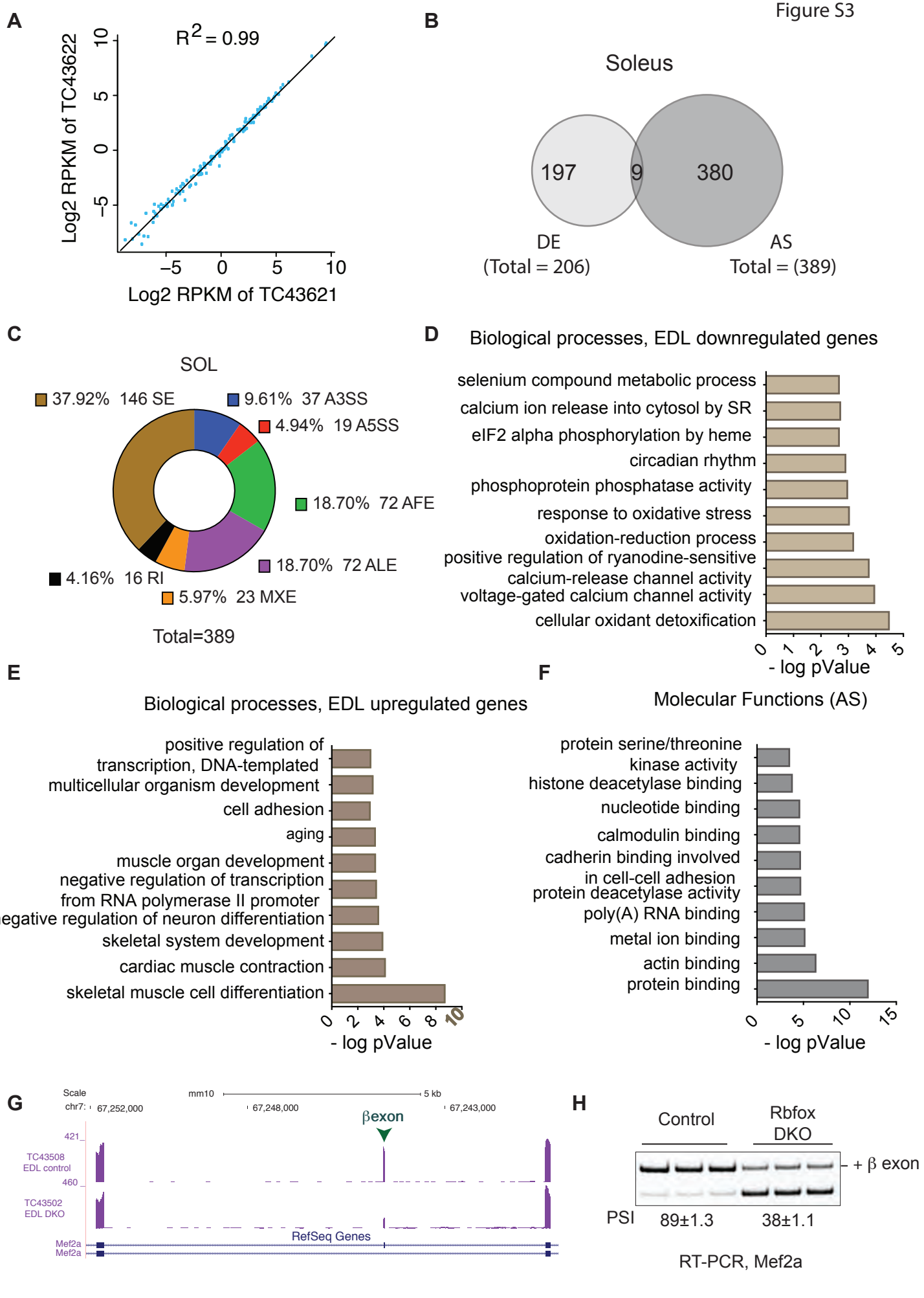
