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Supplemental Information

Skeletal Muscle-Specific Methyltransferase METTL21C Trimethylates p97 and Regulates Autophagy-Associated Protein Breakdown

Janica Lea Wiederstein, Hendrik Nolte, Stefan Günther, Tanja Piller, Martina Baraldo, Sawa Kostin, Wilhelm Bloch, Natalie Schindler, Marco Sandri, Bert Blaauw, Thomas Braun, Soraya Hölper, and Marcus Krüger

Supplemental Information

Supplemental Experimental Procedures

Primer for site directed mutagenesis

Mettl21c inactive mutant (101-108 [LEIGAGAG] to alanine [LEIAAAAA]):

GCTAAAATACTTGAAATTGCTGCTGCAGCAGCCCTTGTTTCC (forward),

GGAAACAAGGGCTGCTGCAGCAGCAATTTCAAGTATTTTAGC (reverse)

p97 K315A:

CCACCTCGCCATGAGTTGCCTCTCTTTTGGGAGCGA

TCGCTCCCAAAAGAGAGGCAACTCATGGCGAGGTGG

Transgenic mouse line

The construct for the targeting vector replacing the *Mettl21c* gene with a LacZ cassette was commercially obtained from Velocigene. After linearization, the construct was transfected into pluripotent embryonal mouse ES cells (F1 hybrid Bl6/129) by electroporation and clones that successfully integrated the construct were selected using G418 antibiotic. ES cells with homologous recombination at the correct locus were injected into C57BL/6 mouse blastocysts and subsequently transplanted into pseudo-pregnant C57BL/6 mice. Resulting chimeras were mated with *Cre*-deleter mice to remove the *loxP* flanked neomycin-resistance cassette. The mice were housed under regular conditions at an ambient temperature of 20-22°C under a 12 hour light/dark cycle, and provided with food and water ad libitum. All animal procedures were performed in accordance with institutional guidelines.

Forced running tests

The forced running exercise was performed on a treadmill (Columbus Instruments) that provides a low-intensity electric shock to the paws of the mouse when the mouse stops running. Mice were trained for two consecutive days while increasing speed from 0 to 12 m/min for a

total duration of 12 min to become familiar with the environment. On the third day, mice were starved for 4 h, and after a 5 min warm-up period, the mice were exercised at 12 m/min for 60 min. Fulfilling any of the three following criteria lead to termination of the exercise session: 1) spending more than 5 consecutive seconds on the shock grid without attempting to re-engage the treadmill, 2) spending more than 50% of time on the shock grid or 3) the mouse was willing to sustain 2 seconds or more of shock for the third time without attempting to re-engage the treadmill. Following the running exercises, mice were returned to their cage and given access to food and water.

In vitro methyltransferase assay with recombinant proteins

Constructs coding for METTL21C with c-terminal 6xHis-Tag (in pET26b vector) and p97 with n-terminal GST-Tag (in pGEX6pi vector) were transformed into E.coli (BL21(DE3)). Expression was induced by addition of 250 mM IPTG and continued o/n at 18°C. Cells were then harvested and lysed. Proteins were purified using an ÄKTA HPLC system with Ni-NTA- or GSH-columns, respectively. Imidazole gradient elution fractions, containing METTL21C-6xHis, were pooled and further purified by Gelfiltration. The GST-tag was removed by incubation with PreScission Protease (GE Healthcare). Successful purification was validated by immunoblotting with METTL21C and p97 antibodies.

In vitro methyltransferase assay was performed in methylation buffer (100 mM Tris pH 8.5, 10 mM MgCl2, 8 mM DTT) with 2 mM S-adenosyl-*L*-methionine for 1h at 37°C. Concentration of recombinant METTL21C ranged from 10 pmol to 1 nmol. Methylation was analyzed by mass spectrometry and immunoblotting with antibodies raised against mono, di- and trimethylated lysine, as well as the p97-K315 specific trimethyl antibody.

