

1 Supplementary Material for

2 Pharmacological PIK3C2B inhibition rescues XLMTM phenotype in mouse models

3 and identifies molecular markers of disease

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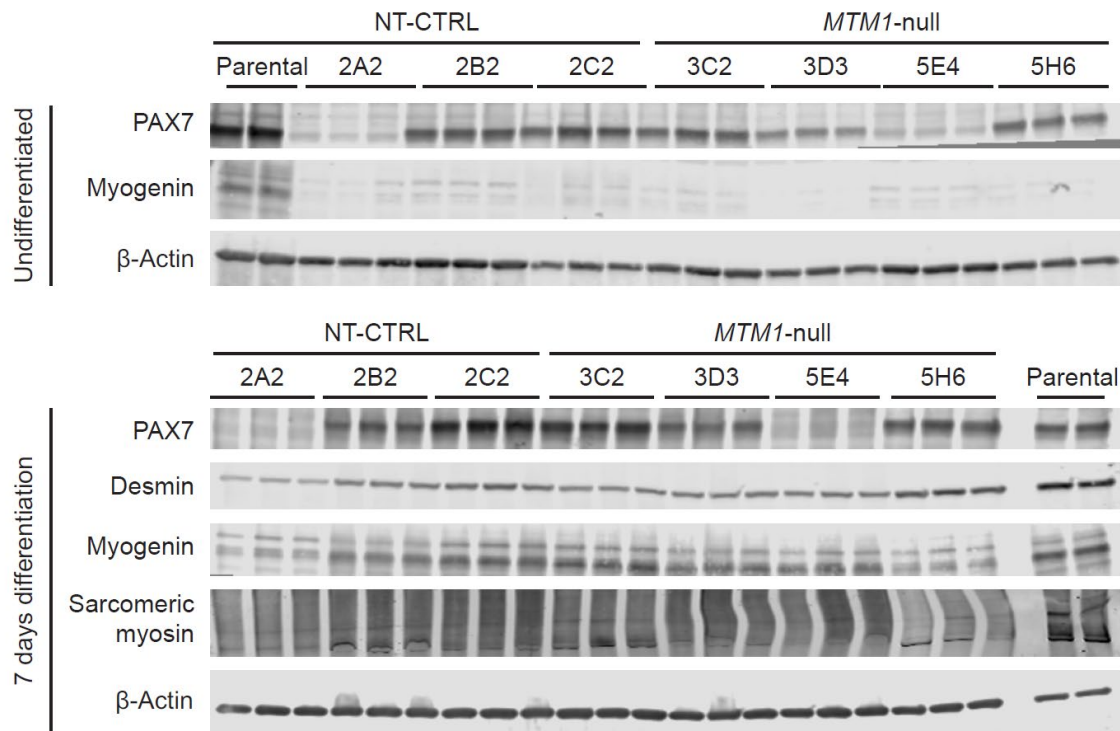
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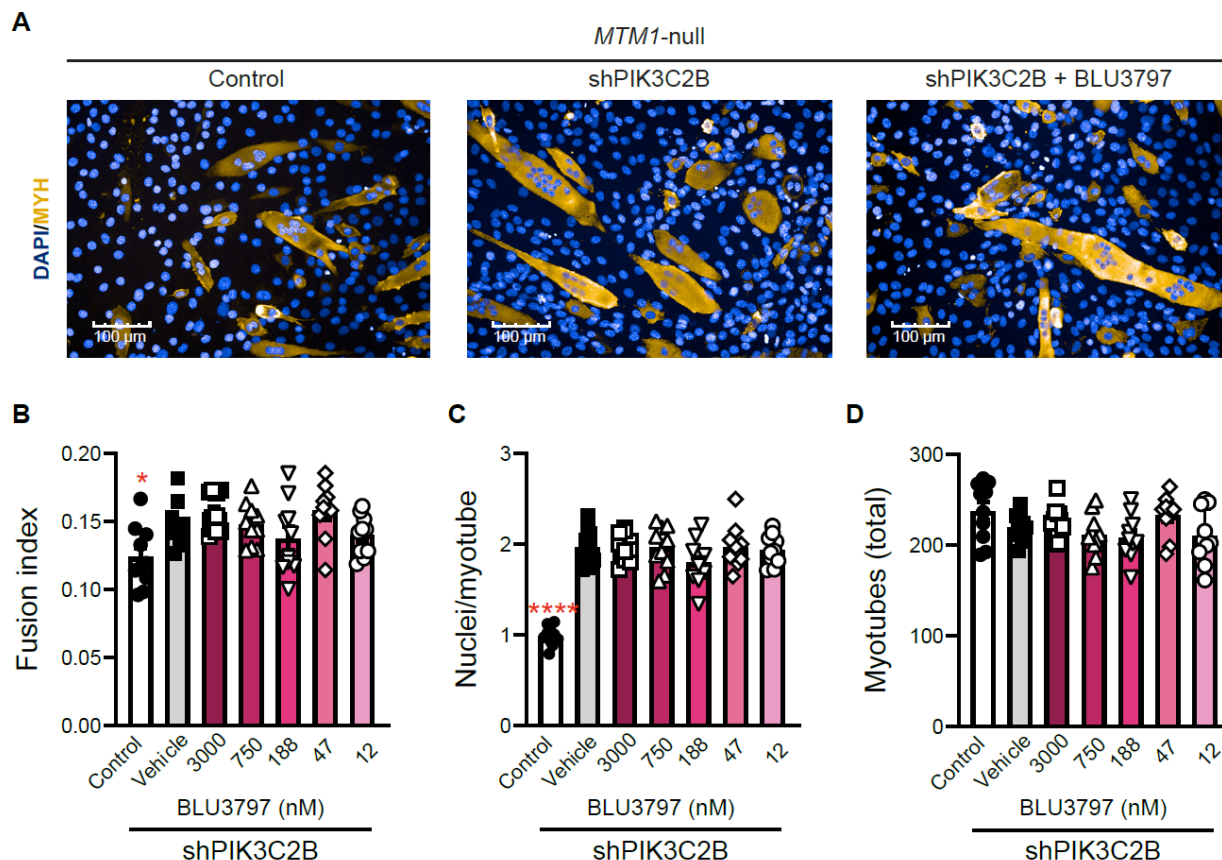
Supplemental figures



