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Supplemental Information

A Key Role for the Ubiquitin Ligase UBR4 in Myofiber Hypertrophy in *Drosophila* and Mice

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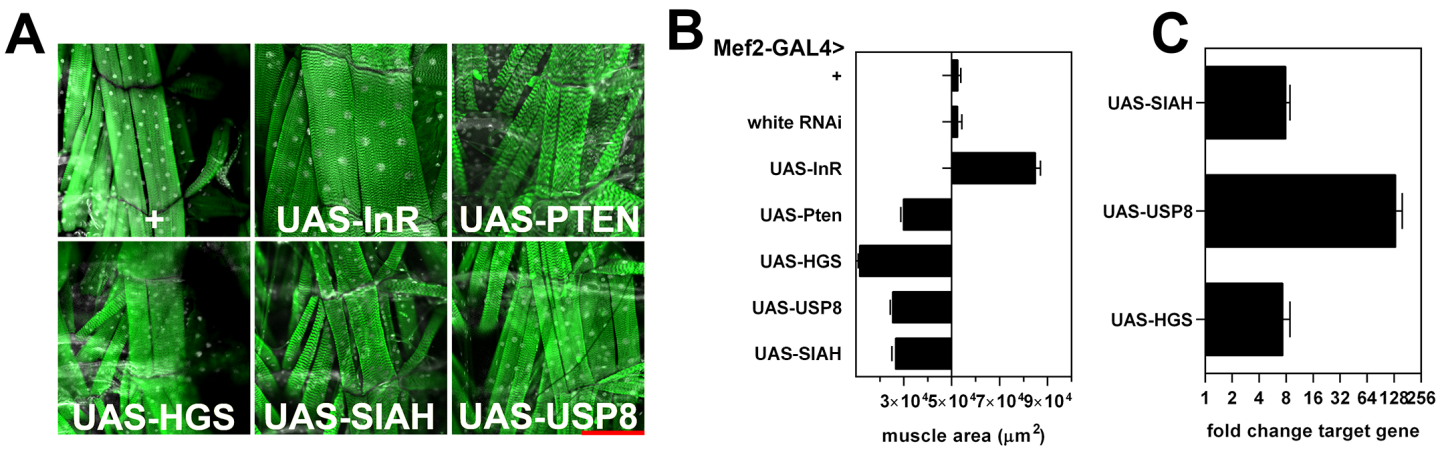
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS



Supplemental Figure S1. Testing Screen Hits by Transgenic Overexpression. Related to Figure 1.

(A) Representative images of larval VL3/4 muscles with Mef2-GAL4 driving overexpression of some screen hits (*Drosophila* homologs of *Hgs*, *Siah*, and *Usp8*) and known regulators of muscle growth (Insulin Receptor, InR; and PTEN). Converse phenotypes to RNAi-mediated knockdown (Figure 1) are obtained. Scale bar (red bar at bottom right) indicates 100 μm.

(B) Quantification of VL3/4 muscle areas for the genotypes shown in (A). All comparisons are significant ($p<0.05$) compared to *Mef2>white*^{RNAi} controls (n≥10 muscles from >3 larvae).

(C) qPCR demonstrates significant increased mRNA levels of *Drosophila* sina, Usp8, and Hrs (respectively *Siah*, *Usp8*, and *Hgs*; screen hits shown in A-B) upon transgenic overexpression; $p<0.05$ and n=3 biological replicates consisting of 5 larvae each.

