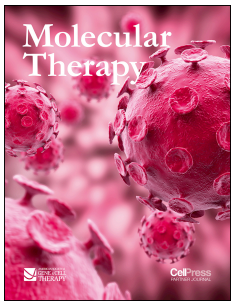

Glycine enhances satellite cell proliferation, cell transplantation and oligonucleotide efficacy in dystrophic muscle

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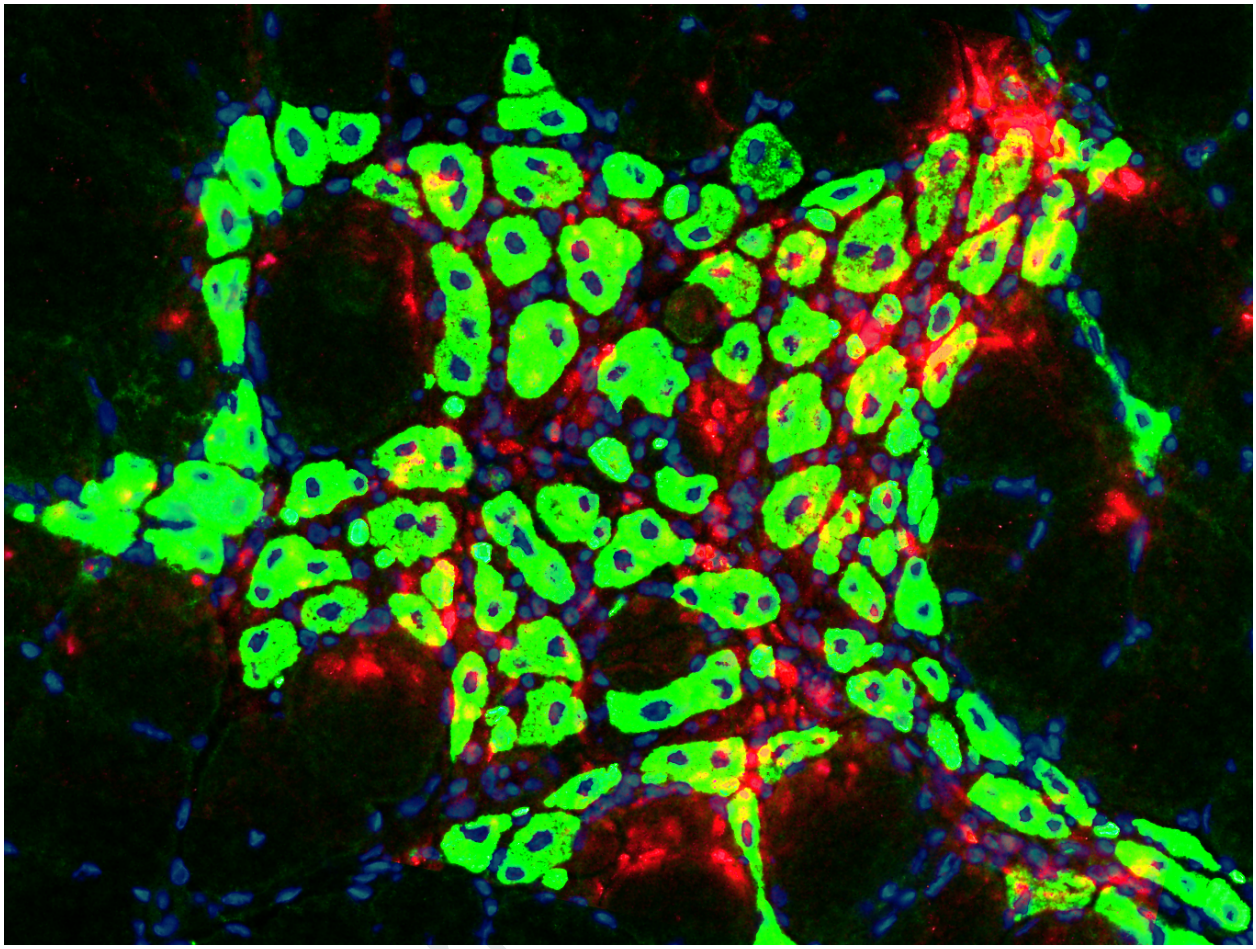

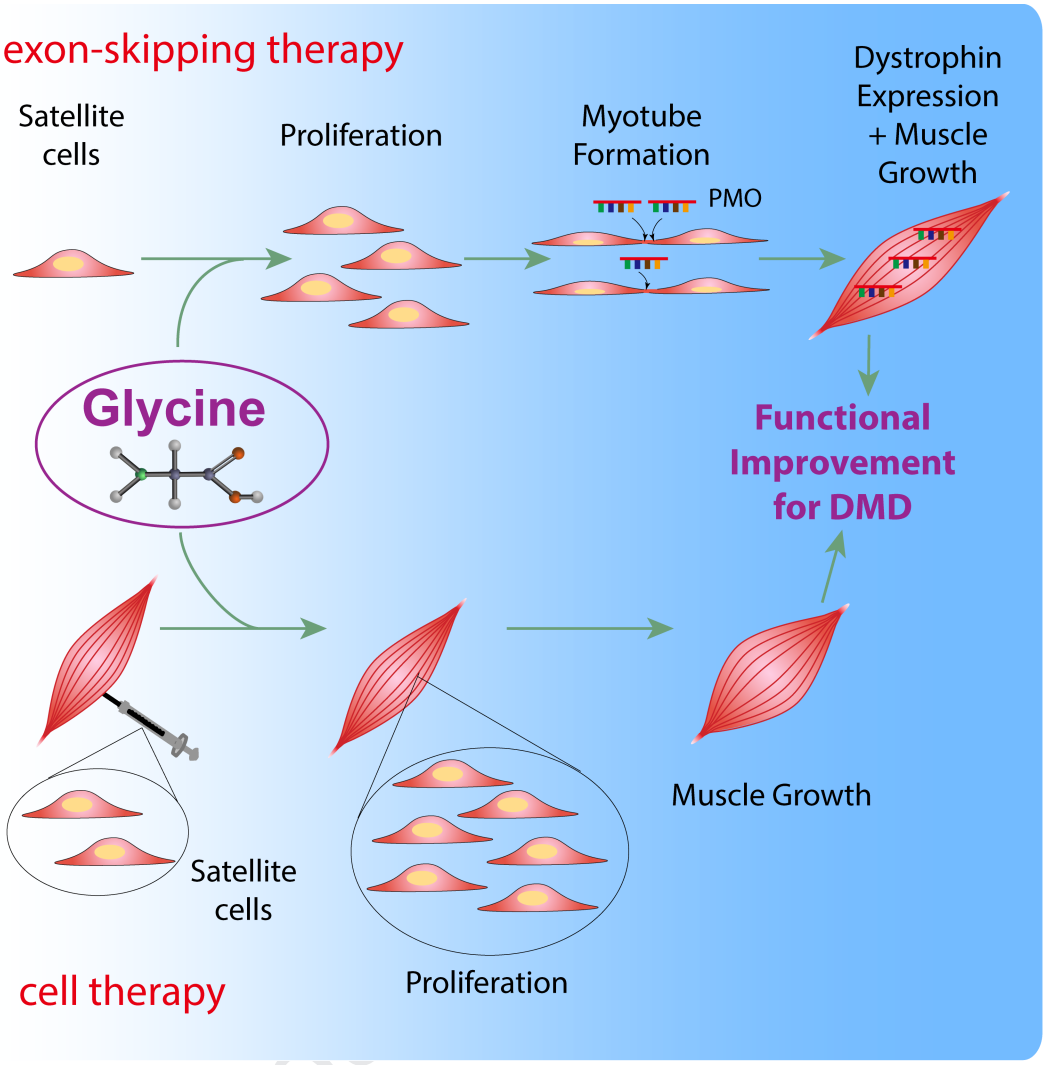
exon-skipping therapy
Satellite cells
Proliferation
Myotube Formation
PMO
Dystrophin Expression + Muscle Growth
Glycine
Functional Improvement for DMD
Muscle Growth
Satellite cells
Proliferation
cell therapy

Glycine enhances satellite cell proliferation, cell transplantation and oligonucleotide efficacy in dystrophic muscle

Running title: Effect of glycine on exon-skipping and cell therapy

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Abstract

The need to distribute therapy evenly systemically throughout the large muscle volume within the body makes Duchenne muscular dystrophy (DMD) therapy a challenge. Cell and exon-skipping therapies are promising but have limited effects, thus enhancing their therapeutic potency is of paramount importance to increase the accessibility of these therapies to DMD patients. Here we demonstrate that co-administered glycine improves phosphorodiamidate morpholino oligomer (PMO) potency in *mdx* mice with marked functional improvement and up to 50-fold increase of dystrophin in abdominal muscles compared to PMO in saline. Glycine boosts satellite cell proliferation and muscle regeneration by increasing activation of mammalian target of rapamycin complex 1 (mTORC1) and replenishing the one-carbon unit pool. The expanded regenerating myofiber population then results in increased PMO uptakes. Glycine also augments the transplantation efficiency of exogenous satellite cells and primary myoblasts in *mdx* mice. Our data provide evidence that glycine enhances satellite cell proliferation, cell transplantation and oligonucleotide efficacy in *mdx* mice, and thus has therapeutic utility for cell therapy and drug delivery in muscle wasting diseases.

Key Words: Duchenne muscular dystrophy, glycine, satellite cell, exon-skipping, cell therapy, mTORC1

Introduction

Treating muscle disorders is a daunting challenge due to the large muscle volume. The predominant systemic muscle wasting disease - Duchenne Muscular Dystrophy (DMD), is caused by frame-disrupting mutations in the *DMD* gene, resulting in the absence of functional dystrophin protein[1]. The first antisense oligonucleotide (AO) drug (Exondys 51) was approved by the US food and drug administration in 2016 and it can be applied to 17.5% of DMD populations[2]. However, the therapeutic potency of this AO drug is inadequate for widespread clinical utilization, which is largely attributed to inefficient bio-distribution to body-wide muscles upon systemic administration[3]. Similarly, cell therapy is another promising therapeutic modality but has limited efficacy due to low transplantation efficiency[4]. Therefore, strategies to enhance the efficacy of cell and exon-skipping therapies can truly improve clinical access of muscle-wasting therapies.