Supplemental Figure 1. Generation of C2C12 *MTM1*-null clones possess varied cellular phenotypes.

Western blot analysis of clones that had been generated by CRISPR-Cas9 targeted ablation of *MTM1* or cells generated using a non-targeting guide.

KO, knock-out; *MTM1*, myotubularin 1; NT-CTRL, non-targeting control; sgRNA, single-guide RNA.



Supplemental Figure 2. PIK3C2B inhibition does not increase myocyte-myotube fusion in PIK3C2B-

depleted *MTM1*-KO C2C12 cells. (A) Example images of *MTM1*^{-/-} C2C12 cells fixed after 5 days

differentiation and 2 days of BLU3797 treatment (experiment conducted twice). **(B)** Quantification of the

fraction of nuclei in MYH positive structures (Fusion Index) from 5 random non-overlapping fields (n =

10). **(C)** Representative quantification of average nuclei in MYH positive structures (myotubes) from 5

random non-overlapping fields (n = 10). **(D)** Representative quantification of the total MYH positive

structures (myotubes) from 5 random non-overlapping fields (n = 10). Error bars show SEM. *p≤0.05,

p≤0.01, *p≤0.001, ****p≤0.0001. Comparison of myocyte fusion, number, and nuclei fraction in

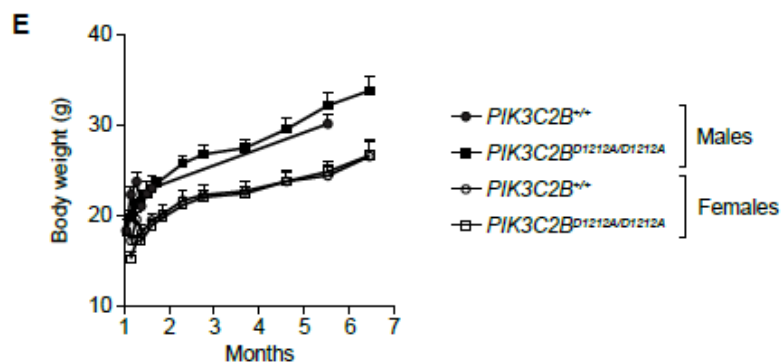
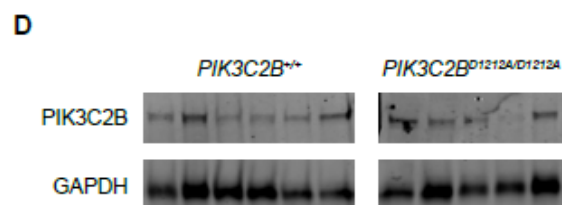
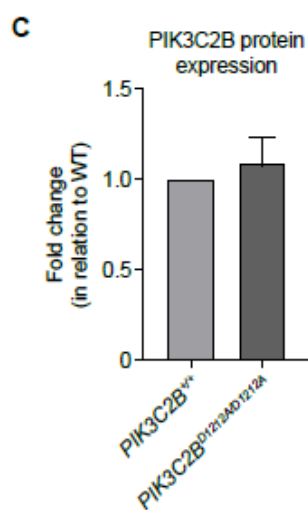
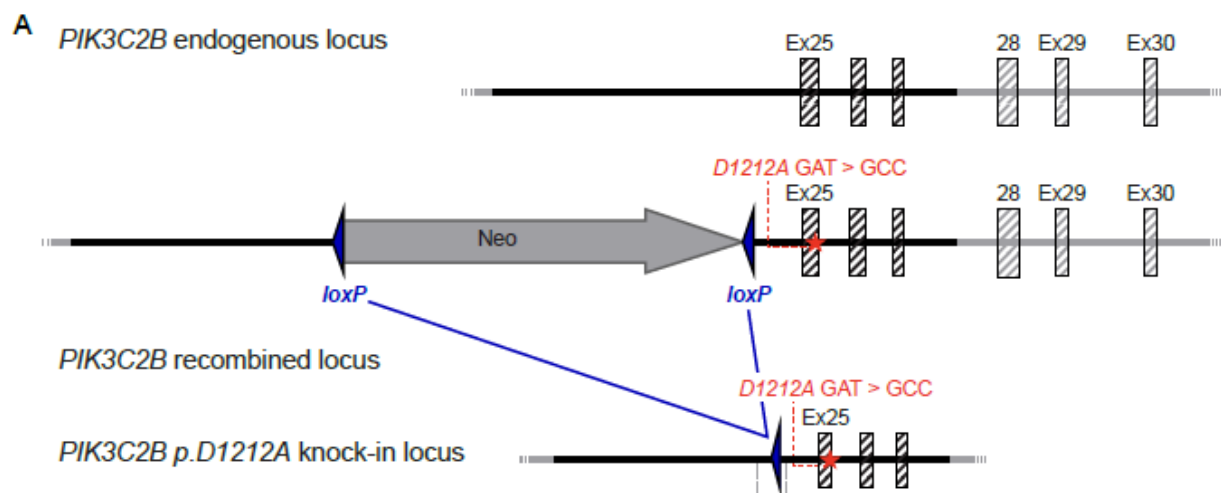
C2C12 cells were done by one-way analysis of variance (ANOVA) with Dunnett's correction for multiple

comparisons.