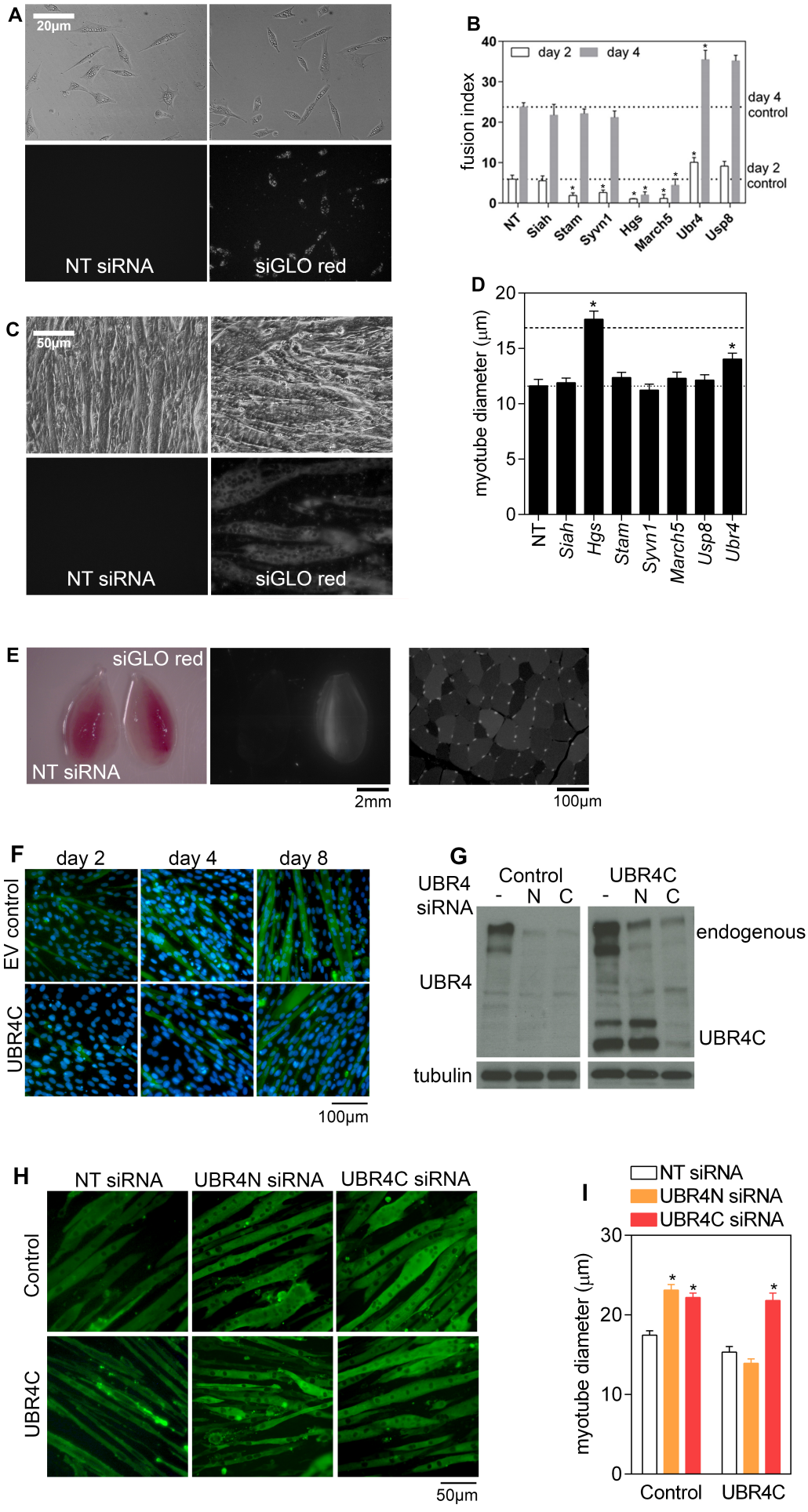

A
20μm
NT siRNA
siGLO red
B
day 2
day 4
fusion index
day 4 control
day 2 control
NT
Siah
Stam
Syvn1
Hgs
March5
Ubr4
Usp8
C
50μm
NT siRNA
siGLO red
D
myotube diameter (μm)
NT
Siah
Hgs
Stam
Syvn1
March5
Usp8
Ubr4
E
siGLO red
NT siRNA
2mm
100μm
F
day 2
day 4
day 8
EV control
UBR4C
100μm
G
Control
UBR4C
UBR4 siRNA
- N C
- N C
endogenous
UBR4
UBR4C
tubulin
H
NT siRNA
UBR4N siRNA
UBR4C siRNA
Control
UBR4C
50μm
I
NT siRNA
UBR4N siRNA
UBR4C siRNA
myotube diameter (μm)
Control
UBR4C

Fig. S2

Supplemental Figure S2. Testing the Efficacy of siRNAs in C2C12 Myoblasts and Myotubes, and in TA Muscles in Mice. Related to Figures 2 and 5.

(A) Representative images of C2C12 myoblasts transfected with siGLO red demonstrate cellular uptake of the fluorescent siRNAs, as also observed in cultures of C2C12 myotubes (C).

(B) Longitudinal fusion indexes measured over 4 days for myoblasts with candidate gene knockdown ($p<0.05$ with n=3 biologically replicated cultures). Statistical significance refers to the comparison to the NT siRNA control for that timepoint.

