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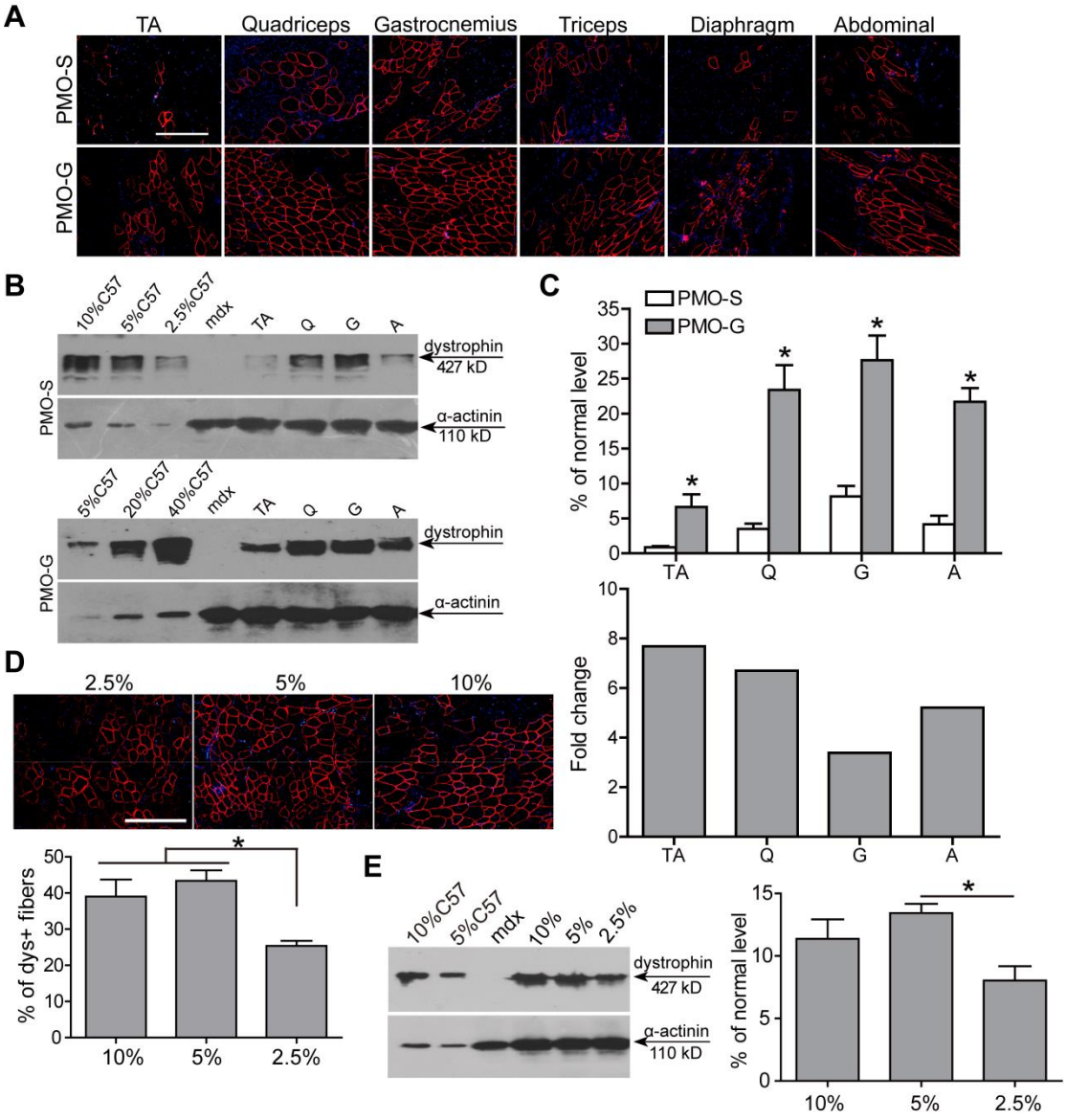
Supplemental Information

Glycine Enhances Satellite Cell Proliferation, Cell Transplantation, and Oligonucleotide Efficacy in Dystrophic Muscle

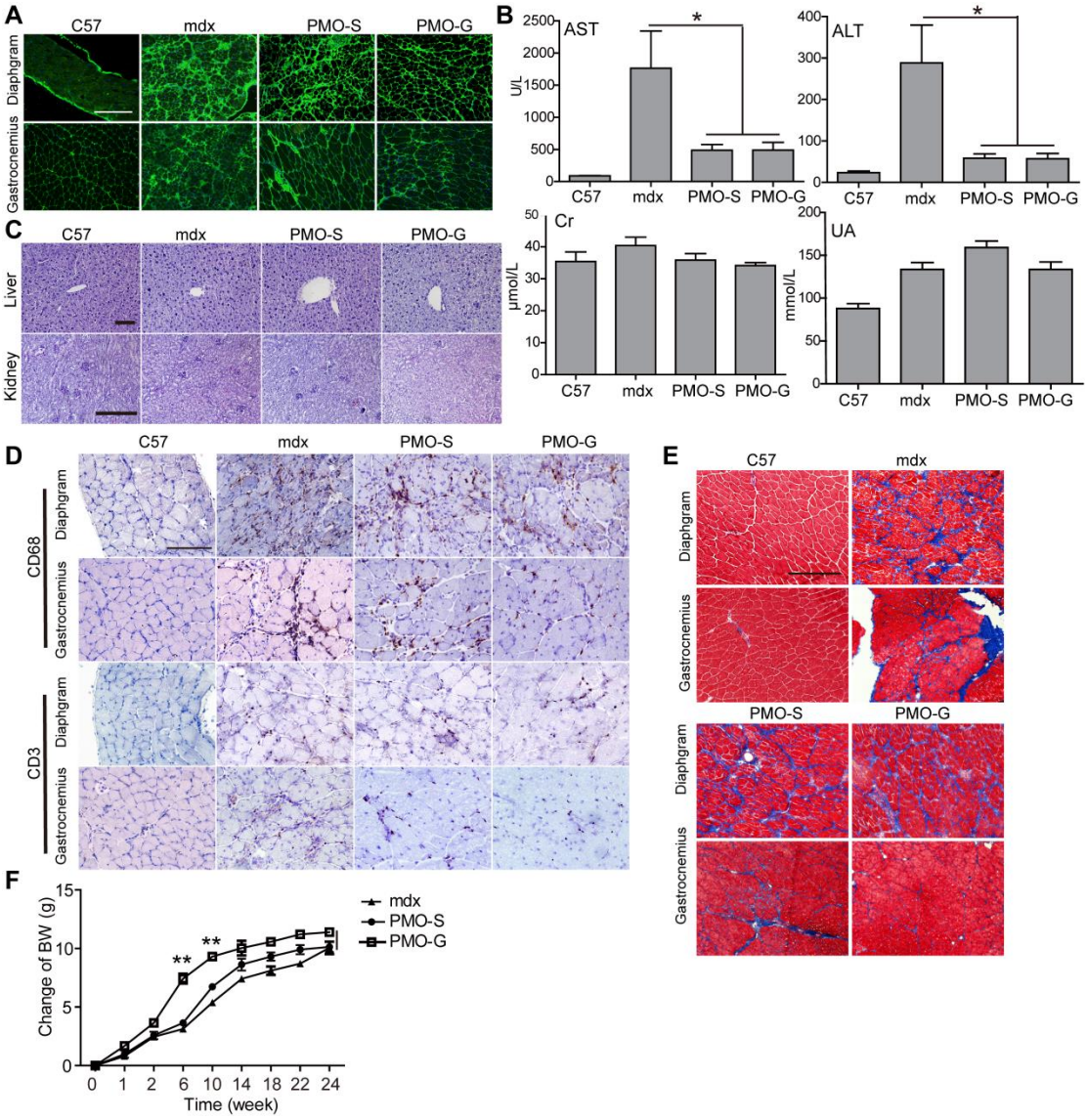
Caorui Lin, Gang Han, Hanhan Ning, Jun Song, Ning Ran, Xianfu Yi, Yiqi Seow, and HaiFang Yin

Supplementary Figures

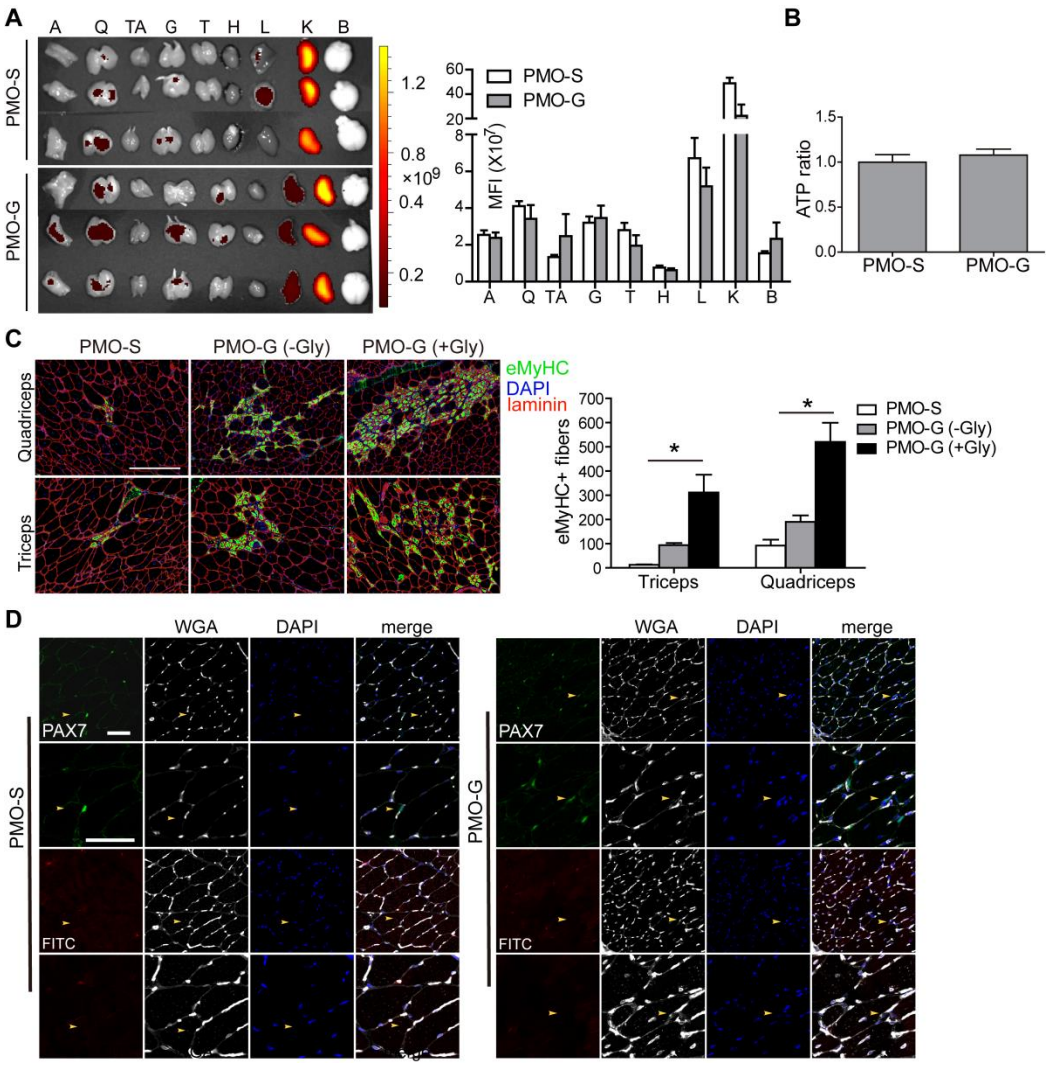


Supplementary Figure 1. Systemic evaluation of glycine with PMO at 50 mg/kg dose and local test of different concentrations of glycine with PMO in *mdx* mice. PMO in glycine or saline was administered intravenously into adult *mdx* mice at 50 mg/kg/week for 3 weeks and muscles were harvested 2 weeks after last injection. (**A**) Immunohistochemistry for dystrophin-positive fibers in body-wide muscles from *mdx* mice treated with PMO in saline (PMO-S) or glycine (PMO-G) at 50 mg/kg/week for 3 weeks intravenously (scale bar=100 μm). Western blot (**B**) and quantitative analysis (**C**) for dystrophin expression in body-wide muscles from *mdx* mice treated with PMO-G or PMO-S at 50 mg/kg/week for 3 weeks intravenously (n=3; two-tailed t test). 1.25 μg, 2.5 μg, 5 μg, 10 μg and 20 μg total protein from *C57BL/6* and 50 μg muscle samples from untreated and treated *mdx* mice were loaded. α-actinin was used as the loading control. The fold change refers to PMO-G relative to PMO-S. TA-tibialis anterior, Q-quadriceps, G-gastrocnemius, A-abdominal muscle. (**D**)

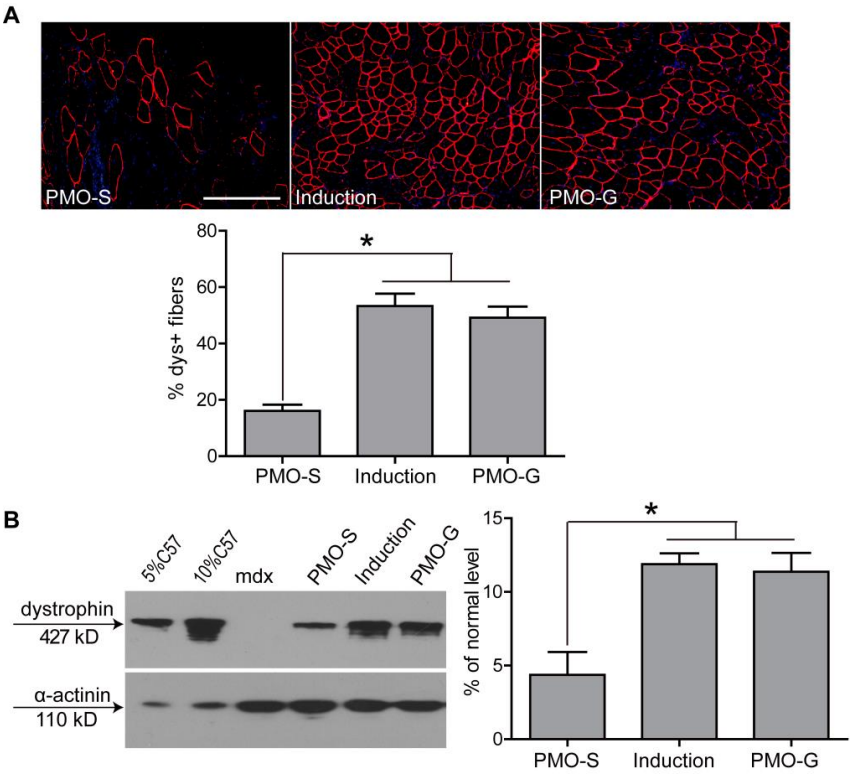
Immunohistochemistry and quantitative analysis of dystrophin-positive fibers in TA muscles from *mdx* mice treated with single intramuscular injection of PMO in different concentrations of glycine (scale bar=100 μm) (n=3; One way -ANOVA post hoc Student-Newman-Keuls test). PMO (2 μg) in different concentrations of glycine was administered into TA muscles of adult *mdx* mice. (**E**) Western blot and quantitative analysis for dystrophin expression in TA muscles from *mdx* mice treated with single intramuscular injection of PMO in different concentrations of glycine (n=3; One way-ANOVA post hoc Student- Newman-Keuls test). 2.5 μg and 5 μg total protein from *C57BL/6* and 50 μg of muscle samples from untreated and treated *mdx* mice were loaded. Data are presented as means ±s.e.m. (*$p<0.05$).