Size exclusion chromatography

Cells were lysed in ice-cold size exclusion chromatography (SEC) buffer (50 mM KCl, 50 mM sodium acetate, pH 7.2), supplemented with protease and phosphatase inhibitor cocktail and sonicated. The cleared lysate was concentrated using 10K Amicon filters. Gel filtration was performed on a BioSep 5 µm SEC column (LC Column, 300 × 4.6 mm, Phenomenex) at a flow rate of 0.3 mL/min at 12°C, and fractions were collected every minute.

Figure S1

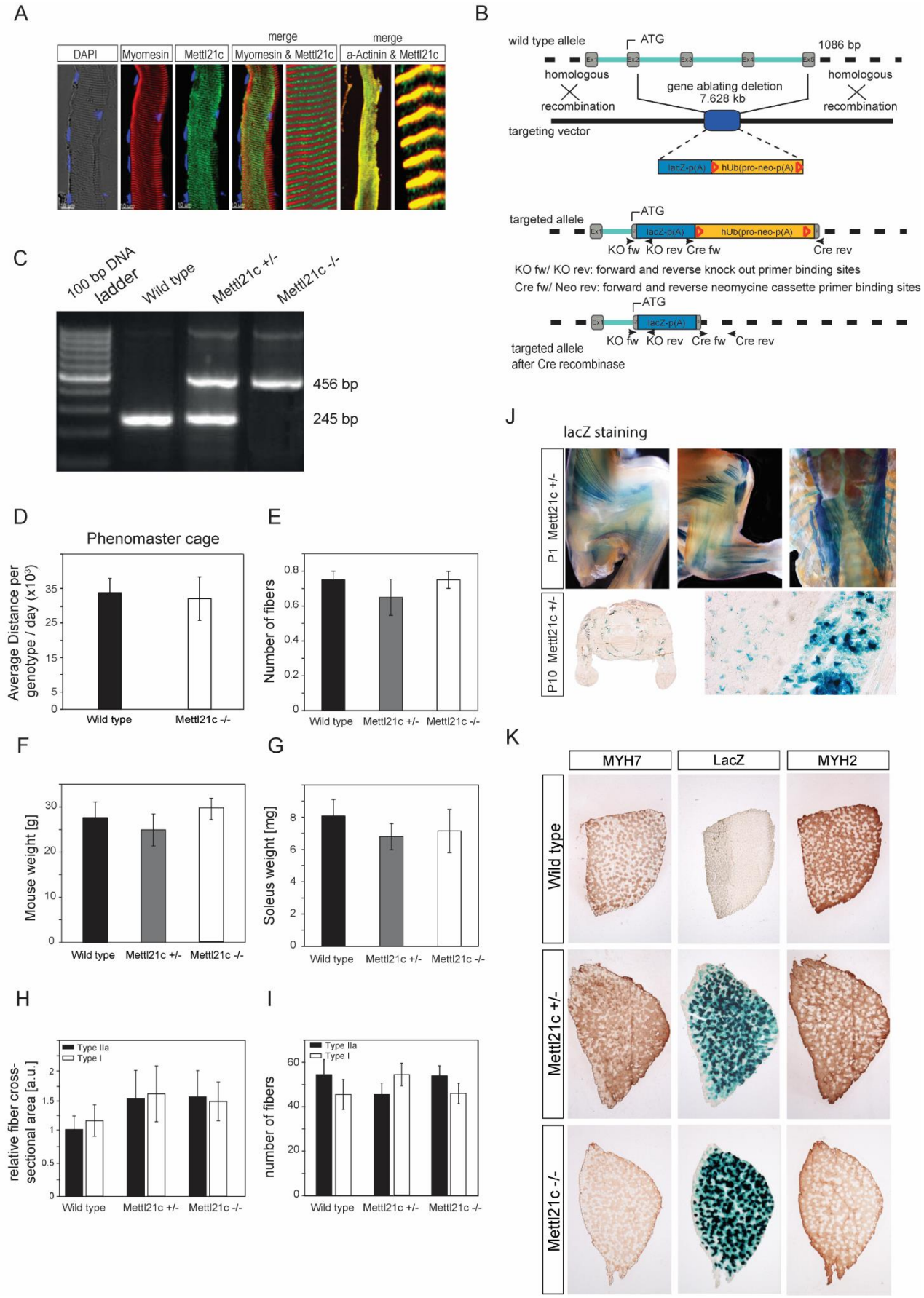
A
DAPI
Myomesin
Mettl21c
merge
Myomesin & Mettl21c
merge
a-Actinin & Mettl21c
B
wild type allele
ATG
1086 bp
homologous recombination
gene ablating deletion 7.628 kb
homologous recombination
targeting vector
lacZ-p(A)
hUb(pro-neo-p(A)
targeted allele
KO fw
KO rev
Cre fw
Cre rev
KO fw/ KO rev: forward and reverse knock out primer binding sites
Cre fw/ Neo rev: forward and reverse neomycine cassette primer binding sites
targeted allele after Cre recombinase
C
100 bp DNA ladder
Wild type
Mettl21c +/-
Mettl21c -/-
456 bp
245 bp
D
Phenomaster cage
Average Distance per genotype / day (x10³)
Wild type
Mettl21c -/-
E
Number of fibers
Wild type
Mettl21c +/-
Mettl21c -/-
F
Mouse weight [g]
G
Soleus weight [mg]
H
relative fiber cross-sectional area [a.u.]
Type IIa
Type I
I
number of fibers
J
lacZ staining
P1 Mettl21c +/-
P10 Mettl21c +/-
K
MYH7
LacZ
MYH2
Wild type
Mettl21c +/-
Mettl21c -/-