Amino acids are important building blocks of protein and potent regulators of protein synthesis in healthy skeletal muscle and muscle wasting conditions[5-8]. Essential amino acids have been shown to stimulate the net muscle protein balance in healthy volunteers after resistance exercise[9] with branched-chain amino acids demonstrated to alleviate muscle pathologies in DMD mice[10]. In addition, the abundance of essential amino acids within the local environment has clearly been demonstrated to trigger the activation of mammalian target of rapamycin complex 1 (mTORC1) and consequently promote protein synthesis and cell growth[11]. Recently, nonessential amino acids have

also drawn much attention as food supplementation or activators of mTORC1 in preventing muscle loss[12-15]. In particular, glycine supplementation was indicated to protect muscles in different muscle wasting models such as cancer cachexia, sepsis and reduced caloric intake[6, 16, 17]. Earlier studies also demonstrated that dietary supplementation of glycine to DMD patients provided beneficial effect on their physical activities though the overall effect is limited[18]. However, an interesting observation from earlier clinical studies was that glycine is more beneficial for children than adult patients[18], implying that glycine might play other roles.

Here we show that repeated co-administration of glycine with phosphorodiamidate morpholino oligomer (PMO) (PMO-G) increased dystrophin expression 50-fold in peripheral muscles of *mdx* mice compared to PMO in saline (PMO-S). Therapeutic levels of dystrophin were restored in *mdx* mice treated with a low dose of PMO (25 mg/kg) in glycine with marked functional improvement in the absence of detectable toxicity. This improvement can be attributed to increased uptake of PMOs in regenerating myofibers potentiated by glycine, which in turn is mediated by enhanced mTORC1 activation and one-carbon unit pool replenishment. Importantly, glycine promoted not only endogenous satellite cell proliferation but also cell transplantation efficiency of exogenous satellite cells and primary myoblasts. The findings presented here provide insights for the role of glycine and have important therapeutic implications for cell therapy and nucleic acid therapeutics in muscle wasting diseases and other muscle related disorders.

Results

Glycine enhances PMO-mediated exon-skipping in muscle

As hexose was shown to promote the uptake and activity of PMO in peripheral muscles of *mdx* mice by replenishing cellular energy stores[19], we examined if other nutrients could similarly improve PMO-mediated exon-skipping in dystrophic muscles (Figure 1A). Unsurprisingly, consistent with previous observations with hexose[19], alternative energy sources such as citric acid, sodium pyruvate and succinic acid induced improvements in PMO activities as reflected by significantly increased dystrophin-positive fibers and levels of dystrophin restoration in tibialis anterior (TA) muscles from *mdx* mice treated with PMO in citric acid, sodium pyruvate and succinic acid compared to PMO in saline (Figure 1B). However, it was surprising that amino acids, particularly serine and glycine, induced a significant increase in PMO-induced dystrophin-positive fibers (Figure 1B) and dystrophin expression (Figure 1C) in TA muscles of treated *mdx* mice. To determine systemic effect of glycine and serine on PMO activity, we intravenously administered 5% glycine (equivalent to clinically available 50 mg/ml infusion buffer) or serine with PMO at the dose of 25 mg/kg/week for 3 weeks in *mdx* mice. Substantial numbers of dystrophin-positive myofibers (Figure 1D) and significantly elevated levels of dystrophin (Figure 1E) were detected in most peripheral muscles of *mdx* mice treated with PMO in glycine (PMO-G) compared to PMO in serine and PMO in saline (PMO-S). To examine if there is any dose-dependent effect for PMO or glycine, we increased the dose of PMO from 25

mg/kg/week to 50 mg/kg/week for 3 weeks or the administration frequency of glycine from weekly to every other day for 5 weeks with the same dose of PMO (25 mg/kg/week) for 3 weeks. As expected, glycine also enhanced the number of dystrophin-positive fibers and dystrophin expression compared to saline when co-delivered with 50 mg/kg of PMO (Figure S1A-C). In contrast, dystrophin-positive fibers (Figure 1F) and dystrophin expression (Figure 1G) dramatically rose in peripheral muscles of PMO-G treated *mdx* mice with additional glycine infusion compared to PMO-G alone. Importantly, up to 50-fold higher level of dystrophin was detected in abdominal muscles from *mdx* mice treated with PMO-G with additional glycine administration compared to PMO-S under identical conditions (Figure 1H). Measurement of serum glycine revealed slightly higher levels after infusion with additional glycine than PMO-G alone (Figure 1I), suggesting that increased glycine availability enhances PMO-mediated exon-skipping. However, increasing glycine from 5% to 10% did not improve PMO activity (Figure S1D and S1E), implying that the effect is saturable. The fact that increased glycine concentration at the point of injection did not improve uptake, but increased frequency of administration did, suggests that the improvements seen in mice are not solely due to short-lived effects such as increasing uptake of circulating PMO. These data demonstrate that glycine potentiates PMO activities in muscle and the potentiating effect can be enhanced by sustained supply of glycine.

PMO-G elicits long-term efficacy and functional improvements in *mdx* mice

Since PMO-G with additional glycine infusion further enhanced PMO activities in *mdx* mice, we adopted this dosing regimen at 25 mg/kg PMO for a period of 6 months (Figure 2A). Repeated administration of PMO-G induced uniform dystrophin expression across most peripheral muscles except for diaphragm with no dystrophin expressed in the heart (Figure 2B). Dystrophin levels were consistently elevated in peripheral muscles of PMO-G treated *mdx* mice compared to PMO-S under identical conditions (Figure 2C and 2D). Importantly, molecular correction of dystrophin resulted in functional improvements in PMO-G treated *mdx* mice as dystrophin-associated protein complex (DAPC), which mis-localizes in the absence of dystrophin[20], re-localized to the sarcolemma (Figure 2E); serum creatine kinase (CK) levels usually elevated in *mdx* mice[21] significantly decreased (Figure 2F); IgG staining also declined (Figure S2A). Significant force recovery and endurance improvements were also observed as reflected by significantly increased grip strength and number of rounds measured with running wheel test in *mdx* mice treated with PMO-G compared to PMO-S and untreated *mdx* mice (Figure 2G and 2H). Levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concordantly declined, whereas no change was found for creatinine and urea (Figure S2B), and histology revealed no evidence of liver or renal toxicity (Figure S2C). Strikingly, inflammatory cells and fibrosis dramatically decreased in peripheral muscles from PMO-G treated *mdx* mice compared to PMO-S and untreated age-matched *mdx* controls (Figure S2D and S2E). Interestingly, bodyweight was significantly increased in PMO-G treated *mdx* mice at earlier time-points compared to

PMO-S and age-matched untreated *mdx* controls though the difference declined at later time-points (Figure S2F), suggesting that glycine might have some beneficial effects on muscle growth although the caveat is that hypertrophy observed in *mdx* mice may also increase muscle volume. These findings indicate that long-term administration of PMO-G leads to significant functional improvements in *mdx* mice at extremely low doses without any detectable toxicity.

Glycine synergizes with PMO therapy by improving muscle regeneration which results in greater PMO uptake and efficacy

To investigate if the enhancement elicited by glycine was due to greater exon-skipping frequency[22] or through enhanced PMO uptake, we administered PMO (2 μg) in saline into TA muscles of adult *mdx* mice followed by injection of glycine to the same muscle 16 hours later, to allow for complete uptake of PMO into the muscle prior to glycine introduction[22]. However, separating glycine from PMO negated the enhancement observed with PMO-G (Figure 3A and 3B), suggesting that glycine is involved in PMO uptake rather than enhancing exon-skipping frequency. To test if glycine functions by increasing PMO uptake, we intravenously administered lissamine-labelled PMO in glycine into adult *mdx* mice at 25 mg/kg/day for 3 days. However, no difference was observed in the fluorescence intensity of body-wide tissues from PMO-G and PMO-S treated *mdx* mice 4 days after injection under identical conditions (Figure S3A). Comparable levels of ATP were consistently found in quadriceps from *mdx* mice treated with PMO-G and PMO-S (Figure S3B),

indicating that glycine did not increase energy availability. Since PMO-G with additional glycine infusion outperformed PMO-G alone, we re-examined the samples and noticed that significantly higher numbers of small-calibre embryonic myosin heavy chain (eMyHC)-positive[23] regenerating myofibers were present in quadriceps and triceps from PMO-G treated *mdx* mice with additional glycine injection compared to PMO-G alone and PMO-S (Figure S3C), implying that glycine promotes muscle regeneration. Given that muscle regeneration was shown to contribute to PMO uptake in *mdx* mice[24], we hypothesized that glycine might promote muscle regeneration and thus enhance PMO uptake. To verify this, we intravenously injected glycine into adult *mdx* mice every other day for one week to induce muscle regeneration, followed by single intravenous injection of carboxyl- fluorescein (FITC)-labelled PMO at the dose of 50 mg/kg, and examined the uptake of PMO 48 hours later. Muscle satellite cells (MuSCs), indicated by paired box transcription factor (PAX7), a known satellite cell marker[25], and the percentage of proliferating $PAX7^{+}/Ki67^{+}$ MuSCs[26], significantly rose in TA and gastrocnemius of glycine-induced *mdx* mice compared to the saline control (Figure 3C and 3D), demonstrating that glycine promotes MuSC proliferation. Significantly higher amounts of FITC-labelled PMO were consistently found in increased numbers of eMyHC-positive regenerating fiber in TA and gastrocnemius of glycine-induced *mdx* mice than the saline control (Figure 3E and 3F), although no co-localization of FITC-labelled PMOs with MuSCs was found in gastrocnemius of glycine-induced or saline control mice (Figure S3D). Evident exon23 skipping was consistently observed in TA and gastrocnemius from glycine-induced *mdx* mice

compared to the saline and untreated *mdx* controls (Figure 3G). Notably, glycine injection into TA muscles followed by PMO 3 days after significantly increased numbers of dystrophin-positive fibers and levels of dystrophin expression (Figure S4A and S4B). Correspondingly, glycine could also be shown to enhance PMO uptake in differentiating myotubes in culture but not in proliferating myoblasts (Figure 3H). The results support the notion that glycine promotes muscle regeneration which in turn results in enhanced PMO uptake. Based on the observation, we adopted the glycine induction regimen and repeated the tissue distribution experiment with FITC-labelled PMO. As expected, significantly higher fluorescence was detected in peripheral muscles from glycine-induced *mdx* mice compared to the saline and untreated *mdx* controls 48 hours after injection (Figure 3I). These data strengthen the conclusion that glycine synergizes PMO therapy by promoting muscle regeneration which in turn results in greater PMO uptake and efficacy.