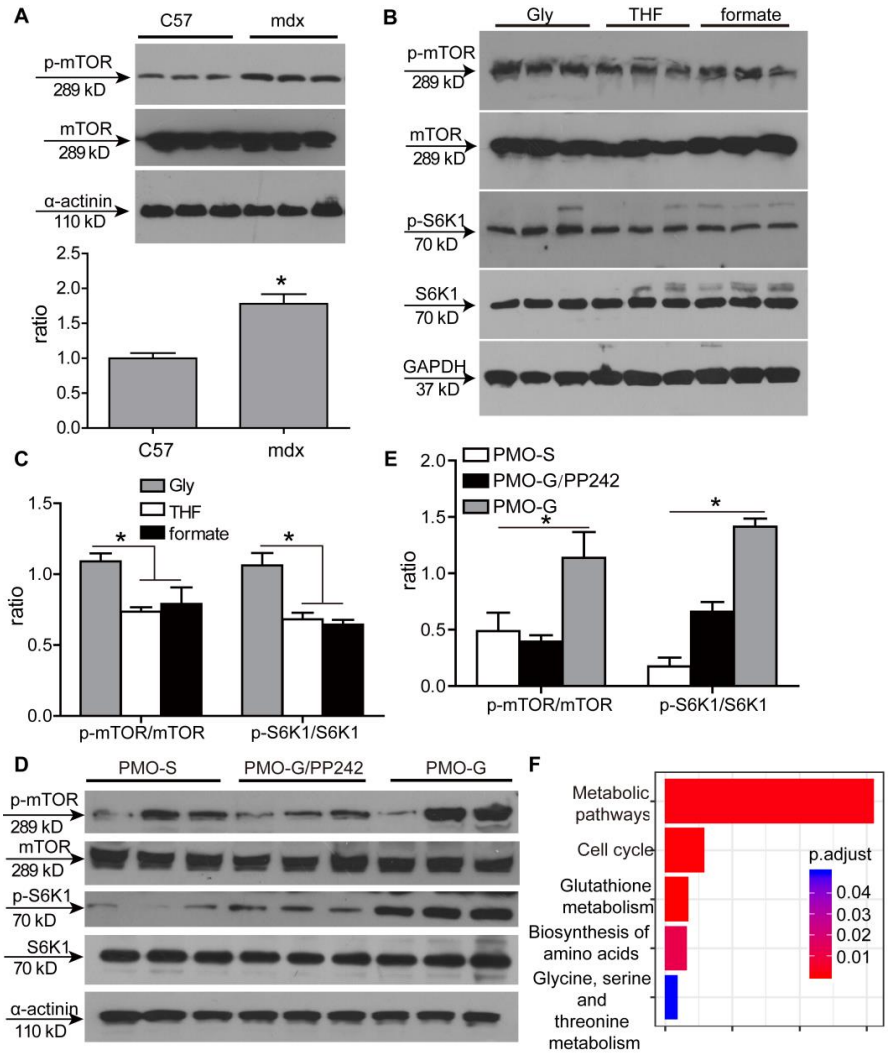
Supplementary Figure 2. Pathological and toxicological assessment of *mdx* mice treated with long-term repeated administration of PMO-G at 25 mg/kg. PMO-G was administered intravenously into adult *mdx* mice at 25 mg/kg/week for 3 weeks with additional supply of glycine every other day, followed by 50 mg/kg/month for 5 months with additional supply of glycine every one week intravenously. Muscles were harvested 2 weeks after last injection. (**A**) Immunohistochemistry for IgGs in gastrocnemius and diaphragm from *mdx* mice treated with PMO-S or PMO-G (scale bar=100 μm). (**B**) Measurement of serum indices from treated *mdx* mice to reflect liver and kidney functions (n=4; One way -ANOVA post hoc Student -Newman-Keuls test). (**C**) Morphological examination of liver and kidney from treated *mdx* mice (scale bar=50 μm). (**D**) Immunohistochemistry of immune cells in gastrocnemius and diaphragm from treated *mdx* mice (scale bar=50 μm). (**E**) Masson Trichrome staining for gastrocnemius and diaphragm from treated *mdx* mice (scale bar=100 μm). (**F**) Measurement of body-weight changes of treated *mdx* mice (n=4; One way -ANOVA post hoc Student-Newman-Keuls test). Data are presented as means ±s.e.m. (*p<0.05; **p<0.001).