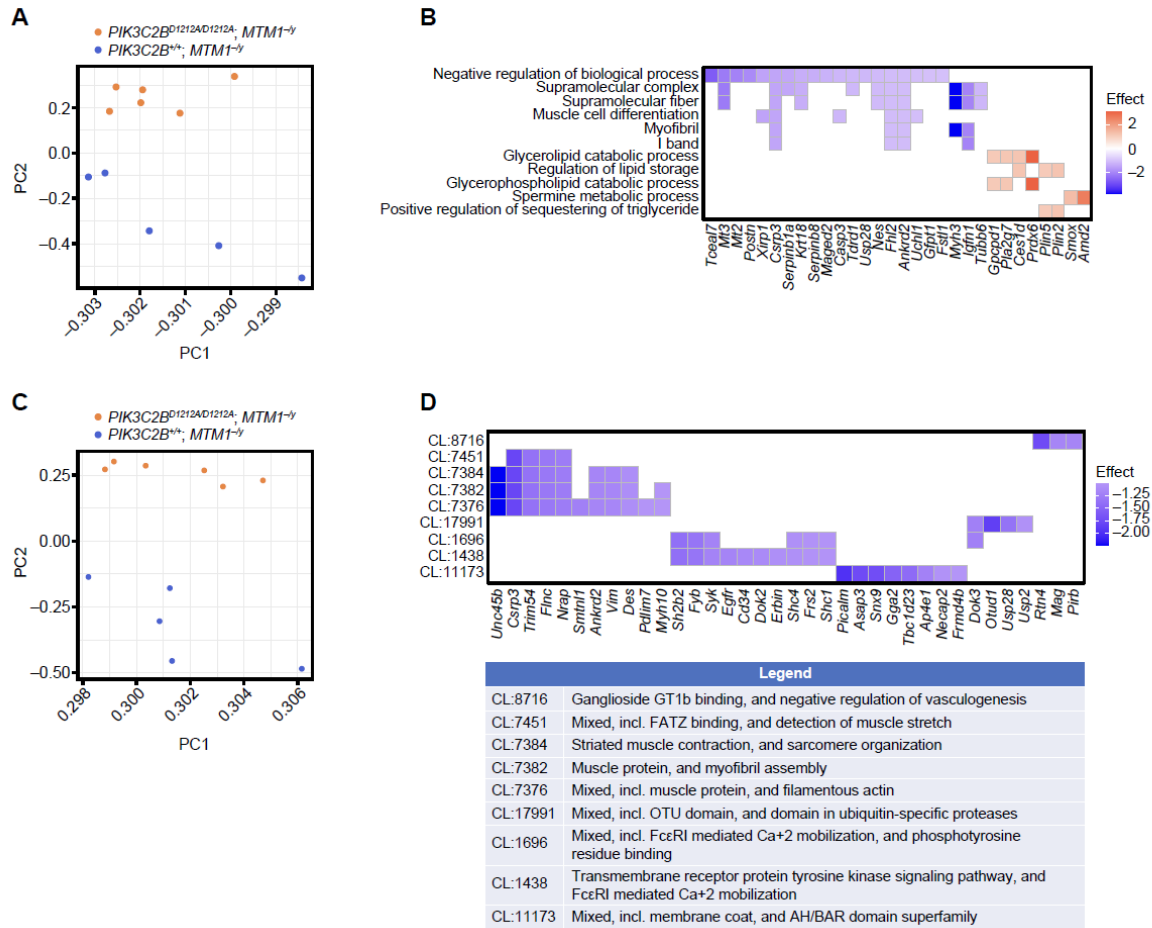
DAPI, 4',6-diamidino 2-phenylindole; MTM1, myotubularin 1; MYH, myosin heavy chain; PIK3C2B,

phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta; SEM, standard error of the

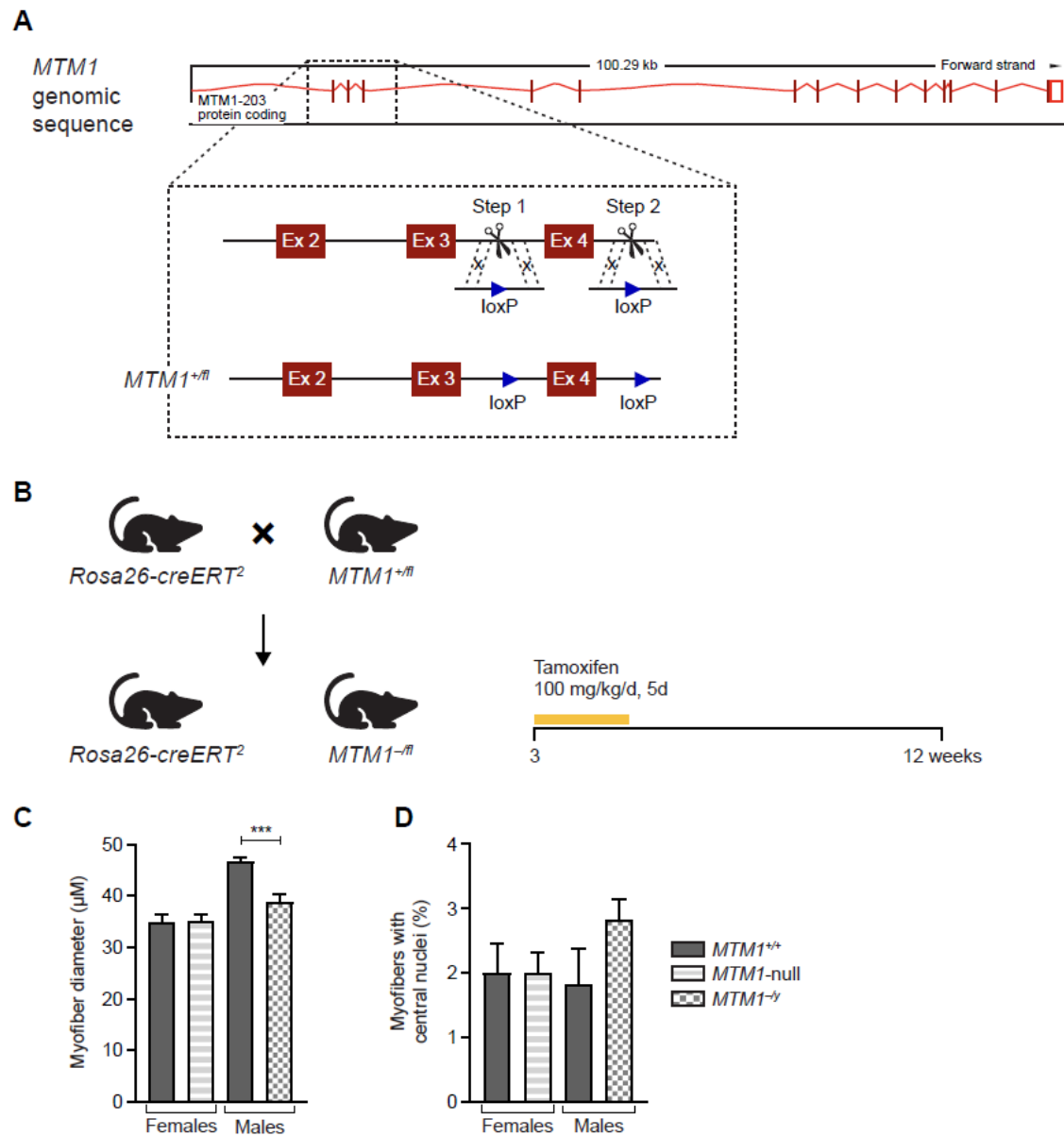
mean.