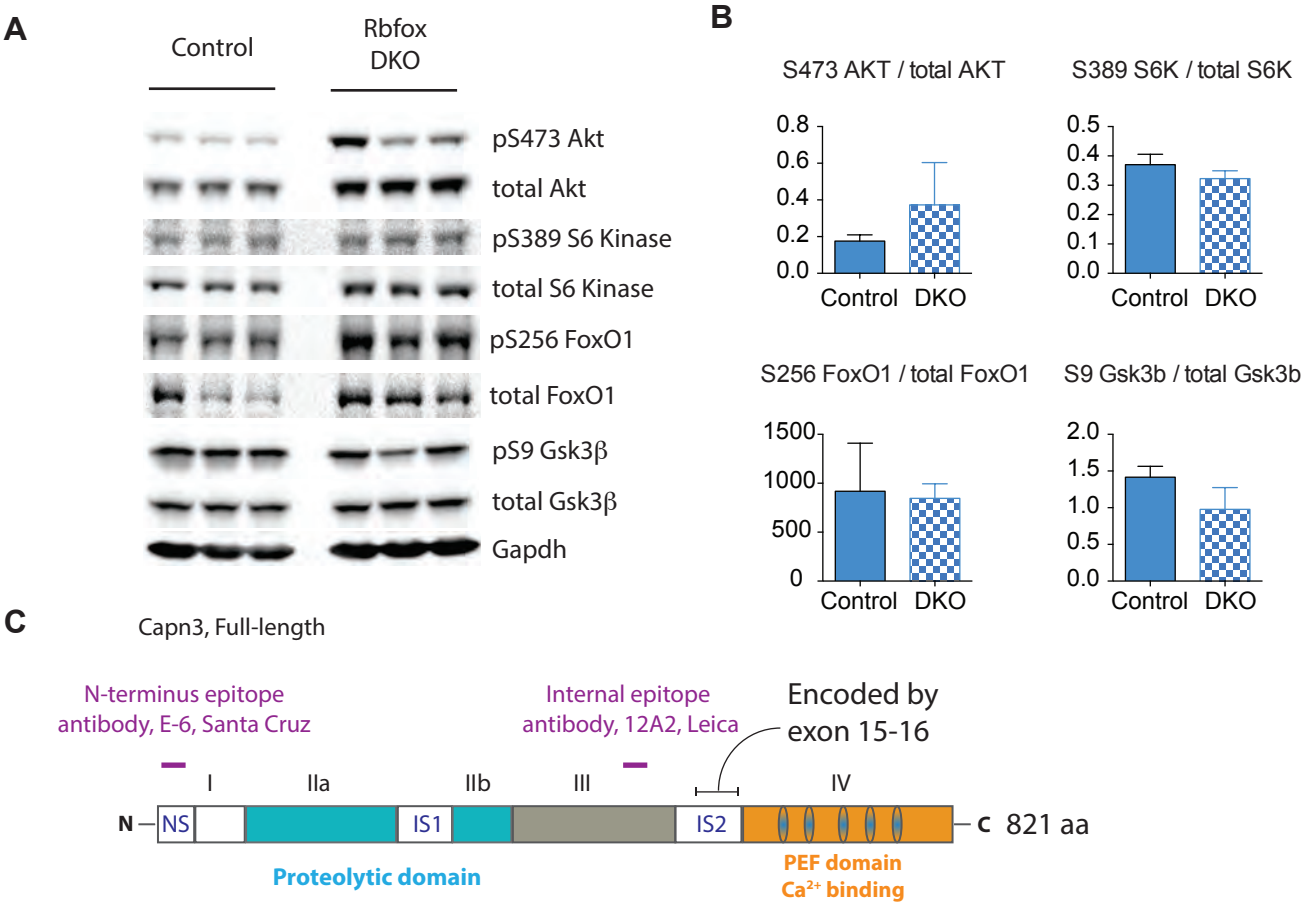