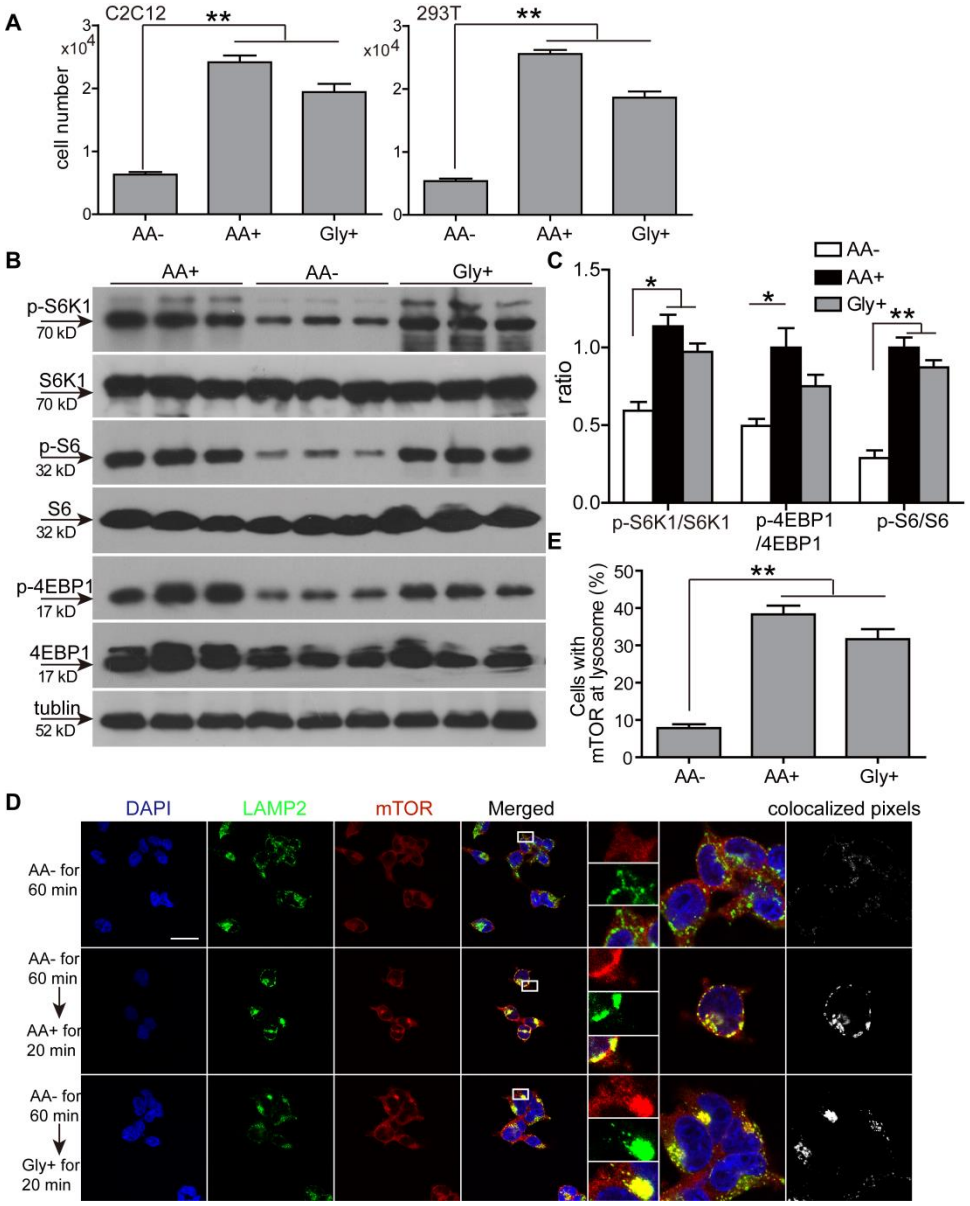
Supplementary Figure 3. Investigation of the mechanism underlying glycine's functionality in *mdx* mice. (**A**) Tissue imaging to examine the fluorescence of lissamine-labelled PMO in body-wide tissues of treated *mdx* mice. Adult *mdx* mice were treated with lissamine-labelled PMO in glycine or saline at 25 mg/kg/day for 3 days intravenously and tissues were harvested 4 days later (n=3). (**B**) Measurement of ATP levels in quadriceps from *mdx* mice treated with PMO-G or PMO-S at 25 mg/kg/week for 3 weeks (n=3). (**C**) Immunohistochemistry and quantitative analysis for eMyHC^{+} regenerating myofibres in quadriceps and triceps from treated *mdx* mice (scale bar=100 μm) (n=4, *P<0.05; One way-ANOVA post hoc Student-Newman-Keuls test). Adult *mdx* mice were treated with PMO-G with additional glycine injections (+Gly) or without additional glycine (-Gly) at 25 mg/kg/week for 3 weeks and glycine was administered every other day intravenously. (**D**) Immunohistochemistry of PAX7^{+} MuSCs and FITC-labelled PMO in gastrocnemius from treated *mdx* mice (scale bar=50 μm). *Mdx* mice were treated with single intravenous injection of FITC-labelled PMO in glycine (PMO-G) or saline (PMO-S) at the dose of 50 mg/ kg and muscles were harvested 48 hr later. Fluorescently tagged wheat germ agglutinin (WGA) was used for the visualization of connective tissues. Data are presented as means ±s.e.m.