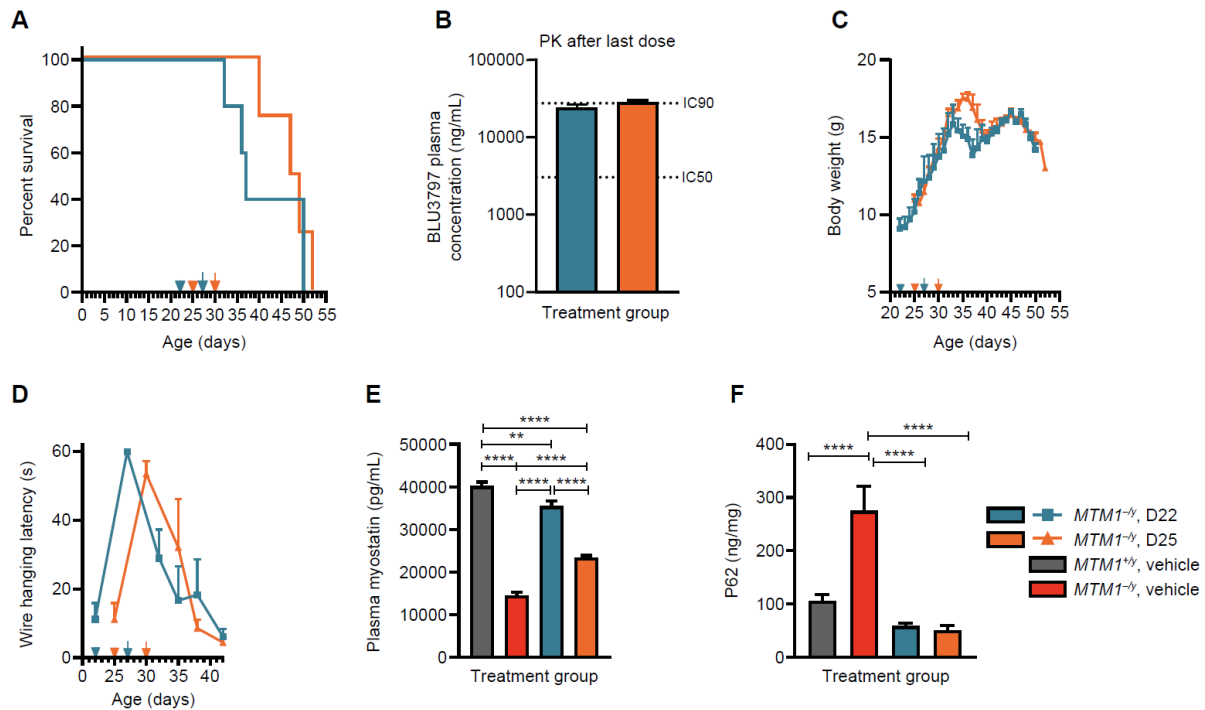
Supplemental Figure 3. The *D1212A* mutation was introduced to generate a kinase dead mouse model of PIK3C2B. (A) Generation of the D1212A kinase dead mouse model by GAT→GCC insertion within exon 25 of C57BL/6 ES cells. A floxed Neomycin resistance cassette was utilized for efficient selection of ES cells. Breeding was established with C57BL/6N Cre-deleter mice. This breeding strategy resulted in the generation of heterozygous mice carrying the *D1212A* knock-in allele. (B) Two CC mutations introduced are shown above in gray for each read, while the corresponding WT sequence is shown in green and red and spelled out at the bottom. (C) Comparison of PIK3C2B protein levels from mouse muscle lysate. (D) Comparison of body weight between male mice with the *D1212A* mutation (*PIK3C2B*^{D1212A/D1212A}) and those without the mutation (*PIK3C2B*^{+/+}), as well as between female mice with the *D1212A* mutation (*PIK3C2B*^{D1212A/D1212A}) and those without the mutation (*PIK3C2B*^{+/+}). Error bars show SEM. ES, embryonic stem; PIK3C2B, phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta; SEM, standard error of the mean; WT, wild-type.