A
R2 = 0.99
Log2 RPKM of TC43622
Log2 RPKM of TC43621
B
Soleus
197
9
380
DE
(Total = 206)
AS
Total = (389)
C
SOL
37.92% 146 SE
9.61% 37 A3SS
4.94% 19 A5SS
18.70% 72 AFE
18.70% 72 ALE
4.16% 16 RI
5.97% 23 MXE
Total=389
D
Biological processes, EDL downregulated genes
selenium compound metabolic process
calcium ion release into cytosol by SR
eIF2 alpha phosphorylation by heme
circadian rhythm
phosphoprotein phosphatase activity
response to oxidative stress
oxidation-reduction process
positive regulation of ryanodine-sensitive calcium-release channel activity
voltage-gated calcium channel activity
cellular oxidant detoxification
- log pValue
E
Biological processes, EDL upregulated genes
positive regulation of transcription, DNA-templated
multicellular organism development
cell adhesion
aging
muscle organ development
negative regulation of transcription from RNA polymerase II promoter
negative regulation of neuron differentiation
skeletal system development
cardiac muscle contraction
skeletal muscle cell differentiation
- log pValue
F
Molecular Functions (AS)
protein serine/threonine kinase activity
histone deacetylase binding
nucleotide binding
calmodulin binding
cadherin binding involved in cell-cell adhesion
protein deacetylase activity
poly(A) RNA binding
metal ion binding
actin binding
protein binding
- log pValue
G
Scale
chr7:
67,252,000
mm10
67,248,000
5 kb
67,243,000
βexon
TC43508
EDL control
TC43502
EDL DKO
RefSeq Genes
Mef2a
Mef2a
H
Control
Rbfox DKO
+ β exon
PSI
89±1.3
38±1.1
RT-PCR, Mef2a