Glycine increases muscle regeneration by replenishing the one-carbon unit pool

To understand how glycine promotes muscle regeneration and if active transport of glycine is critical for its functionality, we blocked glycine transporter 1 (GlyT-1), the ubiquitous glycine transporter[27], with bitopertin (BP), an inhibitor for GlyT-1[28]. Strikingly, muscle regeneration was completely compromised in the presence of BP as $PAX7^+$ or $PAX7^+Ki67^+$ MuSCs (Figure 4A and 4B) and eMyHC-positive regenerating myofibers (Figure 4C) significantly declined in TA muscles co-administered with glycine and BP. PMO activities were consistently negated when

BP was co-administered with PMO-G as dystrophin-positive fibers (Figure 4D) and dystrophin expression (Figure 4E) significantly decreased, suggesting that active transport is vital for glycine's functionality. As intracellular glycine is primarily metabolized as a one-carbon unit donor[29], we examined if glycine promotes muscle regeneration by providing one-carbon units which are building blocks for nucleic acid and protein biosynthesis required for active MuSC proliferation[30]. Blockade of dihydrofolate reductase, a key enzyme required for reducing dihydrofolic acid to tetrahydrofolic acid for one-carbon transfer[31], with methotrexate (MTX), an inhibitor of dihydrofolate reductase[32], completely abolished glycine-induced effects on muscle regeneration as $PAX7^+$ or $PAX7^+/Ki67^+$ MuSCs (Figure 4F and 4G) and eMyHC-positive regenerating myofibers (Figure 4H) significantly declined. To further validate this, we replaced glycine with formate, the only non-tetrahydrofolate (THF)-linked intermediate in one-carbon metabolism[33], or tetrahydrofolate (THF), an intermediate carrier for one-carbon unit[34]. $PAX7^+$ or $PAX7^+/Ki67^+$ MuSCs (Figure 4F and 4G) and eMyHC-positive regenerating fibers (Figure 4H) significantly rose in formate- or THF-treated TA muscles compared to the saline control, suggesting that other one-carbon group donors or carriers can also enhance muscle regeneration, though to a lesser extent than glycine. If glycine functions as a one-carbon donor, a synergistic effect would be expected when glycine and THF were co-administered into TA muscles of *mdx* mice. A dramatic enhancement on MuSC proliferation (Figure 4F and 4G) and muscle regeneration (Figure 4H) was observed in TA muscles of *mdx* mice co-administrated with glycine and THF compared to glycine alone. A positive

correlation between muscle regeneration and PMO activities was concordantly established as dystrophin-positive fibers (Figure 4I) and dystrophin expression (Figure 4J) significantly increased in TA muscles of *mdx* mice treated with PMO in formate, THF or the combination of glycine and THF, whereas a significant decline was observed in *mdx* mice treated with glycine and MTX. However, glycine-induced muscle regeneration was significantly compromised when glycine decarboxylase (GLDC) P-protein, the key enzyme for glycine decarboxylation in the glycine cleavage system[35], was down-regulated with GLDC-targeted shRNA delivered with AAV2/8 (Figure 5A) as $PAX7^+$ or $PAX7^+/Ki67^+$ MuSCs (Figure 5B and 5C) and eMyHC-positive regenerating fibers (Figure 5D) were significantly reduced in TA muscles from treated *mdx* mice. Corroborating with reduced MuSC proliferation and muscle regeneration, dystrophin-positive fibers (Figure 5E) and dystrophin expression (Figure 5F) significantly decreased. These results support the conclusion that glycine contributes to MuSC proliferation and muscle regeneration by supplying one-carbon units.

Glycine strengthens mTORC1 activation and contributes to muscle regeneration

It is known that mTORC1 is activated in muscle regeneration in response to injury[36], thus to investigate if mTORC1 is involved in muscle regeneration process of *mdx* mice, we measured levels of phosphorylated mTOR (p-mTOR) in *mdx* mice. Up-regulation of p-mTOR was observed in TA muscles of *mdx* mice compared to age-matched wild-type *C57BL/6* controls (Figure S5A). Strikingly, levels of p-mTOR,

phosphorylated ribosomal protein S6 kinase 1 (p-S6K1), a hallmark of specific activation by mTORC1, and phosphorylated ribosomal protein S6 (p-S6), a key downstream target of S6K1[37], were elevated in quadriceps of *mdx* mice treated with PMO-G at 25 mg/kg/week for 3 weeks with additional glycine infusion compared to the saline control (Figure 6A and 6B). In contrast, there was no increase of p-mTOR and p-S6K1 in THF- or formate-treated TA muscles compared to glycine (Figure S5B and S5C), highlighting the glycine-specific hyperactivation of mTORC1. Blockade of mTORC1 activation with PP242 (Figure S5D and S5E), a small-molecule protein kinase inhibitor targeting the adenosine triphosphate (ATP)-binding site of mTOR[38], abolished the enhancement potentiated by glycine as $PAX7^+$ or $PAX7^+/Ki67^+$ MuSCs (Figure 6C and 6D) and eMyHC-positive regenerating myofibers (Figure 6E) significantly declined. PMO activity was also negated as dystrophin-positive fibers (Figure 6F) and dystrophin expression significantly decreased (Figure 6G) in TA muscles from treated *mdx* mice, confirming the relevance of mTORC1 in glycine's functionality. Analysis of the gene expression profiles of glycine-treated primary myoblasts, isolated from adult *mdx* mice, revealed that glycine significantly up-regulated two annotated groups of genes - cell cycle and metabolism (Figure 6H and S5F), with up-regulation of cyclin-dependent kinase 1 (*CDK1*), cell division cycle 20 (*CDC20*) and cyclin B2 (*CCNB2*) genes validated by quantitative real-time RT-PCR (Figure 6I), demonstrating that glycine promotes cell proliferation. These results demonstrate that glycine strengthens mTORC1 activation, which in turn promotes MuSC proliferation and muscle regeneration.

Glycine triggers mTORC1 via v-ATPase

To elucidate how glycine regulates mTORC1, we used murine myoblasts (C2C12), which was shown to be responsive to glycine[39, 40], and human embryonic kidney (HEK) 293T cells, a commonly used cellular model[41]. To verify if C2C12 and 293T cells are responsive to glycine stimulation, we examined cell growth in the absence or presence of glycine *in vitro*. Deprivation of total amino acids significantly inhibited C2C12 and 293T cell growth, whereas the supplementation of glycine or total amino acids 24 hours after starvation stimulated cell growth in glycine- or total amino acid-depleted cells (Figure S6A), indicating that C2C12 and 293T cells respond to glycine. Levels of p-S6K1 and phosphorylated eukaryotic initiation factor 4E-binding protein 1 (p-4E-BP1), two best-characterized downstream targets of mTORC1 and a read-out for mTORC1 activity[42], and the downstream target p-S6 were concordantly elevated in glycine- or total amino acid-supplemented C2C12 cells compared to total amino acid-depleted cells (Figure 7A and 7B). As amino acids were shown to transfer signals to mTOR by promoting its translocation to the surface of lysosomes where mTORC1 is activated[43], we starved C2C12 cells for 60 minutes and re-stimulated with glycine or total amino acids for 20 minutes. Localization of mTOR in conditions of amino acid starvation was predominantly cytoplasmic and presented a diffuse staining pattern, with little co-localization was observed with lysosome-associated membrane protein 1 (LAMP1), a lysosomal marker[44], whereas glycine or total amino acid stimulation led to mTOR clustered with lysosomes (Figure 7C and 7D). A similar

pattern was observed in total amino acid-starved 293T cells followed by glycine or total amino acid supplementation (Figure S6B-E). These results proved that glycine can promote recruitment of mTOR to the lysosome. To substantiate the notion that glycine enables the translocation of mTOR to the lysosome, we blocked the vacuolar H^+-adenosine triphosphatase ATPase (v-ATPase), a component necessary for amino acids to activate mTORC1[43], with bafilomycin A1 (BAF)[45], a highly specific inhibitor of the V-ATPase. Glycine-induced mTORC1 activation was substantially blocked with BAF in 293T cells as co-localization of mTOR with lysosomes (Figure 7E and 7F) and levels of p-S6K1 and p-S6 (Figure 7G) significantly reduced compared to glycine. As the Ras-related GTPase (Rag) is known to mediate the amino acid signal to mTOR and facilitate the binding of mTOR to lysosomes[46], we disrupted RagB expression in 293T cells with the CRISPR-Cas9 system. Phosphorylation of S6K1 and S6 was completely abolished in total amino acid-depleted RagB knock-out cells after stimulation with glycine or total amino acids for 10, 20 or 30 minutes (Figure 7H), demonstrating that RagB is responsible for the recruitment of mTOR to the surface of lysosomes.