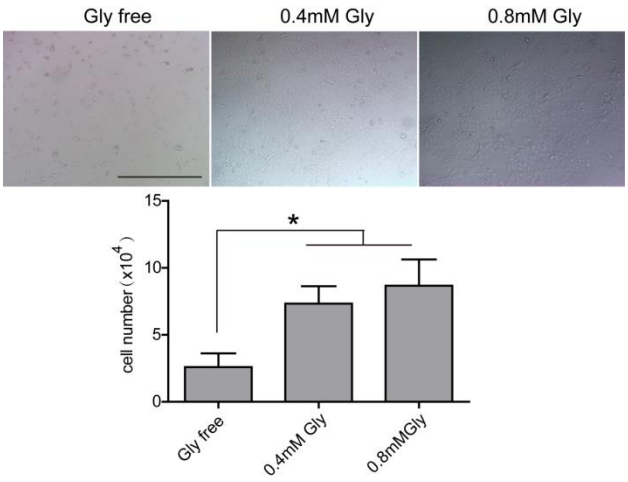
Supplementary Figure 4. **Effect of glycine pre-treatment on PMO activities in TA muscles of adult *mdx* mice.** Glycine (5%) was intramuscularly injected into TA muscle of adult *mdx* mice for once and followed by administration of PMO (2 μg) in saline into the same TA muscles 3 days later. TA muscles were harvested 10 days after PMO injection. (**A**) Immunohistochemistry and quantitative analysis for dystrophin-positive fibers in *mdx* TA muscles treated with PMO in saline (PMO-S), PMO in glycine (PMO-G) and glycine induction (induction) (scale bar=100 μm) (n=3, *P<0.05; One way-ANOVA post hoc Student-Newman-Keuls test). (**B**) Representative Western blot and quantitative analysis for dystrophin expression in TA muscles from treated *mdx* mice (n=3, *P<0.05; One way -ANOVA post hoc Student-Newman-Keuls test). 2.5 μg and 5 μg total protein from *C57BL/6* and 50 μg of muscle samples from untreated and treated *mdx* mice were loaded. α-actinin was used as the loading control. Data are presented as means ±s.e.m.



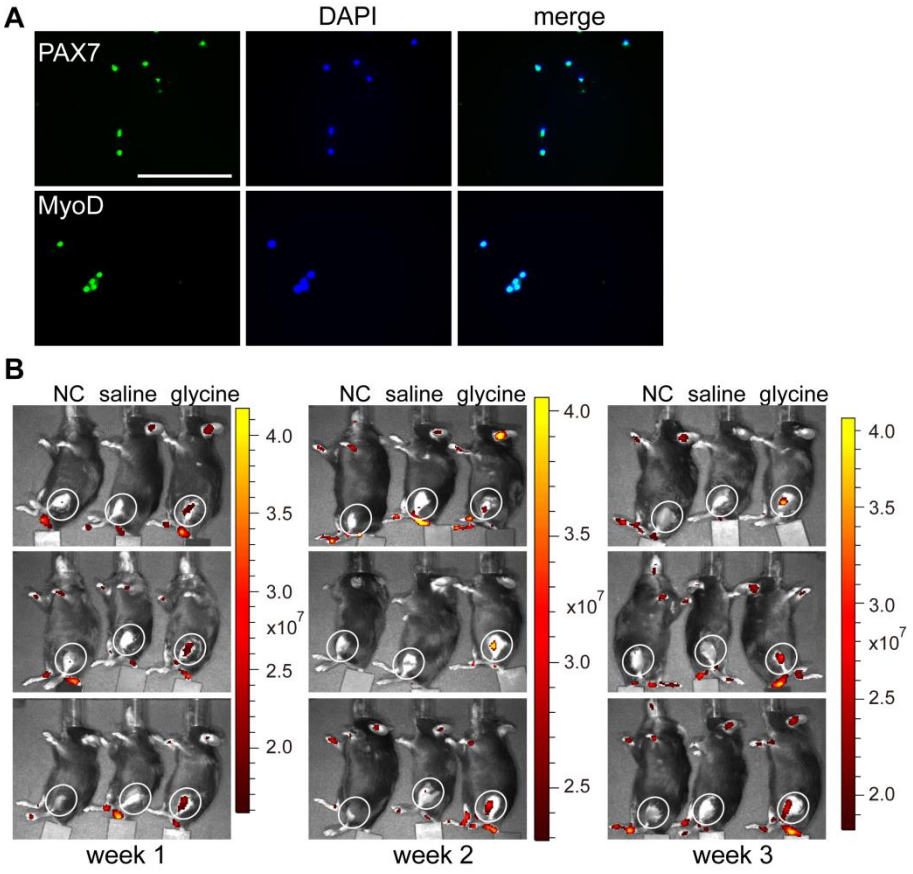
Supplementary Figure 5. Examination on the mTORC1 signaling pathway in *C57BL/6*, untreated and treated *mdx* mice. (**A**) Western blot to detect phosphorylated mTOR expression in TA muscles from *C57BL/6* and *mdx* mice (n=3; two-tailed t test). α-actinin was used as the loading control. 70 μg total protein were loaded. (**B**) Western blot to detect phosphorylated mTOR and S6K1 expression in TA muscles from *mdx* mice treated with glycine, THF or formate intramuscularly. GAPDH was used as the loading control. 50 μg total protein were loaded. (**C**) Quantitative analysis of the ratio of phosphorylated mTOR and S6K1 to total mTOR and S6K1 expression, respectively (n=3; One way -ANOVA post hoc Student-Newman-Keuls test). (**D**) Western blot to detect phosphorylated mTOR and S6K1 expression in TA muscles from *mdx* mice treated with PMO-S, PMO-G or PMO in the mixture of glycine and PP242 (PMO-G/PP242) intramuscularly. α-actinin was used as the loading control. 