Figure S1: Genetic ablation of *Mettl21c* by homologous recombination and tissue-specific detection of lacZ reporter. Related to Figure 1 (**A**) Immunostaining for METTL21C in human longitudinal muscle sections, with co-staining for the Z-disk marker α-actinin-2 and M-band marker myomesin-2. DAPI (blue) was used to counterstain nuclei; scale bars = 10 µm. (**B**) Targeted disruption of the *Mettl21c* genomic locus. Schematic representation of the *Mettl21c* locus (wild-type allele), the targeting vector, and the disrupted allele produced by homologous recombination. Insertion of the β-galactosidase cassette and neomycin resistance gene deleted the coding sequence from exon 2 to exon 5. The targeting vector introduces two *loxP* sites flanking the selection cassette and was deleted by crossing to a *cre*-recombinase expressing mouse strain. (**C**) PCR amplification of genomic DNA from the *Mettl21c* gene locus to genotype mice. (**D-I**) Various parameters were determined for mice that participated in voluntary and forced running experiments, including (D) average distance moved by wild-type and *Mettl21c*$^{-/-}$ mice in a phenomaster cage, (E) soleus fiber length, (F) mouse weight, (G) soleus weight, (H) relative fiber area, (I) and relative number of type I and type IIa fibers in the soleus of wild-type and *Mettl21c*$^{-/-}$ mutants per 100 fibers. All data in D-I are mean ± SD (n = 3). (**J**) β-galactosidase staining of *Mettl21*$^{+/-}$ mice during the early postnatal stages. (**K**) Immunohistochemical staining of consecutive sections from wild-type, *Mettl21*$^{+/-}$ and *Mettl21c*$^{-/-}$ soleus muscle using MYH7 (left), β-galactosidase (middle) and MYH2 (right).