Glycine enhances cell transplantation in *mdx* mice

Given the dramatic effect of glycine on endogenous MuSC proliferation, we examined if glycine has any benefit on exogenous cell transplantation. To this end, we adopted the commonly used cardiotoxin (CTX) injury model by injecting CTX to TA muscles of *mdx* mice 1 day prior to transplantation[47]. Pre-administration of glycine

was shown to suppress immunological reactions in liver transplantation[16, 48], therefore we intravenously administered glycine into *mdx* mice 7 days prior to transplantation and supplied glycine during the experimental period as illustrated in Figure 8A. MuSCs ($1x10^5$) derived from wild-type *C57BL/6* mice were transplanted into CTX-injured TA muscles of immunosuppressed adult *mdx* mice (Figure 8A). Dystrophin-positive fibers (Figure 8B) and muscle weight (Figure 8C) were significantly increased in TA muscles 3 weeks after cell transplantation in the glycine group compared to the saline control. Increased cell transplantation efficiency was consistently observed when wild-type *C57BL/6* primary myoblasts ($1x10^6$) were transplanted into TA muscles of glycine-induced *mdx* mice under identical conditions (Figure 8D). In line with the previous observation[39], glycine promoted murine primary myoblast proliferation (Figure S7). To further confirm the beneficial effect of glycine on cell transplantation, we transplanted EGFP-positive $PAX7^+MyoD^+$ MuSCs derived from transgenic *C57BL/6* EGFP mice (Figure S8A) into CTX-injured TA muscles of glycine-induced *mdx* mice and monitored the fluorescence in a real-time manner. Stronger fluorescence was detected in TA muscles from glycine-induced *mdx* mice than the saline control at different time-points after transplantation with live animal imaging (Figure S8B). Significantly stronger fluorescence signals were consistently detected in TA muscles isolated from glycine-induced *mdx* mice compared to the saline control at week 3 after transplantation (Figure 8E). Co-localization of EGFP-positive fibers with significantly increased numbers of dystrophin-positive fibers (Figure 8F and 8G) confirmed the benefits of glycine on

exogenous MuSC transplantation. These findings support the conclusion that glycine enhances cell transplantation.

Discussion

Glycine is known to have a wide spectrum of functions against different injuries and diseases[16]. Here we demonstrate that glycine promotes the potency of PMOs and cell transplantation in dystrophic mice. Therapeutic levels of dystrophin and functional improvement were achieved in *mdx* mice with extremely low doses of PMO (25 mg/kg) without any detectable toxicity. Importantly, up to 50-fold higher level of dystrophin was produced in abdominal muscles of PMO-G treated *mdx* mice compared to PMO-S, showing the potency of glycine in improving this low dose of PMO. Dissection of glycine functionality revealed that glycine promotes muscle regeneration which in turn results in increased PMO uptake and consequent dystrophin expression in muscle. Mechanistically, glycine potentiates muscle regeneration by strengthening mTORC1 activation via RagB and v-ATPase and replenishing the one-carbon unit pool. Strikingly, glycine improves not only endogenous MuSC proliferation but also exogenous cell transplantation efficiency in dystrophic mice. Our study provides a simple approach for improving the efficacy of nucleic acids therapeutics and cell therapy and also insight for the role of glycine.

Glycine is vital for mammalian metabolism and nutrition and is considered a conditionally essential amino acid, therefore has been extensively used as food

additive[49]. In our current study, we further confirmed its safety and biocompatibility as repeated administrations of physiological concentration of glycine (120µl of 50 mg/ml into adult *mdx* mice, which would have a total blood volume of 1.46ml) with PMO elicited significant functional rescue without any detectable toxicity. Although therapeutic levels of dystrophin were restored in body-wide peripheral muscles with low doses of PMO when administered with glycine, we failed to detect any dystrophin expression in the heart and dystrophin typically becomes detectable in dystrophic hearts only when the dose of PMO is 300mg/kg[50]. Interestingly, marginal improvements in cardiac functions were observed in the heart from repeated treatments of PMO-G for 6 months compared to PMO-S and untreated age-matched *mdx* controls (data not shown). Further studies with peptide-modified PMOs[51] are warranted to see if heart delivery can be achieved as the heart is particularly challenging to deliver to.

Interestingly, unlike hexose[19], glycine promotes PMO uptake by activating MuSC proliferation and muscle regeneration rather than directly improves PMO uptake in dystrophic muscles. Thus, it might be unnecessary to administer glycine together with PMO for future clinical uses. Glycine can be taken via dietary supplementation, whereas PMO drugs can be administered via the delivery route approved by FDA, which can enhance the therapeutic efficacy of approved DMD AO drugs without the requirement for further clinical assessment. Glycine not only provides necessary nutrition for DMD patients and promotes satellite cell proliferation but also serves as

an enhancer for approved AO drugs and eases DMD patients' economic burdens by reducing the dose and related costs; therefore glycine may kill four birds with one stone. Notably, a significant bodyweight gain was observed in PMO-G treated *mdx* mice compared to PMO-S in the long-term study, which is likely attributed to the increased muscle mass stimulated with glycine in combination with dystrophin restoration as no bodyweight gain was observed in *mdx* mice with glycine alone (unpublished observation). In addition, we investigated the effect of PMO-G in aged *mdx* mice, which showed more severe inflammation and fibrosis with much less muscle preserved[52], and a similar potency was achieved with enhanced muscle regeneration and PMO activities (data not shown), further highlighting the benefits of glycine in treating DMD patients irrespective of ages. Worth mentioning, recent studies with glycine alone or glycine together with prednisolone extended lifespan of mice or alleviated the pathological progression in dystrophic mice[53, 54], further confirming the beneficial effect of glycine in general.

Another important finding from our current study is that we demonstrated that glycine can activate mTORC1, a central nutrient-sensing pathway [55], in dystrophic muscle. Amino acids are shown to communicate with mTORC1 via a lysosome-based signalling system; however it remains unclear about how amino acids regulate mTORC1 as the existence of different transport mechanisms for amino acids complicates the situation[56]. In our study, although we proved that glycine-specific mTORC1 activation was involved in muscle regeneration in dystrophic mice and

glycine likely activates mTORC1 via v-ATPase and RagB, two key components for the shuttle of mTORC1 from peripheral to lysosomes[46], and further studies are warranted to address how mTORC1 senses glycine in muscle.

One major hurdle for the clinical implementation of cell therapy is the low transplantation efficiency, which is likely due to poor retention and survival of transplanted cells[57]. Here we show that glycine not only promotes endogenous MuSC proliferation but also increases exogenous cell transplantation efficiency in dystrophic mice, suggesting that glycine can improve cell therapy. We adopted the protocol of pre-administration based on the assumption that glycine can prevent the activation of immune cells as shown for liver transplantation[16], and monitored the fluorescence of EGFP-positive MuSCs after transplantation in CTX-injured TA muscles of *mdx* mice in a real time manner. Although the results showed stronger fluorescence signals in TA muscles from glycine-induced mice compared to the saline control at different time-points after transplantation, we were uncertain about whether the presence of glycine increases cell retention and survival or promotes its proliferation due to the low fluorescence intensity and auto-fluorescence from muscle. Further detailed studies to understand how glycine impacts on cell transplantation are warranted. Nevertheless, our study demonstrated a proof-of-concept that glycine can enhance cell transplantation.

In summary, we demonstrate that glycine potentiates AO efficacy and cell

transplantation and thus open a fundamentally new avenue to overcome the challenge of *in-vivo* systemic delivery of nucleic acids and cell therapy. Moreover, our findings unveil the role of glycine as an activator of mTORC1 pathway in muscular dystrophies.

Materials & Methods

Animals and injections

Adult *mdx* mice (6~8-week old) and age-matched *C57BL/6* mice were used in all experiments (the number of mice per group was specified in corresponding Figure legends). Mice were housed under specific pathogen-free conditions in a temperature -controlled room. The experiments were carried out in the Animal unit, Tianjin Medical University (Tianjin, China), according to procedures authorized by the institutional ethical committee (Permit Number: SYXK 2009-0001). For local intramuscular injection, 2 μg PMO was dissolved in saline or other solutions. For intravenous injections, various amounts of PMO in 120 μl saline or amino acids solutions were injected into tail vein of *mdx* mice at 25 or 50 mg/kg per week for 3 weeks. For the long-term systemic study, PMO in glycine or saline was repeatedly injected at 25 mg/kg per week for 3 weeks with 50 mg/ml glycine administered every other day for the first 3 weeks, followed by 25 mg/kg per month for 5 months with 50 mg/ml glycine injected weekly in *mdx* mice intravenously. For mechanistic studies, 2 μg PMO was dissolved in glycine with 0.2 μg tetrahydrofolate, 0.2 μg methotrexate (Sigma, USA) [58] or 3 μg Bitopertin or 5 μg PP242 (Selleck, USA)[59]. Or 2 μg PMO

was dissolved in 50 mg/ml formate (Sigma, USA) or 0.4 μg tetrahydrofolate[58]. Mice were killed by CO_2 inhalation at 2 weeks after the last injection unless otherwise specified, and muscles and other tissues were snap frozen in liquid nitrogen-cooled isopentane and stored at -80 °C.

Oligonucleotides

Unlabeled and FITC- or lissamine-labeled PMO were synthesized by GeneTools LLC (Corvallis, Oregon, USA). PMO (5'-ggccaaacctcggcttacctgaaat-3') sequences were targeted to murine *dmd* exon 23 / intron 23 boundary site[60].

Protein extraction and Western blot

For the detection of dystrophin in muscle tissues, Western blot was performed as described previously[19]. Briefly, total protein was extracted from frozen muscles and the protein concentration was quantified by Bradford assay (Sigma, USA). Various amounts of protein from wild-type *C57BL/6* mice were used as positive controls, and corresponding amounts of protein from muscles of treated or untreated *mdx* mice were loaded onto SDS-polyacrylamide electrophoresis gels (4% stacking and 6% resolving), followed by transfer to the PVDF membrane. The membrane was then washed and blocked with 5% skimmed milk and probed overnight with primary mouse monoclonal antibodies including DYS1 (1:200, NovoCastra, UK) and α-actinin (1:4000, Sigma-Aldrich, USA). For the detection of mTOR and other related proteins, 4% stacking and 6% resolving or 4% stacking and 10% resolving

SDS-polyacrylamide electrophoresis gels were used, respectively. The following primary antibodies were used at the indicated dilution for Western blot analysis: rabbit monoclonal antibodies including mTOR (1:1000), phospho-mTOR (1:500), S6 ribosomal protein (1:1000), phospho-S6 ribosomal protein (1:1000), phospho-4E-BP1 (1:500), RagB (1:1000), p70S6K (1:500), phospho-p70S6K (1:500), GAPDH (1:1000; Cell Signaling Technology, USA); 4E-BP1 (1:4000, Abcam, UK); and alpha tubulin mouse monoclonal antibody (1:1000; Abcam, UK). Secondary HRP-tagged antibody (1:4000; Sigma, USA) was used and signals were visualized with ECL Western blotting analysis system (Amersham Pharmacia Biosciences). Bands were quantified by densitometric analysis using ImageJ software program (USA). The dystrophin/ α-actinin ratios of treated samples were normalized to the average *C57BL/6* dystrophin /α-actinin ratios (from serial dilutions). The ratio of phosphorylated mTOR and other proteins relative to corresponding total protein expression was calculated based on the mean value of band intensity with Image J software. At least three biological samples were examined unless otherwise specified.