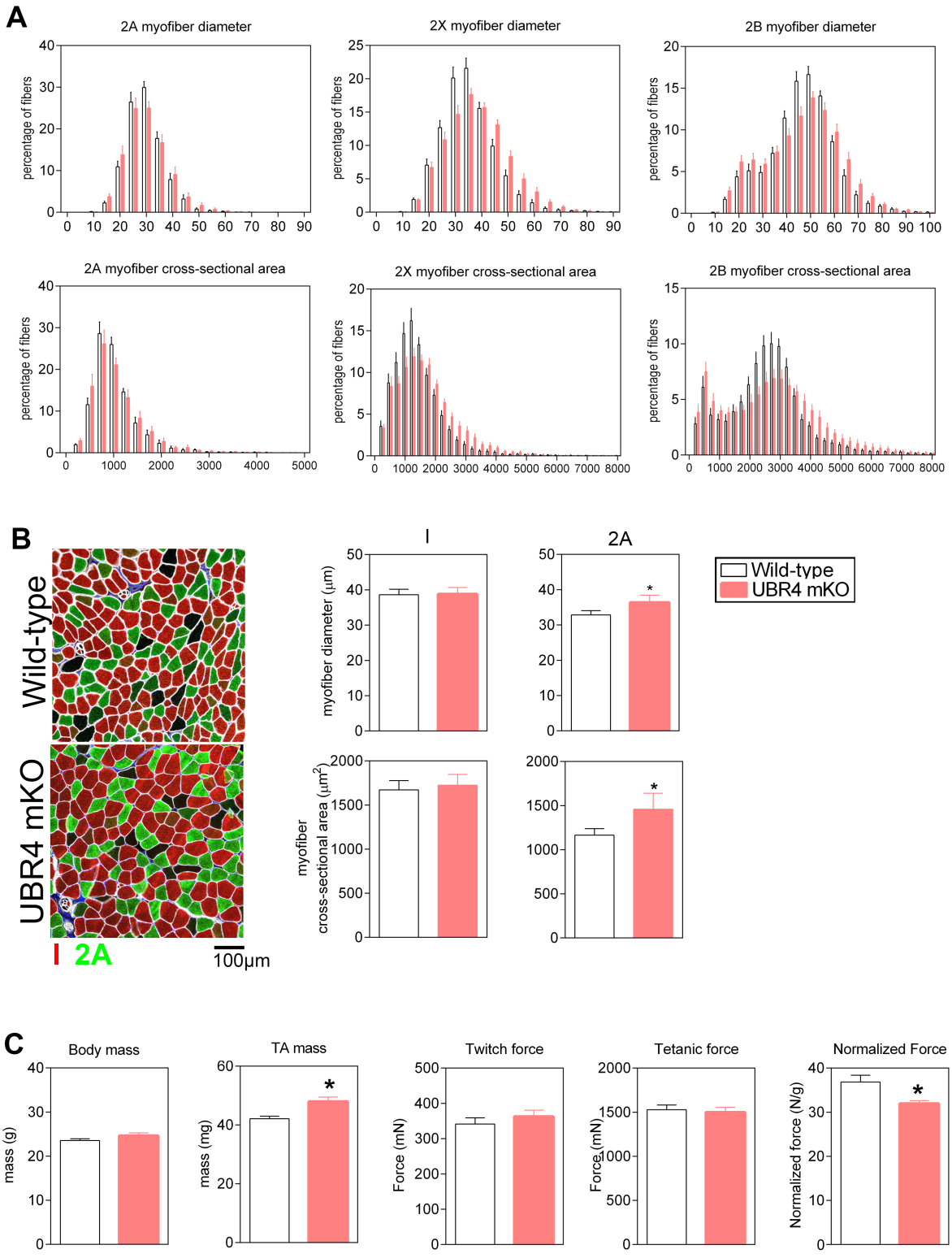
(D) Size of C2C12 myotube cultures transfected with candidate gene siRNAs and starved to induce atrophy. The top dashed line indicates the myotube size for NT control siRNAi under fed conditions. Note that Hgs and Ubr4 siRNAs prevent starvation-induced atrophy. $p<0.05$ with n=3 biological replicates.

(E) Representative images of tibialis anterior (TA) skeletal muscles following electroporation with siGLO red demonstrate the incorporation of siRNAs into the muscle.