Supplemental Figure 4. Differential proteomics and phosphoproteomics abundance in PIK3C2B kinase dead *MTM1*^{-/-} mice compared with *PIK3C2B*^{+/+}; *MTM1*^{-/-} identifies genes involved in enrichment of muscle-related pathways. (A) Principal components analysis of protein abundance in *PIK3C2B*^{D1212A/D1212A}; *MTM1*^{-/-} mice and *PIK3C2B*^{+/+}; *MTM1*^{-/-} mice. (B). Heatmap of genes involved in the 10 GO most enriched pathways identified in differential abundance analysis of the proteome in *PIK3C2B*^{D1212A/D1212A}; *MTM1*^{-/-} vs. *PIK3C2B*^{+/+}; *MTM1*^{-/-} mice. (C) Principal components analysis of phosphoprotein abundance in *PIK3C2B*^{D1212A/D1212A}; *MTM1*^{-/-} and *PIK3C2B*^{+/+}; *MTM1*^{-/-} mice. (D) Heatmap of genes involved in the 10 most enriched GO pathways identified in differential abundance analysis of the phosphoproteome in *PIK3C2B*^{D1212A/D1212A}; *MTM1*^{-/-} vs. *PIK3C2B*^{+/+}; *MTM1*^{-/-} mice with *MTM1*^{-/-}. GO, gene ontology; MTM1, myotubularin 1; PIK3C2B, phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta.

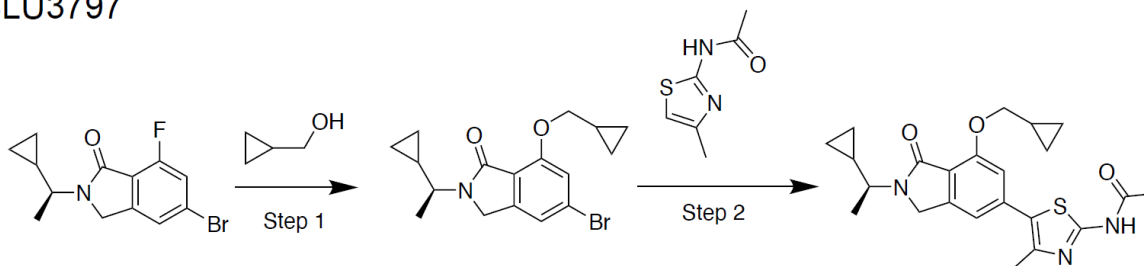


59 **Supplemental Figure 5. The conditional knock-out of the *MTM1* gene in mice resulted in a subtle**
60 **muscle phenotype specifically observed in male individuals. (A)** Generation of the *MTM1*^{+/fl} mouse,
61 loxP sites were introduced upstream and downstream of *MTM1* exon 4 using CRISPR/Cas9. **(B)**
62 Recombination of the floxed allele was induced by crossing *MTM1*^{-/fl} animals with *Rosa26creERT2* mice.
63 Resulting offspring were treated with tamoxifen at a dose of 100 mg/kg/d for 5 consecutive days. **(C)**
64 Summary of histological analysis of myofiber diameter in 12-week-old male and female mice. **(D)**
65 Summary of histological analysis of the percentage of myofibers with central nuclei for both male and
66 female muscle. Error bars show SEM. ***p≤0.001. Comparisons of myofiber diameter were done by
67 Kruskal-Wallis test with Dunn's correction for multiple comparisons.
68 *MTM1*, myotubularin 1; SEM, standard error of the mean.