Immunohistochemistry

Immunohistochemistry for dystrophin and embryonic myosin heavy chain was performed as previously described [19]. Briefly, 8μm of cryosections were fixed in DPBS for 10 min and blocked for 1 hr in DPBS supplemented with 20% goat serum and 10 mg/ml BSA (Sigma-Aldrich, MO, USA). Rabbit polyclonal antibody 2166 against the dystrophin C- terminal region (1: 50; the antibody provided by Professor

Kay Davies, Department of Physiology, Anatomy and Genetics, University of Oxford), mouse monoclonal antibody BF-45 (1:30; Developmental studies hybridoma bank, University of Iowa, USA) and laminin (1:400; rabbit monoclonal antibody, Abcam, UK) were used. For the detection of DAPC components, serial sections were stained with a panel of polyclonal and monoclonal antibodies as described previously [19]. Briefly, rabbit polyclonal antibody to neuronal nitric oxide synthase (1:50) and mouse monoclonal antibodies to β-dystroglycan, α-sarcoglycan and β-sarcoglycan were used according to the manufacturer's instructions (1:200, Novocastra, UK). Polyclonal antibodies were detected by goat-anti-rabbit IgG Alexa 594 and the monoclonal antibodies by goat-anti-mouse IgG Alexa 594 (Molecular Probe, UK). The M.O.M. blocking kit (Vector Laboratories, Inc., Burlingame, CA, USA) was applied for the immunostaining of the DAPC. For the immunostaining of PAX7, Ki67 and laminin[61], 8μm of cryosections were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature (RT), followed by washing with 1% Triton X-100 in PBS for 5 min for 3 times. Subsequently, slides were immersed in PH6.0 citrate buffer heated at 100°C for 10 min, followed by blocking with 10% affinipure Fab goat anti mouse IgG (1: 10; Jackson Immunoresearch, USA) in 1% Triton X-100 for 1 hr at RT and replaced with 20% goat serum and 10 mg/ml BSA in 1% Triton X-100 for 30 min at RT prior to the incubation with PAX7 mouse monoclonal antibody (1:300; a gift from the Prof. Hongbo Zhang, ZhongShan University, GuangZhou, China), Ki67 (1:1000; rabbit monoclonal antibody, Abcam, UK). Rabbit monoclonal antibody was detected by goat-anti-rabbit IgG Alexa 594 and mouse monoclonal antibodies were detected by

goat-anti-mouse IgG Alexa 488 (Invitrogen, USA). For the detection of FITC-labelled PMO in muscle tissues, 8 μm of cryosections were fixed with 4% PFA for 15 min at RT, followed by fixation with ice-cold acetone for 10 min. Subsequently, sections were blocked in DPBS supplemented with 20% goat serum and 10 mg/ml BSA for 1 hr, followed by the incubation with anti-fluorescein (1:200, Life Technologies, MA, USA) overnight at 4°C. Wheat germ agglutinin-Alexa Fluor 647 (1:500; Life Technologies, MA, USA) was applied to the sections for 10 min at RT and washed with DPBS for 3 times 5 min each time. Sections were mounted with in fluorescent mounting medium (DAKO, CA, USA) with 0.1% DAPI.

Cell culture

HEK293T cells or murine C2C12 myoblasts were kept in house and cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FBS) and 1% penicillin and streptomycin. HEK293T cells ($7x10^3$) or murine C2C12 myoblasts ($9x10^3$) were seeded in 96-well plates for 12 hrs prior to the replacement with total amino acid-free high glucose DMEM (Thermo Fisher, USA) for 1 hr, followed by the addition of total amino acid mixture consisting of MEM amino acids solution (1:50 vol /vol) and MEM non-essential amino acids solution (1:100 vol/vol) (Thermo Fisher, USA) or 0.8mM glycine (Sigma, USA) for 24 hrs. Cells were counted with hemocytometer. For the amino acid starvation experiments, 10% dialyzed FBS was depleted unless otherwise indicated[46]. For the v-ATPase blocking experiment, 1 μM Baf A1 (MCE, USA) was added into the starved cells for

1 hr prior to stimulation with total amino acids or glycine for 30 min. To examine the effect of different concentrations of glycine on PMO uptake in differentiating myotubes and proliferating myoblasts, FITC-labelled PMO (1μM) and different concentrations of glycine were added into differentiating myotubes (24 hrs after differentiation induction) and proliferating myoblasts. The fluorescence was measured 48 hrs later with plate-reader.

Immunocytochemistry

HEK293T cells ($1x10^4$) or murine C2C12 myoblasts ($1x10^4$) were seeded on matrixgel-coated glass coverslips in 24-well plates for 24 hrs prior to the replacement with total amino acid-free high glucose DMEM (Thermo Fisher, USA) for 1 hr, followed by the addition of total amino acid mixture consisting of MEM amino acids solution (1:50 vol /vol) and MEM non-essential amino acids solution (1:100 vol/vol) (Thermo Fisher, USA) or 0.8mM glycine (Sigma, USA) for 10, 20 or 30 min. Cells were fixed with 4% PFA and permeabilized with 0.5% Triton X-100 in DPBS for 10 min, followed by rinsing with DPBS 3 times and blocking with 20% goat serum in DPBS for 90 min. The following primary antibodies including mTOR rabbit monoclonal antibody (1:100), LAMP1 rat monoclonal antibody (1:200) and mouse monoclonal antibodies LAMP2 (1:100, Abcam, UK), PAX7 (1:300) and MyoD (1:100; Santa Cruz, USA) were incubated with the coverslips overnight at 4°C, followed by washing with PBST (0.1% tween-20 in DPBS) 3 times 5 min each time. Secondary antibodies including goat-anti-mouse IgG Alexa 488 (1:200), goat-anti-rat IgG Alexa

488 (1:200) and goat-anti-rabbit IgG Alexa 594 (1:200; Invitrogen, USA) were incubated for 1 hr at RT. Coverslips were mounted with in fluorescent mounting medium (DAKO) with 0.1% DAPI and visualized with confocal fluorescence microscopy (Zeiss LSM510, Germany) or (Olympus FV1000, Japan). Image J and Image-Pro Plus software (USA) were used for the quantification and co-localization analysis.

Histology

To examine the presence of CD3 T lymphocytes and macrophages in muscle tissues from treated *mdx* or control mice, muscle tissues were fixed in Bouin's solution (Sigma-Aldrich, USA) and embedded with paraffin. CD3+ T lymphocytes or CD68+ macrophages were stained with rabbit polyclonal antibodies CD3 (1:200) or CD68 (1:400, Abcam, UK) and detected by goat-anti-rabbit secondary antibody (Sigma, USA). To measure the fibrotic areas, Masson's trichrome staining kit (Sigma, USA) was applied as per manufacturer's instructions. Routine H&E staining was used to examine the overall liver and kidney morphology and assess the level of infiltrating mononuclear cells. Quantification analysis was performed using Image J software.

Tissue distribution

Lissamine-labelled PMO was diluted in 120 µl saline or glycine and administered into adult *mdx* mice intravenously at 25 mg/kg/day for 3 days and tissues were harvested 4 days later. For the glycine-induction experiment, adult *mdx* mice were treated by 50

mg/ml glycine every other day for one week prior to single intravenous injection of FITC-labelled PMO at 50 mg/kg doses, tissues were harvested 48 hrs later. Perfusion was performed with 50 ml of cold PBS to wash out free oligonucleotides in circulation. Body-wide muscles, liver, kidney and brain were harvested for imaging and quantification with IVIS imaging system (PE, USA).

Generation of *RagB* knockout HEK293T cells

HEK293T cells were transfected with CRISPR/Cas9-expressing lentivirus (Lentiviral CRISPR Toolbox, Zhanglab, USA), followed by 2 µg/mL blasticidin selection for 2 weeks. Cas9-expressing HEK-293T cells were transfected were transfected with sgRNA-expressing lentivirus for 24 hr, followed by 1µg/mL puromycin selection for 2 weeks. The guide sequence targeting Exon 3 of human *RagB* is 5'-CCACCACTAGGGGAACCGGA-3'[62]. The sgRNA was cloned into a LentiGuide-puro vector at *BsmBI* site as previously reported[63].

RNA extraction and RT–PCR analysis

Total RNA was extracted with Trizol (Invitrogen, UK) as per the manufacturer's instructions. For the detection of PMO-mediate exon-skipping of murine *dmd* gene, 200 ng of RNA template was used for 20 ml reverse transcriptase-PCR (RT-PCR) with One Step RT-PCR kit (Qiagen, UK) and nested RT-PCR was performed as described previously[19]. The products were examined by electrophoresis on a 2% agarose gel. For the validation of cell cycle-related gene expression, 1µg RNA as

template was reverse transcribed into cDNA with Transcriptor first strand cDNA synthesis Kit (Roche, Germany) per the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed with SYBR Green (Roche, Germany) on 7500 Fast Real-Time PCR System (Applied Biosystems, USA). Primers used for the study were listed CCNE1: forward 5'-CCTGCAGATGCTGTGCTCTAT-3', Reverse 5'-CATCCCACATTTGCTCACAAC-3'; CDK1: forward 5'- ACACCTTTCCCAAG TGGAAGC-3', Reverse 5'-GCCATTTTGCCAGAGATTCG; CDC20: Forward 5'-TTCCCAGGTGTGCTCCATCC-3', Reverse 5'-CCCGTGCTGTGTGTCCTTTG-3'; CCNB1: forward 5'-GCGTGTGCCTGTGACAGTTA-3', Reverse 5'- CCTAGCGTT TTTGCTTCCCTT-3'; CCNB2: Forward 5'-TGTCAACAAGCAGCCGAAAC-3', Reverse 5'-TCAGAGAAAGCTTGGCAGAGG-3'; CCNA1: Forward 5'-GCTGGC CATGAACTACCTG-3', Reverse 5'-AGAGGCCACGAACATGC-3'; β actin: Forward 5'-TGCACCACCAACTGCTTAG-3', Reverse 5'-GGATGCAGGGATGAT GTTC-3'. All qPCR data were performed with at least three biological replicates. The PCR products were confirmed by proper melting curves.