(F) Representative images of UBR4C overexpression in C2C12 myoblasts demonstrate that UBR4 inhibits myotube formation and inhibits myotube growth.

(G) Western blots demonstrating that truncated UBR4 (UBR4C ~100 kDa) is distinguishable from endogenous UBR4 (~600 kDa) and differentially targeted by N- and C-terminal targeting siRNAs for UBR4. For a scheme of UBR4C, see Fig. 5D.

(H) Representative images of *UBR4C*-overexpressing myotubes which are smaller in size in comparison to controls, and are resistant to a N-terminal targeting UBR4 siRNA but not to a C-terminal targeting UBR4 siRNA. These results indicate that UBR4C rescues the myotube hypertrophy induced by UBR4N siRNAs. However, UBR4C is targeted by UBR4C siRNAs and therefore does not rescue the myotube hypertrophy induced by UBR4C siRNAs. Quantification of myotube diameters is shown in (I), with n=3 biological replicates and $p<0.05$.

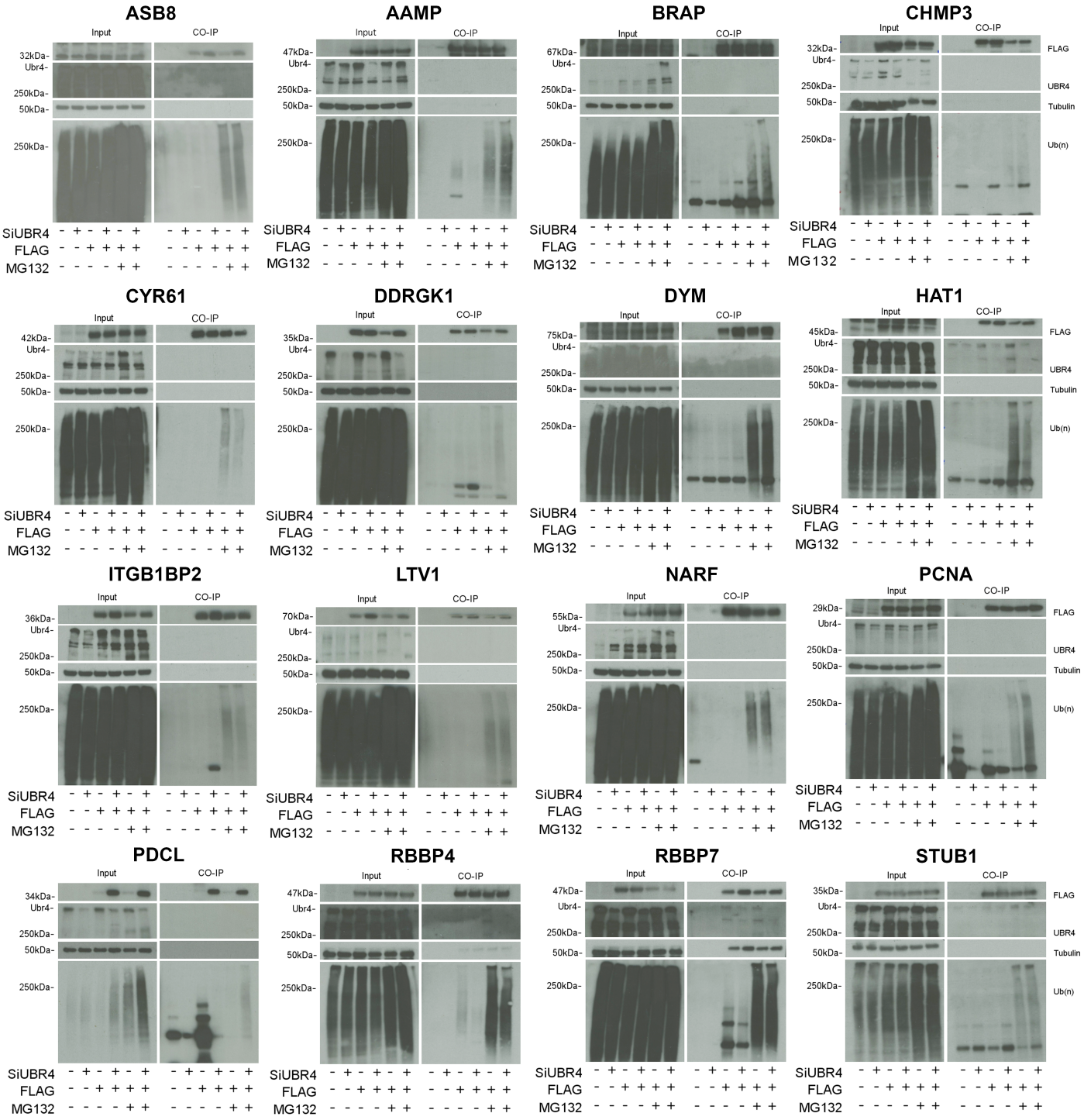


Supplemental Figure S3. Analysis of Muscle Strength in UBR4 mKO Mice and Controls. Related to Figures 3 and 4.