Figure S3. Rbfox knockout in skeletal muscle causes wide-spread transcriptome changes, related to Figure 3. A) Gene expression from two biological replicates was compared with each other. B) Venn diagram showing differential gene expression (DE) and alternative splicing (AS) changes in Rbfox knockout soleus muscles when compared to control animals at 2 weeks after knockout induction. C) Donut chart showing description of transcriptome changes in soleus muscle. Abbreviation: SE- Cassette exons, A3SS- alternative 3' splice site, A5SS - alternative 5' splice site, AFE- alternative first exon, ALE- alternative last exon, MXE - mutually exclusive exons, RI - retained intron. D-F) DAVID GO analysis to identify biological processes that are enriched for genes that are downregulation (D) or upregulation (E), and molecular functions (F) that are enriched in transcripts that are alternatively spliced in EDL muscles after Rbfox knockout. G) RNA-seq tracks showing Mef2a in control and Rbfox DKO muscles. Inclusion of β exon is dependent on Rbfox proteins. H) RT-PCR showing reduced PSI for Mef2a β exon in DKO muscle when compared to control muscle.



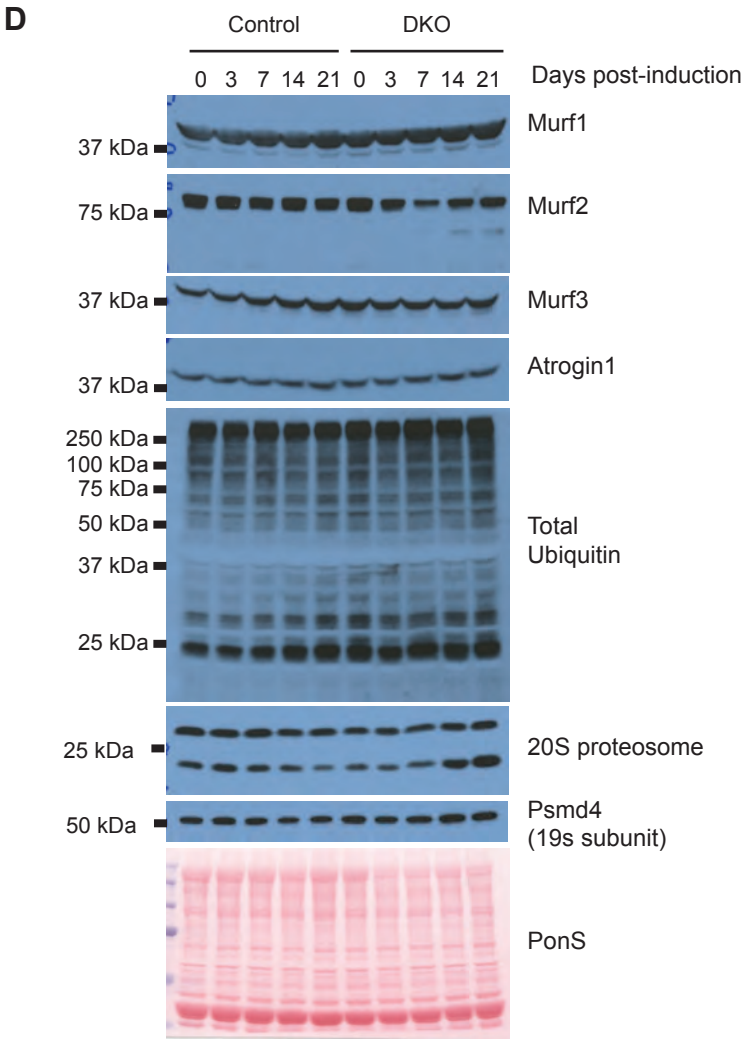


Figure S4. Altered proteostasis in Rbfox knockout muscles, related to Figure 4. A) Western blots for indicated proteins in TA muscle extracts from control and Rbfox DKO mice (3-weeks post induction). B) The bar graphs display the ratios of indicated phospho-protein to total protein from blots in Fig. S4A (mean ± SD, n=3) . C) Schematic showing four domains of calpain 3 protein which are also present in Calpain family members. There are three unique calpain 3-specific insert sequences: N-terminus insert sequence (NS), Insert Sequence 1 (IS1), and 2 (IS2) are also indicated. Note that majority of the IS2 sequence is encoded by exons 15-16. Also indicates representative regions that are recognized by the two antibodies used in this study, the N-terminus (E-6, Santa Cruz) and the internal epitope (NCL-CALP-12A2 , Leica) antibodies. D). Western blots using antibodies against indicated proteins in uninduced (0 day) and 3,7,14, and 21 days induced TA muscles from control and DKO animals.