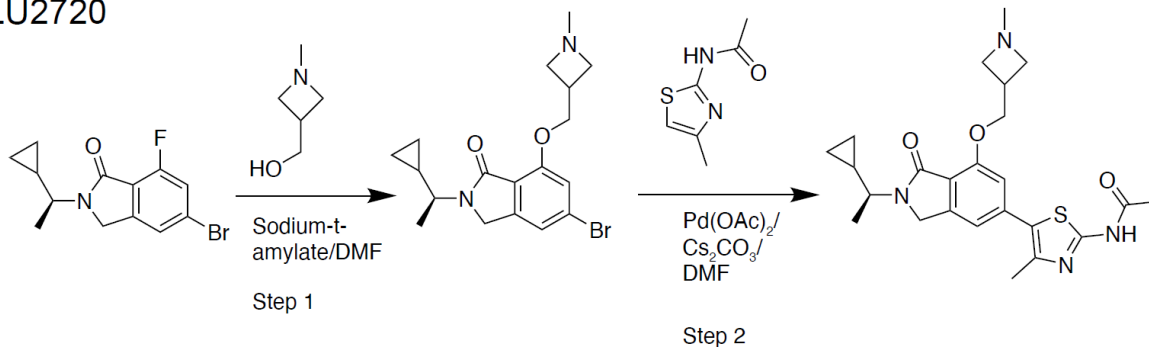


Supplemental Figure 6. BLU3797 effectively increases the lifespan of *MTM1*^{-/-} mice and restores muscle function, regardless of whether treatment is initiated on day 22 or day 25 of age. (A) Kaplan–Meier curve of *MTM1*^{-/-} animals that were given BLU3797 at a dose of 75 mg/kg, twice daily, starting either on day 22 (teal arrowhead) or day 25 (orange arrowhead) of age. Dosing stopped 5 days later (teal and orange arrows). **(B)** Plasma PK after the last dose of BLU3797. FBS-corrected NanoBRET™ IC₅₀ and IC₉₀ lines are indicated. **(C)** Animal weight. **(D)** Analysis of muscle function by hanging latency. **(E)** Measurement of plasma myostatin on the final dosing day, day 5 post first dose. **(F)** SQSTM1/P62 protein levels measured in the muscle at end of the study. Arrowheads represent start of the study; arrows represent the last day of dosing. Error bars show SEM. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. Comparisons of plasma myostatin levels were done by one-way analysis of variance with Tukey's correction for multiple comparisons. Comparisons of muscle P62 expression were done by one-way analysis of variance with Šidák's correction for multiple comparisons. FBS, fetal bovine serum; IC₅₀, half-maximal inhibitory concentration; IC₉₀, 90% inhibitory concentration; MTM1, myotubularin 1; PK, pharmacokinetics; SEM, standard error of the mean.

A. BLU3797



B. BLU2720



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89 **Supplemental Figure S7. Steps in the generation of BLU3797 and BLU2720.** (A) Step 1 involves the
 90 synthesis of (S)-5-bromo-2-(1-cyclopropylethyl)-7-(cyclopropylmethoxy)isoindolin-1-one. Step 2 involves
 91 the synthesis of (S)-N-(5-(2-(1-cyclopropylethyl)-7-(cyclopropylmethoxy)-1-oxoisoindolin-5-yl)-4-
 92 methylthiazol-2-yl)acetamide. (B) Step 1 involves the synthesis of 5-bromo-2-[(1S)-1-cyclopropylethyl]-7-
 93 [(1-methylazetidin-3-yl)methoxy]isoindolin-1-one. Step 2 involves the synthesis of N-[5-[2-[(1S)-1-
 94 cyclopropylethyl]-7-[(1-methylazetidin-3-yl)methoxy]-1-oxoisoindolin-5-yl]-4-methylthiazol-2-
 95 yl]acetamide.

96 DMF, N,N-Dimethylformamide.

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