Figure S2

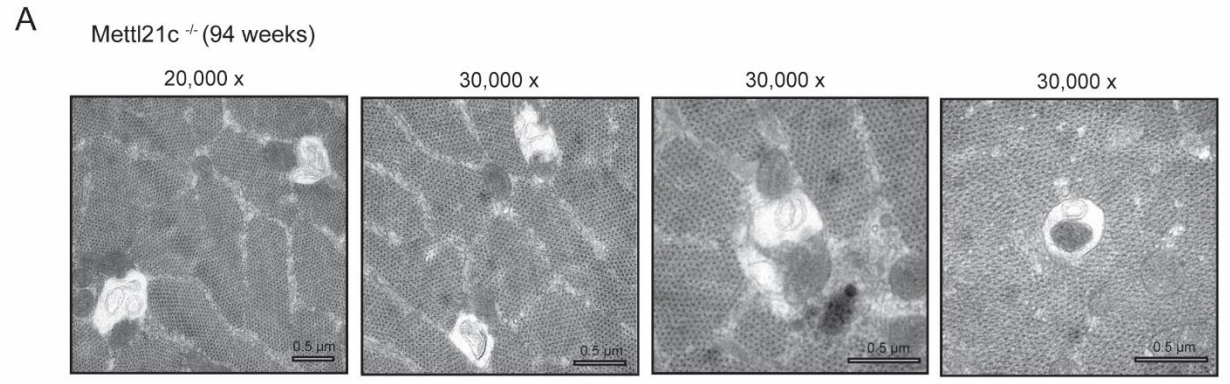

Figure S2: Electron microscopy reveals accumulation of autophagic vacuoles in the soleus of *Mettl21c*$^{-/-}$ mice. Related to Figure 2. (**A**) Representative electron micrographs of the soleus from 94-week-old *Mettl21c*$^{-/-}$ mutants showing non-digested autophagosomes.