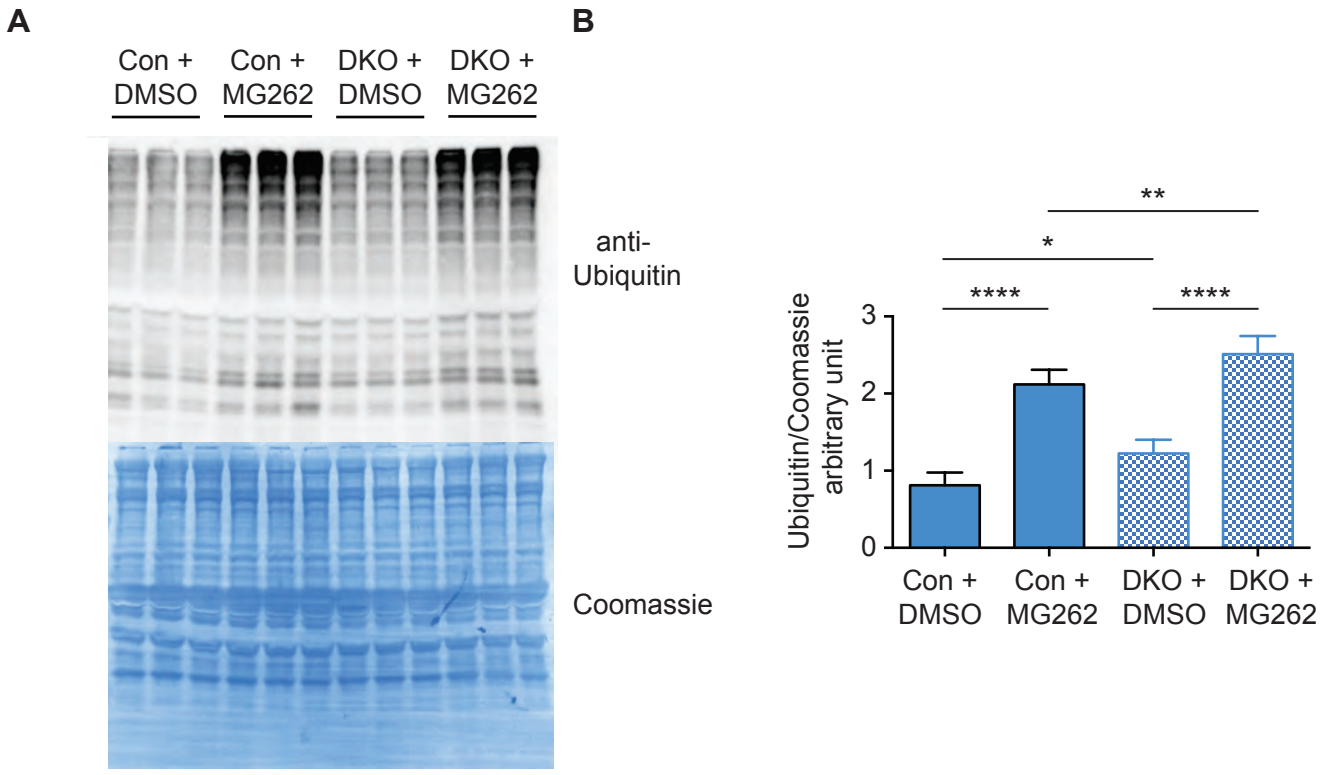


Figure S5. Increased ubiquitinated proteins in Rbfox DKO muscles, related to Figure 5. A) Western blot to detect ubiquitinated proteins from TA muscles from control and DKO mice treated with DMSO or MG262 (5umol/kg) ($n \geq 3$). B) Signals from ubiquitinated proteins were normalized to coomassie signal from each lane in the same blot. The columns and error bars in graphs indicate normalized mean intensity of ubiquitinated proteins ± SD. p values as indicated by asterisk were calculated by ANOVA, *$p \leq 0.05$, **$p \leq 0.01$, and ****$p \leq 0.0001$.

Supplemental Information

Supplemental experimental procedures

Mouse muscle strength and activity assays

Treadmill assays were performed on the Exer 3/6 six treadmill (Columbus Instruments, OH) at a starting speed of 6 meters/min for first two minutes (10 degree incline) increased 2 meters/min every two minutes until exhaustion. The speed and time were used to measure the total distance run by mice in each group. Grip strength was performed using a 1027SM grip strength meter with single sensor (Columbus Instruments, OH). Forelimb and all limb grip strength used 5 measurements of grams of force produced in the t-pk mode and average values were calculated. For the hang test, soft padding was added in cardboard box (14 in x14 in x14 in), a wire grid was placed on top of the box and the mouse was placed upside down; the time until the mouse could no longer hold onto the wire was recorded for each mouse.

Histological analyses

For histology of muscles, skin and facia was removed and whole limbs were fixed overnight in 10% buffered formalin. Muscles were isolated and processed for paraffin embedding, routine sectioning, hematoxylin and eosin staining.

Food Intake, Energy Expenditure, Activity

The Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, OH) was used to measure food intake, activity, and energy expenditure. Mice were adapted to the cages at 9 days after starting the dox diet and various measurements were taken from day 13 to day 17 to quantify energy balance, food intake, and activity as previously described (Crossland et al., 2017).

Glucose Tolerance Test

The glucose tolerance test was performed by the Mouse Metabolism Core at Baylor College of Medicine. Two weeks after starting the dox diet, mice were starved for 6 hours, and then glucose (1.5 g/kg of body weight) was injected intraperitoneally. Blood was drawn at the

indicated times after injection and glucose and insulin levels were measured as described before (Saha et al., 2010).