(A) Frequency distribution of myofiber sizes by isotype from male UBR4 mKO and wild-type tibialis anterior muscles (Figure 3J-K).

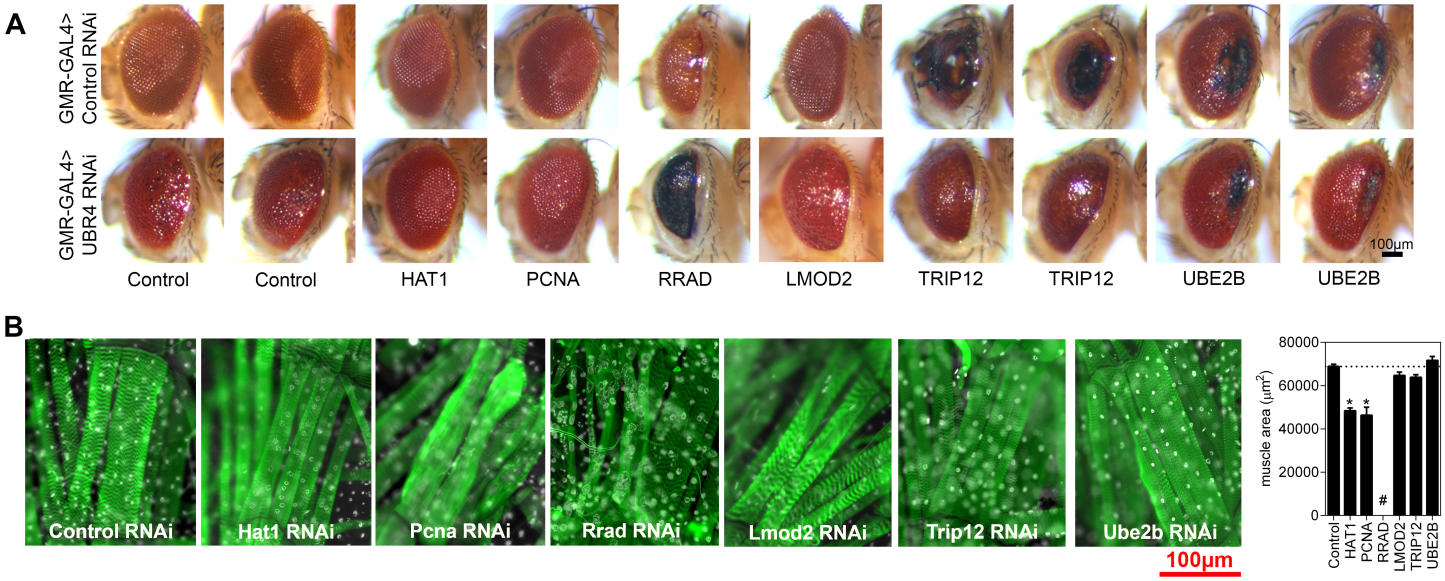
(B) Representative images of soleus muscle cross sections labelled for type 1 (red) and type 2A (green) myofibers. Quantitation of the sizes of different myofiber types indicates that type 2A myofibers are significantly larger in the soleus muscle of UBR4 mKO mice, compared to controls, whereas type 1 myofibers are overall unchanged (n≥7 mice per group).

(C) Body mass, tibialis anterior (TA) muscle mass, and *in situ* functional force measurements for the TA muscles of wild-type (white) and of UBR4 mKO (pink) female mice after 3 months of tamoxifen-induced recombination (n≥8 mice per group).



Supplemental Figure S4. Immunoprecipitation of FLAG-tagged UBR4 Target Proteins for Testing their Physical and Functional Interactions with UBR4. Related to Figure 5.

Full blots for all co-immunoprecipitated FLAG-tagged putative UBR4 target proteins. The interaction with endogenous UBR4 was tested together with assessment on whether UBR4 is required for their poly-ubiquitination, by using control and UBR4 siRNAs (SiUBR4).