50 μg total protein were loaded. (**E**) Quantitative analysis of the ratio of phosphorylated mTOR and S6K1 to total mTOR and S6K1 expression, respectively (n=3; One way -ANOVA post hoc Student-Newman-Keuls test). (**F**) Gene set enrichment analysis (GSEA) of metabolism and cell cycle-related genes for primary myoblasts isolated from *mdx* mice followed by treatment with 0.8mM glycine for 24 hr (n=6). The number of biological replicates in the control group is 3 (n=3). Data are presented as means ±s.e.m. (*$p<0.05$).



Supplementary Figure 6. Glycine enhances activation of the mTOR signaling pathway in 293T cells. (**A**) Measurement of cell growth in starved C2C12 and 293T cells followed by re-stimulation of glycine and total amino acid (n=4; One way -ANOVA post hoc Student-Newman-Keuls test). AA- means the depletion of total amino acid; AA+ or Gly+ refers to the supplementation of total amino acid and glycine into starved cells. (**B**) Western blot to detect phosphorylated S6K1, S6 and 4EBP1 expression in 293T cells under different conditions. 30 μg total protein was loaded and tubulin was used as a loading control. (**C**) Quantitative analysis of the ratio of phosphorylated S6K1, S6 and 4EBP1 to total protein expression of counterparts (n=3; One way -ANOVA post hoc Student- Newman-Keuls test). Immunocytochemistry for mTOR (**D**) and quantitative analysis of cells with mTOR at lysosomes (**E**) in starved 293T cells followed by re-stimulation of total amino acid or glycine (scale bar=10 μm) (n=10; One way -ANOVA post hoc Student-Newman-Keuls test). LAMP2 was used as a lysosome marker. Data are presented as means ±s.e.m. (*p<0.05; **p<0.001).



Supplementary Figure 7. Measurement of the effect of glycine on murine primary myoblasts. Primary myoblasts were isolated from wild-type *C57BL/6* mice and cultured for 24 hrs with different concentration of glycine (scale bar=100μm) (n=4, *P<0. 05; One way -ANOVA post hoc Student-Newman-Keuls test). Data are presented as means ±s.e.m.



Supplementary Figure 8. Immunocytochemistry for isolated MuSCs from transgenic GFP *C57BL/6* mice (A) and real-time monitoring of transplanted GFP-positive MuSCs in TA muscles of adult *mdx* mice (B). *Mdx* mice were imaged for GFP fluorescence at different time-points after cell transplantation (n=3).