Western blotting

Frozen muscles were homogenized with Bullet Blender 24 (Next Advance, Averill Park, NY) using zirconium oxide beads in buffer containing 50 mM Tris–HCL, pH 7.5, 100mM NaCl, 10 mM EDTA, 10 mM EGTA, 10% glycerol, 1% NP40, 50 mM NaF, 10uM MG132, 1mM PMSF, 0.5% Sodium deoxycholate, 1% SDS, protease and phosphatase inhibitor (Roche Diagnostics, Indianapolis, IN). Relative protein amount was quantified using the Pierce BCA Protein Assay Kit and equivalent amount of proteins were loaded on SDS-PAGE gels. Separated proteins were transferred to PVDF membrane. Membranes were stained with Ponceau S to assess even transfer and subsequently probed with specific primary antibodies. Secondary anibodies were conjugated to HRP and blots were imaged with ChemiDoc XRS+ (Bio-Rad, Hercules, CA) or standard autoradiography film. For quantification of western blot, images from ChemiDoc were exported for analysis and analyzed by Carestream software (Rochester, NY).

Inhibition of proteasome experiment

Seven-week-old Rbfox control and DKO mice were put on dox diet, and 4-days later, mice were injected intraperitoneally (IP) with 60% DMSO or 5 umol/kg MG262 (ApexBio, MA). TA muscle was harvested 16 hours after the injection for western blotting with anti-ubiquitin [P4D1] antibody (UBPBio, CO). The same Western blots were stained with the Coomassie for normalization of ubiquitin signal by total protein signal by densitometry to calculate relative levels of ubiquitinated proteins in each group.

Resource Table:

REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
Antibodies		
Anti- Rbfox1	Cooper lab	
Anti-Rbfox2	Cooper lab	
Anti-Capn3 (E-6)	Santa Cruz	sc-365277
Anti-Calpain (12A2)	Leica Microsystems Inc	CALP12A2
Anti-Calpastatin (H-300)	Santa Cruz	sc-20779
Anti-Trim63 (MuRF1) antibody	Proteintech Group, Inc	55456-1-AP

Anti-MuRF2 antibody	Abcam	ab4387
Anti-MuRF3 (N-19) Antibody	Santa Cruz	sc-50252
Anti-phospho Gsk3β (S9)	Cell Signaling Technology, Inc.	5558
Anti-phospho S6 Kinase (S389)	Cell Signaling Technology, Inc.	9234
Anti-S6 Kinase	Cell Signaling Technology, Inc.	9202
Anti-phospho FoxO1	Cell Signaling Technology, Inc.	9461
Anti-FoxO1	Cell Signaling Technology, Inc.	2880
Anti-phopho AKT (S473)	Cell Signaling Technology, Inc.	4060
Anti-Akt (pan)	Cell Signaling Technology, Inc.	4691
Anti-phospho Gsk3β	BD Biosciences	610202
Anti-gapdh	Cell Signaling Technology, Inc.	5174
Anti-p62	Progen Biotechnik	GP62-C
Anti-LC3b	Cell Signaling Technology, Inc.	2775
Anti-Anti-Spectrin alpha chain (nonerythroid), clone AA6	EMD Millipore	MAB1622
Anti-Ubiquitin (P4D1)	UPBio	Y3012
Anti-Psmd4	Abcam	ab137109
Anti-20S Proteasome	UBPBio	Y2011
Anti-Fbx32 antibody (Atrogin 1)-Conjugated to HRP	Abcam	ab198958

Chemicals, Peptides, and Recombinant Proteins		
Colchicine	Sigma Aldrich	C9754
Puromycin . 2HCl	Enzo Life Sciences	BML-GR312-0050

Supplemental References

Crossland, R.F., Balasa, A., Ramakrishnan, R., Mahadevan, S.K., Fiorotto, M.L., and Van den Veyver, I.B. (2017). Chronic Maternal Low-Protein Diet in Mice Affects Anxiety, Night-Time Energy Expenditure and Sleep Patterns, but Not Circadian Rhythm in Male Offspring. PLoS One *12*, e0170127.

Saha, P.K., Reddy, V.T., Konopleva, M., Andreeff, M., and Chan, L. (2010). The triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic-acid methyl ester has potent anti-diabetic effects in diet-induced diabetic mice and Lepr(db/db) mice. J Biol Chem *285*, 40581-40592.