RNA sequencing

Total RNA (3 µg/sample) was extracted from primary myoblasts isolated from *mdx* mice followed by incubation in 0.8mM glycine for 24 hrs. RNA sequencing was performed as described previously[64]. Briefly, sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) per manufacturer's instructions and index codes were added to attribute sequences to each

sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia, USA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2500 platform and 100/50 bp single-end reads were generated. DESeq R package (1.10.1) was used for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. Benjamini and Hochberg's approach was used to adjust the resulting P-values for controlling the false discovery rate. Genes with an adjusted P-value < 0.05 were assigned as differentially expressed. GOseq R package was used for Gene Ontology (GO) enrichment analysis of differentially expressed genes, in which gene length bias was corrected. GO terms with corrected $p < 0.05$ were considered significantly enriched by differentially expressed genes. Clustered heat-maps were produced by cluster 3.0 software. Original data were uploaded to Gene Expression Omnibus database with accession number: GSE130843.

GLDC knockdown in _mdx_ mice

For the *GLDC* knockdown experiment, three different short hairpin RNA (shRNA) target sequences were selected and optimized by Obio Technology Co., Ltd (Shanghai, China). The selected *GLDC* shRNA was packaged in AAV2/8 serotype plasmid. High-titre AAV2/8 particles were produced and supplied by Obio Technology Co., Ltd (Shanghai, China). AAV2/8 particles (40 μl) were injected into TA muscles of *mdx* mice and the knockdown efficiency of GLDC protein was detected at 1 and 3 weeks

post-injection. PMO (2 μg) in glycine was administered into the same TA muscles of *mdx* mice treated with *GLDC* shRNA 1 week after transduction and TA muscles were harvested 2 weeks after injection.

Isolation of primary myoblasts

Hindlimb muscles including gastrocnemius, quadriceps and TA were dissected and washed with pre-cooled D' hanks buffer. All muscles were minced and digested with 400 U/ml of type II collagenase (Sigma, USA) in D' hanks for 2 hrs at 37°C and centrifuged at 750g for 5 min. The digested fiber pellets were vortexed and filtered through 70-μm and 40-μm cell strainers, respectively, followed by centrifugation at 750g for 5 min and seeded in matrigel-coated (1:100, Corning, USA) petri-dishes. Isolated primary myoblasts were cultured in Ham's F-10 (Gibco, USA) supplemented with 20% FBS, 10% horse serum, 1% penicillin-streptomycin (Gibco, USA) and 2.5 ng/ml bovine fibroblast growth factor (FGF) (PeproTech, USA). To examine the effect of glycine on isolated primary myoblasts, isolated primary myoblasts were cultured in growth medium supplemented with different concentrations of glycine in 96-well plates for 24 hrs, followed by cell counting with hemocytometer.

Isolation of satellite cells

Hindlimb muscles were minced and digested with 250 U/ml of type II collagenase (Sigma, USA) in D' hanks for 60 min at 37°C and then centrifuged at 750g for 5 min, followed by digestion with 60 U/ml type II collagenase and 3 U/ml type II dispase

(Sigma, USA) for 45 min at 37°C. The digested fiber pellets were filtered through 70-μm and 40-μm cell strainers, respectively, and washed and then resuspended in D' hanks buffer and immediately stained with anti-mouse antibodies including PE-VCAM (1:500), FITC-CD31 (1:500), FITC-CD45 (1:500) and APC-Sca-1 (1:1000, eBioscience, USA) for 30 min at 4°C, followed by analysis and sorting using the BD FACS Aria II instrument (USA). For the isolation of satellite cells from skeletal muscle explants, it was adopted from previous reports[65]. Briefly, skeletal muscles isolated from hindlimbs were minced into small pieces and digested with 500 U/ml type II collagenase and 3 U/ml type II dispase in D' hanks for 60 min at 37°C, followed by centrifugation at 300g for 5 min. The fiber pellets were resuspended in high glucose DMEM (Gibco, USA) supplemented with 20% FBS, 10% horse serum, 1% penicillin-streptomycin, 1% Antibiotic-Antimitotic and 2.5 ng/ml bFGF. The suspension containing small pieces of muscle tissues was seeded on matrigel-coated flasks at 10-20% surface coverage and incubated at 37°C and 5% CO2 to allow attachment of the tissues to the surface and subsequent migration of cells for 3-5 days. Cells from skeletal muscle explants were detached with 0.25% trypsin and centrifuged at 300g for 5 min. To separate the non-myogenic cells from myoblasts, cells were seeded on 0.2% gelatin-coated flasks and incubated at 37°C and 5% CO_2 for 1 hr and the supernatant was transferred to a matrigel-coated dish for subsequent culture.

Myoblast transplantation in *mdx* mice

Cardiotoxin (CTX) (2 μg) was injected into TA muscles of *mdx* mice and primary

myoblasts ($1x10^6$) or MuscS ($1x10^5$) were transplanted into the same injured TA muscle 1 day later as reported previously[61]. *Mdx* mice were fed with 7.5 mg/l FK506 (Astellas, Japan) in the drinking water every day during the experimental period as reported previously[64, 66]. Glycine (120 μl) was intravenously injected into *mdx* mice every other day for 1 week prior to cell transplantation and continuously injected with the same dosing regimen during the experimental period. Treated *mdx* mice were monitored at week 1, 2 and 3 after transplantation with the IVIS imaging system (PE, USA). TA muscles were harvested 3 weeks after transplantation and frozen in liquid nitrogen for storage.

Functional grip strength and running wheel test

Treated and control mice were tested using a commercial grip strength monitor (Chatillon, West Sussex, UK) as described previously[19]. Briefly, each mouse was held 2 cm from the base of the tail, allowed to grip a protruding metal triangle bar attached to the apparatus with their forepaws and pulled gently until they released their grip. The force exerted was recorded and five sequential tests were carried out for each mouse, averaged at 30s apart. Subsequently, the readings for force recovery were normalized by the body weight. For muscle endurance, mice were placed in cages equipped with voluntary running wheels (Zhenhua, Anhui, China) in a quiet and cleanly room at a temperature of 24±1°C. Running circles were recorded and distance was analyzed.

Clinical biochemistry

Serum and plasma were taken from the jugular vein immediately after killing with CO_2 inhalation. Analysis of serum creatinine kinase, aspartate aminotransferase, alanine aminotransferase, creatinine and urea was performed by the clinical pathology laboratory (Tianjin Metabolic Disease Hospital, Tianjin, China).

Data analysis

All data are reported as mean values ± s.e.m. Statistical differences between different treated groups were evaluated by Sigma Stat (Systat Software Inc., Chicago, IL, USA). Both parametric and non-parametric analyses were applied as specified in figure legends. Significance was determined based in $p<0.05$.

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Author Contributions

H.Y. conceived the project. C. L., G.H. and H.Y. designed the experiments, analyzed the data and wrote the manuscript with input from all authors. C.L., G.H., H.N., J.S. and N.R. carried out the experiments. X.Y. and Y. S. performed the RNA seq data analysis.

Competing interests

H.Y., C.L and G.H. have filed a patent for glycine (Application No. PCT/CN2019/109926).

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and /or the Supplementary Materials. Additional data related to this paper may be obtained from the corresponding author upon reasonable request.

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Figure legends

Figure 1. Screening of amino acids with PMO in adult *mdx* mice. (**A**) Different concentrations of nutrients used for the study. Dystrophin expression following single intramuscular injection of 2 µg PMO into *mdx* TA muscles (**B**-**C**) or intravenous injection of PMO at 25 mg/ kg/week for 3 weeks in different amino acid solutions or saline in *mdx* mice, respectively (**D**-**E**). (**B**) Immunohistochemistry and quantitative analysis for dystrophin-positive fibers in *mdx* TA muscles treated with single intramuscular injection of 2 µg PMO in different nutrients (scale bar=100 µm). The concentration was 5% as illustrated in (A). For glycine and saline groups, the number of animals used per group is 6 (n=6) and the rest is 3 (n=3; One way-ANOVA post hoc Student-Newman-Keuls test). Red staining on fiber membrane shows dystrophin expression. Nuclei were counterstained with DAPI (blue) (the same is for the rest unless otherwise specified). (**C**) Representative Western blot and quantitative analysis for dystrophin expression in TA muscles from *mdx* mice treated with single intramuscular injection of PMO in different solutions. For glycine and saline groups, the number of animals used per group is 6 (n=6) and the rest is 3 (n=3One way-ANOVA post hoc Student-Newman-Keuls test). α-actinin was used as the loading control. 5 µg, 2.5 µg and 0.5 µg total protein from *C57BL/6* and 50 µg from muscle samples from untreated and treated *mdx* mice were loaded. TA muscles from *C57BL/6* were used as normal controls (the same for all Western blots unless otherwise specified). (**D**) Immunohistochemistry for dystrophin expression in body-wide muscles from *mdx* mice treated with PMO in saline or amino acid

solutions (5%) at 25 mg/kg/week for 3 weeks intravenously. TA represents tibialis anterior (scale bar=100 μm). (**E**) Western blot and quantitative analysis of dystrophin expression in body-wide muscles from *mdx* mice treated with PMO in glycine (n=4), serine or saline (n=3) at 25 mg/kg/week for 3 weeks intravenously (One way-ANOVA post hoc Student-Newman-Keuls test). 0.5 µg, 2.5 µg and 5 µg total protein from *C57BL/6* and 50 µg from muscle samples from untreated and treated *mdx* mice were loaded. (**F**) Immunohistochemistry for dystrophin expression in body-wide muscles from *mdx* mice treated with PMO in glycine at 25 mg/kg/week for 3 weeks with (+Gly) or without additional glycine (-Gly) every other day for 5 weeks intravenously (scale bar=100 μm). (**G**) Western blot for dystrophin expression in body-wide muscles from *mdx* mice treated with PMO in glycine at 25 mg/kg/week for 3 weeks with (+Gly) or without additional glycine (-Gly) every other day for 5 weeks intravenously. 0.5 µg, 2.5 µg, 5 µg and 15 µg totalprotein from *C57BL/6* and 50 µg from muscle samples from untreated and treated *mdx* mice were loaded. TA-tibialis anterior, Q-quadriceps, G-gastrocnemius, T-triceps, A-abdominal muscle and D-diaphragm. (**H**) Quantitative analysis of dystrophin expression in body-wide muscles from *mdx* mice treated with PMO in glycine at 25 mg/kg/week for 3 weeks with (+Gly) or without additional glycine (-Gly) every other day for 5 weeks intravenously (n=4; One way-ANOVA post hoc Student-Newman-Keuls test). (**I**) ELISA assay for measurement of glycine in serum from *mdx* mice treated with PMO in glycine at 25 mg/ kg/week for 3 weeks with (+Gly) or without additional glycine (-Gly) every other day for 5 weeks intravenously (n=4). Data are presented as means