Figure S3

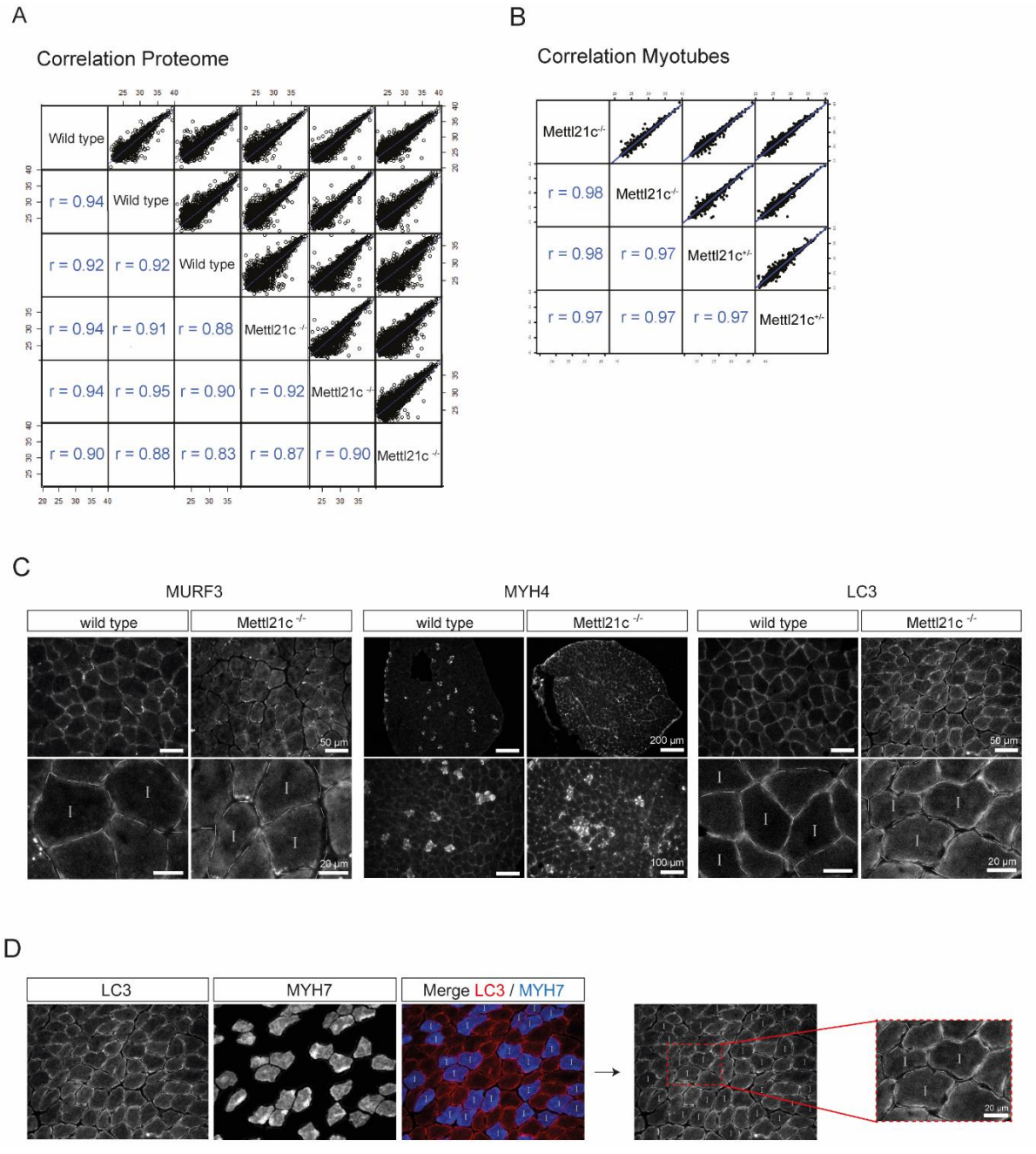

Figure S3: Correlation of muscle proteomes and immunohistochemistry of proteins with enhanced levels in *Mettl21c*$^{-/-}$ mutants. Related to Figure 3. (**A**) Correlation of $\log_2$ protein intensities for *Mettl21*$^{-/-}$ and control wild-type whole soleus muscles. (**B**) Correlation of $\log_2$ protein intensities for isolated β-galactosidase-positive muscle fibers from *Mettl21c*$^{+/-}$ and *Mettl21c*$^{-/-}$ mutants. (**C**) Immunostaining for TRIM54, MYH4 and LC3 in soleus cryosections.

(**D**) Workflow example for fiber type characterization based on co-staining with the MYH7 antibody. Slow type I fibers (blue) are marked with “I”.