Supplemental Figure S5. Epistatic Interactions of UBR4 with Target Proteins During Tissue Growth. Related to Figure 6.

(A) Testing genetic interactions of UBR4 with putative UBR4 target proteins in *Drosophila* eyes with UBR4 RNAi driven by GMR-GAL4 concomitantly to control RNAi or to RNAi for a UBR4 target protein.

UBR4 RNAi causes a rounded and rough eye phenotype consistent with overgrowth that can be suppressed epistatically by HAT1 and PCNA RNAi. Similar to UBR4 RNAi, UBE2B RNAi produced an overgrowth phenotype. Conversely, RRAD, LMOD2, and TRIP12 RNAi inhibited eye growth both independently and in combination with UBR4 RNAi. Two different RNAi lines were used for TRIP12 and UBE2B.

(B) Knockdown of the *Drosophila* homologs of HAT1, PCNA, RRAD, LMOD2, and TRIP12 (Hat1, PCNA, Rgk1, tmod, and ctrip, respectively) driven by Mef2-GAL4 reduces muscle growth whereas RNAi for *Drosophila* UBE2B (Ubc6) increases muscle size.

# indicates that the majority of RRAD RNAi larvae had either missing VL3/4 muscles or that these muscles were too disrupted to accurately be measured. $p<0.05$ with $n\geq3$ larvae.

Supplemental Table S7. List of siRNAs used and their catalogue numbers. Related to the STAR Methods.

ON-TARGET Plus siRNA mouse siRNA	Supplier: Dharmacon	Catalog number
Hgs		L-055516-01
Stam		L-060542-01
Stam2		L-059321-01
Usp8		L-059455-01
Siah1a		L-044891-01
Siah1b		L-044849-01
Siah2		L-041993-01
Ubr4		L-050850-00
March5		L-057048-01
Syvn1		L-041789-01
Narf		L-059561-01
Brap		L-047389-01
Ipo13		L-061248-01
Dnajc21		L-049723-01
Rbbp4		L-047448-01
Trip12		L-053913-01
Usp13		L-047753-01
Chmp3		L-062411-01
Klhl30		L-043614-01
Rbbp7		L-041063-00
Nat9		L-063342-01
Ecd		L-045010-01
Fyb		L-043701-00
Zranb1		L-059888-01
Hat1		L-047665-01
Pyroxd1		L-054188-01
Dym		L-049709-01
Inpp5k		L-040159-01
Pcna		L-048531-00
Ddrgk1		L-167022-00
Ccndbp1		L-043931-01
Cdk5rap3		L-046463-01
Ltv1		L-053984-02
Ufl1		L-058635-01
Arfgap3		L-040580-01
Itgb1bp2		L-047746-01
Stub1		L-063143-01
Pdcl		L-044992-01
Clhc1		L-063638-01
Ppp1r27		L-064502-01
Rrad		L-049536-01
Aamp		L-051212-01
Prkdc		L-040958-00
Fam160b2		L-047972-01
Cyr61		L-043717-01
Gfpt1		L-062877-01
Atxn10		L-043628-01
Prepl		L-059506-01
Mcmbp		L-060010-01
Ipo8		L-057518-00
Lmod2		L-055604-01
Asb8		L-061037-01
Mt2		L-042685-00
Hectd1		L-041047-00
Asns		L-047839-01
Ube2b		L-060426-00
Dnaja1		L-058731-01

Supplemental Table S8. Oligonucleotides used for qRT-PCR. Related to the STAR Methods.