±s.e.m. (*p<0.05; **p<0.001).

Figure 2. Long-term repeated administartions of PMO in glycine (PMO-G) or in saline (PMO-S) in adult *mdx* mice. PMO-G was administered intravenously into adult *mdx* mice at 25 mg/kg/week for 3 weeks with additional glycine administration every other day intravenously followed by 25 mg/kg/month for 5 months with additional glycine administration every one week intravenously. (**A**) Diagram of dosing regimen for the long-term study in *mdx* mice. i.v. refers to intravenous injection. (**B**) Immunohistochemistry for dystrophin expression in body-wide muscles from *mdx* mice treated with PMO-S or PMO-G (scale bar=100 μm). (**C**) Western blot for dystrophin expression in body-wide muscles from *mdx* mice treated with PMO-G or PMO-S. 2.5 μg, 5 μg, 10 μg and 25 μg total protein from *C57BL/6* and 50 μg from muscle samples from untreated and treated *mdx* mice were loaded. TA-tibialis anterior, Q-quadriceps, G-gastrocnemius, T-triceps, A-abdominal muscle and D-diaphragm. (**D**) Quantitative analysis of dystrophin expression in body-wide muscles from treated *mdx* mice (n=4; two-tailed t test). (**E**) Re-localization of DAPC components in treated *mdx* mice to assess dystrophin function and recovery of normal myoarchitecture (scale bar=50 μm). The arrowheads point to identical myofibers. (**F**) Measurement of serum creatine kinase (CK) levels in treated *mdx* mice (n=4; One way-ANOVA post hoc Student-Newman-Keuls test). (**G**) Muscle function was assessed to determine the physical improvement with grip strength test (n=4; One way-ANOVA post hoc Student-Newman-Keuls test). (**H**) Measurement of muscle endurance with the

running wheel test (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).

Figure 3. Glycine promotes PMO uptake in synergizing with muscle regeneration in *mdx* mice. (**A**) Immunohistochemistry and quantitative analysis for dystrophin-positive fibers in *mdx* TA muscles injected with 2 μg PMO followed by separate administration of glycine 16 hr later (scale bar=100 μm) (n=3; One way-ANOVA post hoc Student-Newman-Keuls test). (**B**) Western blot and quantitative analysis for the dystrophin protein in treated *mdx* mice (n=3; One way-ANOVA post hoc Student-Newman-Keuls test). (**C**) Immunohistochemistry for $PAX7^+$ and $Ki67^+$ muscle satellite cells (MuSCs) in TA and gastrocnemius muscles from *mdx* mice treated with saline or glycine every other day for 1 week intravenously (scale bar=100 μm). TA-tibialis anterior. The arrowheads point to $PAX7^+$ and $Ki67^+$ MuSCs. (**D**) Quantitative analysis for $PAX7^+$ and $PAX7^+Ki67^+$ MuSCs in TA and gastrocnemius muscles from treated *mdx* mice (n=4; two-tailed t test). (**E**) Immunohistochemistry for embryonic myosin heavy chain -positive ($eMyHC^+$) regenerating myofibers in gastrocnemius from treated *mdx* mice. FITC-labelled PMO in glycine (PMO-G) or saline (PMO-S) was intravenously administered into adult *mdx* mice at 50 mg/kg for single injection and muscles were harvested 48 hr later (scale bar=100 μm). Fluorescently tagged wheat germ agglutinin (WGA) was used for the visualization of connective tissues. The arrowheads point to the $eMyHC^+$ regenerating myofibers and FITC-labelled PMO. (**F**) Quantitative analysis for the

fluorescence intensity of eMyHC$^+$ regenerating myofibers in TA and gastrocnemius muscles from treated *mdx* mice (n=3; two-tailed t test). (**G**) Representative RT-PCR to detect the exon-skipping efficiency, which is shown by shorter exon skipped bands (indicated by Δexon 23-exon 23 skipped). G- gastrocnemius. (**H**) Measurement of the uptake of FITC-labelled PMO in differentiating (Differentiating) and proliferating myoblasts (Proliferating) treated with different concentrations of glycine (n=4; One way-ANOVA post hoc Student-Newman-Keuls test). NC refers to untreated differentiating myotubes or proliferating myoblasts. (**I**) Tissue distribution of FITC-labelled PMOs in *mdx* mice and quantitative analysis of fluorescence intensity in body-wide tissues. Body-wide tissues were harvested 48 hr after single intravenous injection of FITC-labelled PMO in glycine (PMO-G) or saline (PMO-S) at the 50 mg/ kg doses (n=3; two-tailed t test). A-abdominal muscle, Q-quadriceps, TA-tibialis anterior, G-gastrocnemius, T-triceps, H-heart, K-kidney, L-liver and B-brain. Data are presented as means ±s.e.m. (*$p<0.05$).

Figure 4. Glycine potentiates PMO activity by replenishing one-carbon unit pool. (**A**) Immunohistochemistry for PAX7$^+$ and Ki67$^+$ MuSCs in TA muscles from *mdx* mice treated with PMO-G and bitopertin (BP) (scale bar=100 μm). Glycine or the mixture of glycine and BP was administered into *mdx* TA muscles and muscles were harvested 3 days later. The arrowheads point to PAX7$^+$ and Ki67$^+$ MuSCs. (**B**) Quantitative analysis for PAX7$^+$ and PAX7$^+$Ki67$^+$ MuSCs in TA muscles from treated *mdx* mice (n=3; One way-ANOVA post hoc Student- Newman-Keuls test). (**C**)

Immunohistochemistry and quantitative analysis for eMyHC$^+$ regenerating myofibers in TA muscles from treated *mdx* mice (scale bar=100 μm) (n=3; One way-ANOVA post hoc Student-Newman-Keuls test). (**D**) Immunohistochemistry and quantitative analysis for dystrophin-positive fibers in TA muscles from treated *mdx* mice (scale bar=100 μm) (n=3; One way-ANOVA post hoc Student-Newman-Keuls test). PMO (2 μg) in saline and other solutions was injected into *mdx* TA muscles and muscles were harvested 2 weeks later. (**E**) Western blot and quantitative analysis for dystrophin expression in TA muscles from treated *mdx* mice (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 from muscle samples from untreated and treated *mdx* mice were loaded. (**F**) Immunohistochemistry for PAX7$^+$ and Ki67$^+$ MuSCs in TA muscles from treated *mdx* mice (scale bar=100 μm). Glycine, formate, THF or the mixture of glycine with MTX or THF was injected in *mdx* TA muscles and muscles were harvested 3 days later. THF and MTX refer to tetrahydrofolate or methotrexate, respectively. The arrowheads point to PAX7$^+$ and Ki67$^+$ MuSCs. (**G**) Quantitative analysis for PAX7$^+$ and PAX7$^+$Ki67$^+$ MuSCs in TA muscles from treated *mdx* mice (n=3; One way-ANOVA post hoc Student-Newman-Keuls test). (**H**) Immunohistochemistry and quantitative analysis for eMyHC$^+$ regenerating myofibers in TA muscles from treated *mdx* mice (scale bar=100 μm) (n=3; One way-ANOVA post hoc Student-Newman-Keuls test). (**I**) Immunohistochemistry and quantitative analysis for dystrophin-positive fibers in TA muscles from treated *mdx* mice (scale bar=100 μm) (n=3; One way-ANOVA post hoc Student-Newman-Keuls test). (**J**) Western blot and quantitative analysis for

dystrophin expression in TA muscles from treated *mdx* mice (n=3; One way-ANOVA post hoc Student-Newman-Keuls test). 0.5 µg, 5 µg and 10 µg total protein from *C57BL/6* and 50 µg from muscle samples from untreated and treated *mdx* mice were loaded. Data are presented as means ±s.e.m. (*p<0.05; **p<0.001).

Figure 5. Disruption of glycine decarboxylase (GLDC) compromise glycine's functionality in *mdx* mice. (**A**) Western blot analysis to determine *GLDC* knock-down efficiency at the protein level in TA muscles transfected with *GLDC* shRNA-expressing AAV2/8 viruses in *mdx* mice 3 weeks later (n=3). SC refers to AAV2/8 expressing scramble shRNA. (**B**) Immunohistochemistry for $PAX7^+$ MuSCs in TA muscles from treated *mdx* mice (scale bar=100 µm). The arrowheads point to $PAX7^+$ MuSCs. (**C**) Quantitative analysis for $PAX7^+$ MuSCs in TA muscles from treated *mdx* mice (n=3). (**D**) Immunohistochemistry and quantitative analysis for $eMyHC^+$ regenerating myofibers in TA muscles from treated *mdx* mice (n=3). (**E**) Immunohistochemistry for dystrophin-positive fibers in TA muscles from treated *mdx* mice (scale bar=100 µm). (**F**) Western blot and quantitative analysis for dystrophin expression in TA muscles from treated *mdx* mice (n=4). 2.5 µg and 5 µg total protein from *C57BL/6* and 50 µg from muscle samples from untreated and treated *mdx* mice were loaded. Two-tailed t test was used for statistical analysis (*p<0.05; **p<0.001). Data are presented as means ±s.e.m.