Figure S4

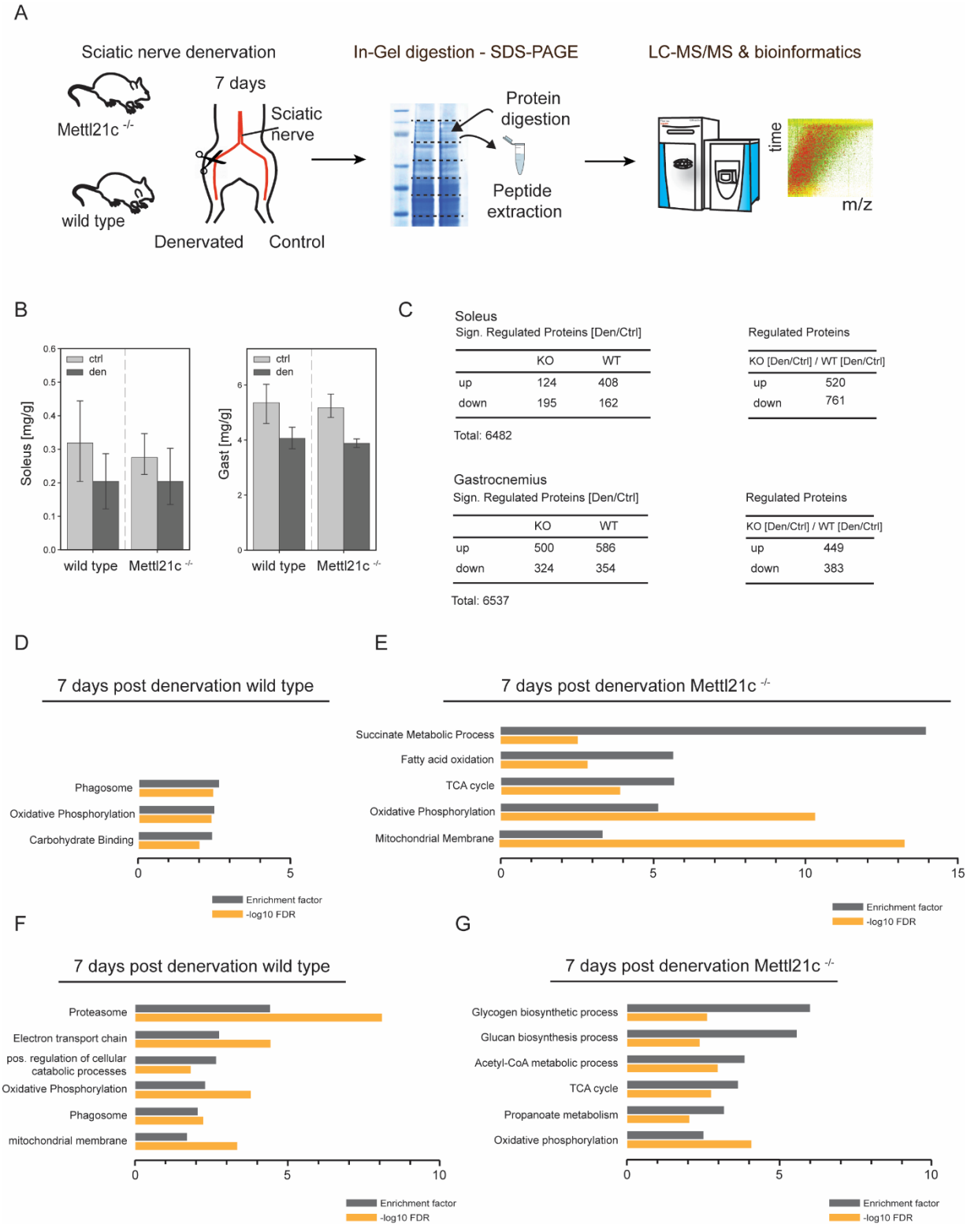

Figure S4: Experimental workflow for the sciatic nerve denervation experiments and evaluation of MS data. Related to Figure 4. (**A**) Schematic illustration of denervation of the sciatic nerve and the proteomic workflow. Unilateral denervation via sciatic nerve section was

performed; after seven days, the animals were sacrificed and muscles dissected for analysis. The contralateral hind limb served as a control. **(B)** Weight of muscles from denervated (den) and control (ctrl) legs in relation to total body weight at 7 days after denervation for wild-type and *Mettl21c*$^{-/-}$ mice. Data are mean ± SD (n = 3). **(C)** Table of the numbers of regulated proteins (t-test significant after permutation-based FDR cutoff of 0.05) in wild-type and *Mettl21c*$^{-/-}$ soleus and GAST at 7 days after denervation (left panel). The $\log_2$ fold changes (den/ctrl) were compared between wild-type and *Mettl21c*$^{-/-}$ mice and the number of differentially regulated proteins ($\log_2$ fold change > 0.58) was determined (right panel). **(D-G)** Fisher exact tests of enriched GO annotations in wild-type and *Mettl21c*$^{-/-}$ soleus (D, E) and GAST at 7 days after denervation **(**F, G**)**.