Oligonucleotides			
Gene	**Forward sequence**	**Reverse sequence**	**Species**
Sina	CGGCCACCATTTTATGCTCG	CCGTTCAACTCCAGGCGATA	*Drosophila melanogaster*
Hrs	GGCACCTCCAGTTACCGAAA	TAATCAGTTCCGCCGTAGCC	*Drosophila melanogaster*
Stam	TTCACGGGATTTCGAGACGG	GCACTTGGCGCATTTTCAGT	*Drosophila melanogaster*
Sip3	AGACCTGCTTGGCCTTTACC	GGGGGAACGCTCCATAAAGT	*Drosophila melanogaster*
CG9855	GCTATTCCAGTGGGGCTTGT	TCCTGGCTGAGGTTCGGATA	*Drosophila melanogaster*
CG17991	CCAGGCTTGGGTGAATCCAT	CGTTTGGCATTGTGGACAGG	*Drosophila melanogaster*
Ubpy/Usp8	TGTCAGCTGAACCAGTGCAT	ACGAGCCAGAGTTACTGGGA	*Drosophila melanogaster*
Poe/UBR4	TCGGACCTGTCCTCGGTTAT	CGCCAGGATTTGTTCAGTGC	*Drosophila melanogaster*
Ubc6/UBE2B	GAGTACCCAAACAAACCGCC	TCGCTCAGCAGTGACTGTAT	*Drosophila melanogaster*
AlphaTub84b	GTTTGTCAAGCCTCATAGCCG	GGAAGTGTTTCACACGCGAC	*Drosophila melanogaster*
Siah1	ACCTTCCTGGTGCTGTTGAC	ATCGCCGCCTATGACCATTT	*Mus musculus*
Siah2	CCTGGAAGCTGTGATGTCCC	TGCATCATCACCCAGTCCAC	*Mus musculus*
Hgs	GGAACTACTGGGAGAAGAA	ATGGGCTGAGAGTCTGTCTC	*Mus musculus*
Stam	ACTAACCACCAGCACGAAGG	CCACCAGTTGGGATCACTGT	*Mus musculus*
Stam2	AGAGGTTGAGACAGCAACGG	GGTCTTGGGAGTCGGGTTTT	*Mus musculus*
Syvn1	GTGGTGGCTCATGCCTACTA	GATGTACAGGACTGCCATGCT	*Mus musculus*
March5	AGTGCAACGCCGAGTACTTA	ATGGCCTACAACCTGCATCA	*Mus musculus*
Usp8	ACAGGGAGCCATCGAAACTG	CATGGTGGCTTGTTTTCCCG	*Mus musculus*
Ubr4	TGAGTGAGGACAAGGGCAAC	GGGTTGGATCGAACGAAGGT	*Mus musculus*
Map1lc3b	GATAATCAGACGGCGCTTGC	CCAGGAGGAAGAAGGCTTGG	*Mus musculus*
Sqstm1	GAATGTGGGGGAGAGTGTGG	CCTCAATGCCTAGAGGGCTG	*Mus musculus*
Trim63	ACCTTCCTCTCAAGTGCCAAG	TCCCAAAGTCAATGGCCCTC	*Mus musculus*
Fbxo32	TGAGCGACCTCAGCAGTTAC	GCGCTCCTTCGTACTTCCTT	*Mus musculus*
Ubb	GGACGCTTAACCGATGGAGA	GCATTTTGACCTCTTTTCTGCCT	*Mus musculus*
Ubc	CTGCCCTCCCACACAAAGC	TCTGCATCGTCTCTCTCACG	*Mus musculus*
Psma1	GGTTGCACTGAAGAGAGCAC	CATCAGCAGTTAGACCCGCA	*Mus musculus*
Psma5	ACCAACATCGAGCTAGCCAC	TCGAGGACGGCTCCTTCTTA	*Mus musculus*

Hat1	ATGCCCTGACCTTTCATCCC	TCATTCACCCGTTTGCCGTA	*Mus musculus*
Rbbp4	ACGGGCCGCAGGAAACAATA	CAAAGGCCGCTTCCTTGTCAG	*Mus musculus*
Rbbp7	GAGATCGCGGCGTCTGG	TCAAACATCTCTTTACTCGCCA	*Mus musculus*
Cd101	AGCCGGCAGCAAGAGATTTT	GAAGCTGCGGGTTACCATCT	*Mus musculus*
Sln	TCTTCAGGAAGTGAAGACAAGCC	TGGTAGGACCTCACGAGGAG	*Mus musculus*
Mt2	TCGACCCAATACTCTCCGCT	AGGATCCATCGGAGGCACA	*Mus musculus*
Aldh3a1	GCGCAAGAATGAATGGACCTC	TCCTGGGTCTGACGAGTCTT	*Mus musculus*
Ppia	GGCCGATGACGAGCCC	TGTCTTTGGAACTTTGTCTGCAA	*Mus musculus*
Gapdh	CCAGAACATCATCCCTGCATCC	ATACTTGGCAGGTTTCTCCAGG	*Mus musculus*
Hprt	GATTAGCGATGATGAACCAGGTT	TCCAAATCCTCGGCATAATGAT	*Mus musculus*