Figure S5

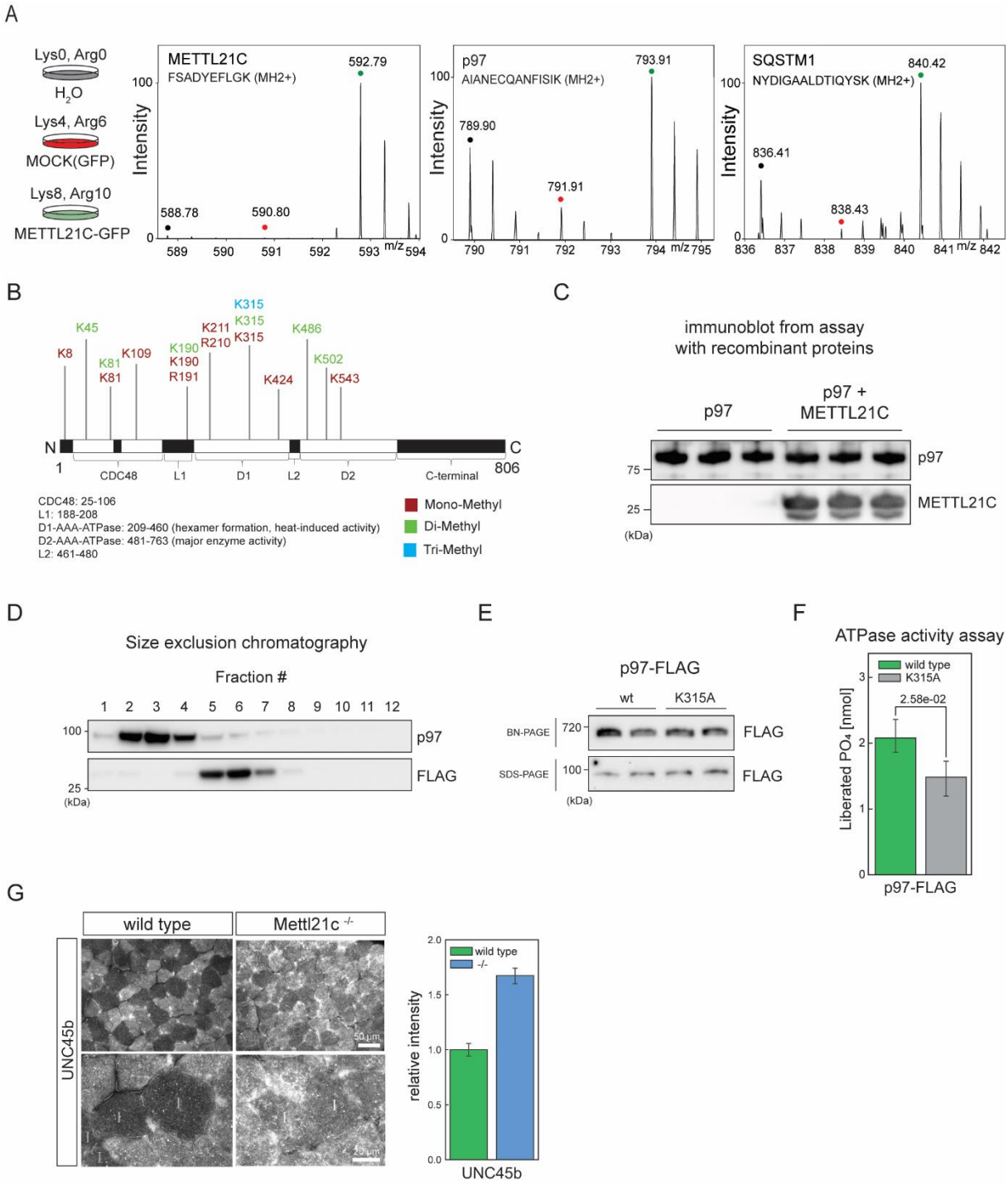

Figure S5: Overview of immunoprecipitation experiments, p97 methylation sites, size exclusion chromatography, p97 hexamer assembly and activity. **Related to Figure 5.** (**A**) Triple SILAC labeling of 293 HEK cells. Control transfection with H_2O (light = Lys0, Arg0), the GFP expression vector (middle = Lys4, Arg6) or Mettl21c-GFP expression construct (heavy = Arg10, Lys8). After immunoprecipitation with GFP-beads, the three samples were mixed in

a 1:1:1 ratio and analyzed by mass spectrometry. Representative SILAC pairs are shown for peptides from METTL21C (left panel), p97 (middle panel) and p62 (right panel). (**B**) Methylation sites identified on p97 after incubation with METTL21C. (**C**) Immunoblot of recombinant p97 and METTL21C proteins utilized for in vitro methylation assay. (**D**) Immunoblotting of p97 and METTL21C-FLAG from HEK cell lysates fractionated by size exclusion chromatography. (**E**) BN-PAGE and SDS-PAGE of HEK lysates after expression of p97 wild type or K315A FLAG-fusion using anti-FLAG antibody for detection. SDS-PAGE is used for determination of total p97 levels and serves as loading control. (**F**) Bar plot of ATPase activity assay. p97 wild type and K315A FLAG-fusion were overexpressed in HEK cells and pulled down with FLAG beads. Levels of liberated phosphate after 1 hour of incubation were determined using a malachite green-based colorimetric assay. Bar plots represent mean ± 0.95 CI (n = 4). (**G**) Immunostaining of UNC45b and quantitative analysis of fluorescent signals in slow type I fibers. Slow fibers were identified by co-staining with MYH7. Bar plots represent mean ± 0.95 CI (n = 15-20).