Figure 6. Glycine augments PMO activities by heightening mTORC1 activation

in *mdx* mice. (**A**) Western blot to detect phosphorylated mTOR, S6K1 and S6 expression in quadriceps from *mdx* mice treated with PMO-S or PMO-G at 25 mg/kg/week for 3 weeks with additional supply of glycine every other day for 5 weeks intravenously. α-actinin was used as the loading control. 50 µg total protein were loaded. (**B**) Quantitative analysis of the ratio of phosphorylated mTOR, S6K1 and S6 to mTOR, S6K1 and S6 total protein expression, respectively (n=3; two-tailed t test). (**C**) Immunohistochemistry for $PAX7^+$ and $Ki67^+$ MuSCs in TA muscles from treated *mdx* mice (scale bar=100 µm). Glycine (Gly) or the mixture of glycine with PP242 (Gly/PP242) was injected into *mdx* TA muscles and muscles were harvested 3 days later. The arrowheads point to $PAX7^+$ and $Ki67^+$ MuSCs. (**D**) Quantitative analysis for $PAX7^+$ and $PAX7^+Ki67^+$ MuSCs in TA muscles from treated *mdx* mice (n=3; One way-ANOVA post hoc Student- Newman-Keuls test). (**E**) Immunohistochemistry and quantitative analysis for $eMyHC^+$ regenerating myofibers in TA muscles from treated *mdx* mice (n=3; One way-ANOVA post hoc Student-Newman-Keuls test). (**F**) Immunohistochemistry and quantitative analysis for dystrophin-positive fibers in TA muscles from treated *mdx* mice (scale bar=100 µm) (n=3; One way -ANOVA post hoc Student-Newman-Keuls test). PMO (2 µg) in saline (PMO-S), glycine (PMO-G) or the mixture of glycine with PP242 (PMO-G/PP242) was injected into *mdx* TA muscles and muscles were harvested 2 weeks later. (**G**) Western blot and quantitative analysis for dystrophin expression in TA muscles from treated *mdx* mice (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

from muscle samples from untreated and treated *mdx* mice were loaded. (**H**) Hierarchical clustering analysis of cell cycle-related gene expression profiles in primary myoblasts isolated from *mdx* mice and then treated with 0.8mM glycine for 24 hrs. Expression levels (fold) are depicted in colors in which red represents up-regulation and green means down-regulation. (**I**) Real-time quantitative RT-PCR analysis of cell cycle-related gene expression in TA muscles from *mdx* mice treated with glycine every other day for 1 week intravenously (n=3; two-tailed t test). CDK1-cyclin-dependent kinase 1; CDC20- cell division cycle 20; CCNB2-cyclin B2; CCNB1-cyclin B1; CCNA1-cyclin A1; CCNE1- cyclin E1. Data are presented as means ±s.e.m. (*p<0.05; **p<0.001).

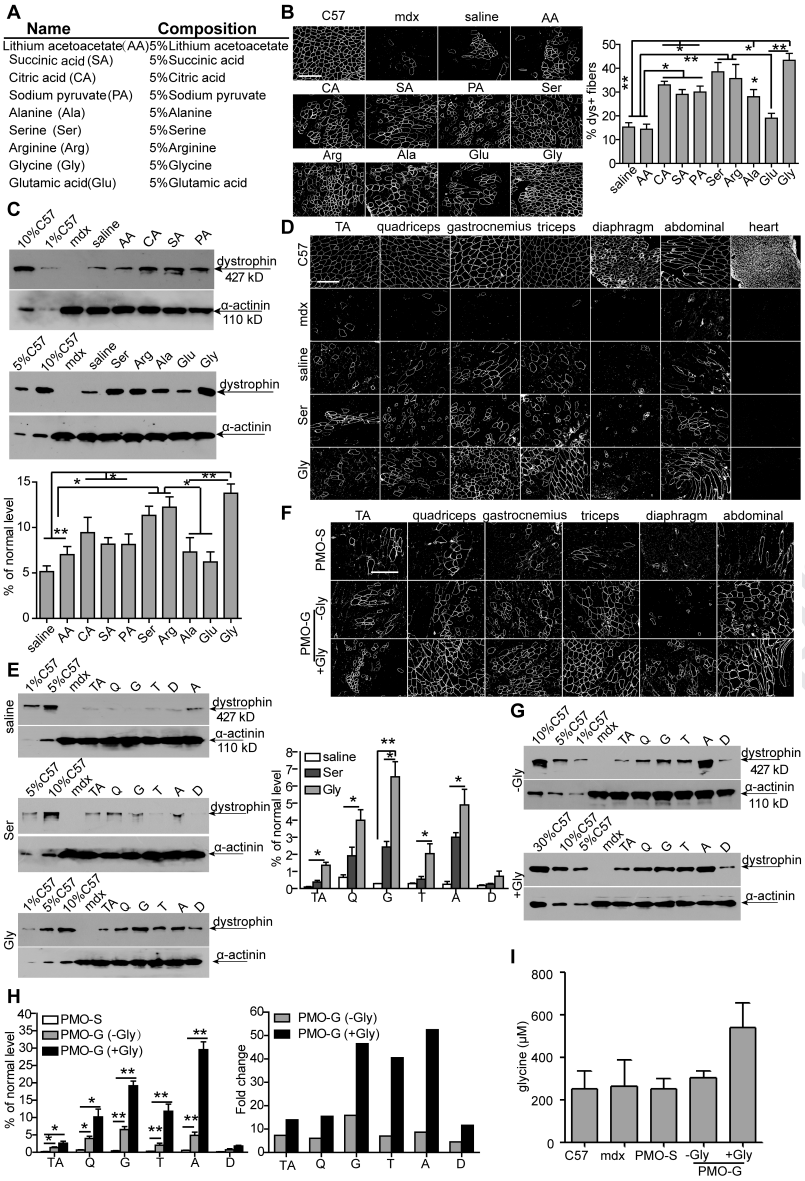
Figure 7. Glycine promotes mTORC1 translocation via v-ATPase and RagB. (**A**) Western blot to detect phosphorylated S6K1, S6 and 4EBP1 expression in starved C2C12 cells followed by re-stimulation of total amino acids and glycine. 30 μg total protein was loaded and tubulin was used as a loading control. AA^+ or Gly^+ refers to the supplementation of total amino acids or glycine into starved cells; AA^- represents the depletion of total amino acids. (**B**) Quantitative analysis of the ratio of phosphorylated S6K1, S6 and 4EBP1 to total expression of counterparts (n=3; One way-ANOVA post hoc Student-Newman-Keuls test). Immunocytochemistry for mTOR (**C**) and quantitative analysis of cells with mTOR at lysosomes (**D**) in starved C2C12 cells followed by re-stimulation of total amino acids or glycine (scale bar=10 μm) (n=10; One way-ANOVA post hoc Student-Newman-Keuls test). LAMP1 and

LAMP2 were used as lysosome markers. Immunocytochemistry for mTOR (**E**) and quantitative analysis of cells with mTOR at lysosomes (**F**) in 293 cells treated with glycine and BAF (scale bar=10 μm) (n=10; two-tailed t test). (**G**) Western blot to detect phosphorylated S6K1 and S6 expression in 293 cells treated with glycine or glycine and BAF. 30 μg total protein was loaded and tubulin was used as a loading control. (**H**) Western blot to detect phosphorylated S6K1 and S6 expression in RagB knock-out 293T cells treated with glycine or total amino acids. 30 μg total protein was loaded and GAPDH was used as a loading control. RagB CON refers to normal 293T cells; RagB KO means RagB knock-out 293T cells. Data are presented as means ±s.e.m. (*p<0.05; **p<0.001).

Figure 8. Effects of glycine on cell transplantation in *mdx* mice. (**A**) Schematics for the cell transplantation in immunosuppressed *mdx* mice. i.v. refers to intravenous injection; -7 and -1 means 7 days or 1 day prior to cell transplantation. *Mdx* mice were immunosuppressed during the experimental period unless otherwise specified. (**B**) Immunohistochemistry and quantitative analysis for dystrophin-positive fibers in TA muscles from glycine-treated *mdx* mice transplanted with wild-type MuSCs and muscles were harvested 3 weeks later (scale bar=100μm) (n=4; two-tailed t test). (**C**) Measurement of TA muscle weight from *mdx* mice transplanted with MuSCs 3 weeks after transplantation. *Mdx* refers to *mdx* controls without cell transplantation (n=4). (**D**) Immunohistochemistry and quantitative analysis for dystrophin-positive fibers in TA muscles from glycine-treated *mdx* mice transplanted with wild-type primary

myoblasts 3 weeks after transplantation (scale bar=100 μm) (n=4; two-tailed t test). (**E**) Tissue imaging to examine the GFP fluorescence and quantitative analysis of fluorescence intensity in glycine-treated *mdx* mice transplanted with wild-type GFP-positive MuSCs 3 weeks after transplantation (scale bar=100 μm) (n=6; One way- ANOVA post hoc Student-Newman-Keuls test). Immunohistochemistry (**F**) and quantitative analysis for dystrophin- and GFP-positive fibers (**G**) in TA muscles from glycine-treated *mdx* mice transplanted with GFP-positive MuSCs 3 weeks later (scale bar=100 μm) (n=6; two-tailed t test). Data are presented as means ±s.e.m. (*$p<0.05$; **$p<0.001$).

Yin and colleagues demonstrate that intravenous or dietary glycine enhances satellite cell proliferation, cell transplantation efficiency and oligonucleotide-mediated dystrophin restoration in Duchenne muscular dystrophy (DMD) mouse model, portending more efficacious cell and exon-skipping therapies for DMD.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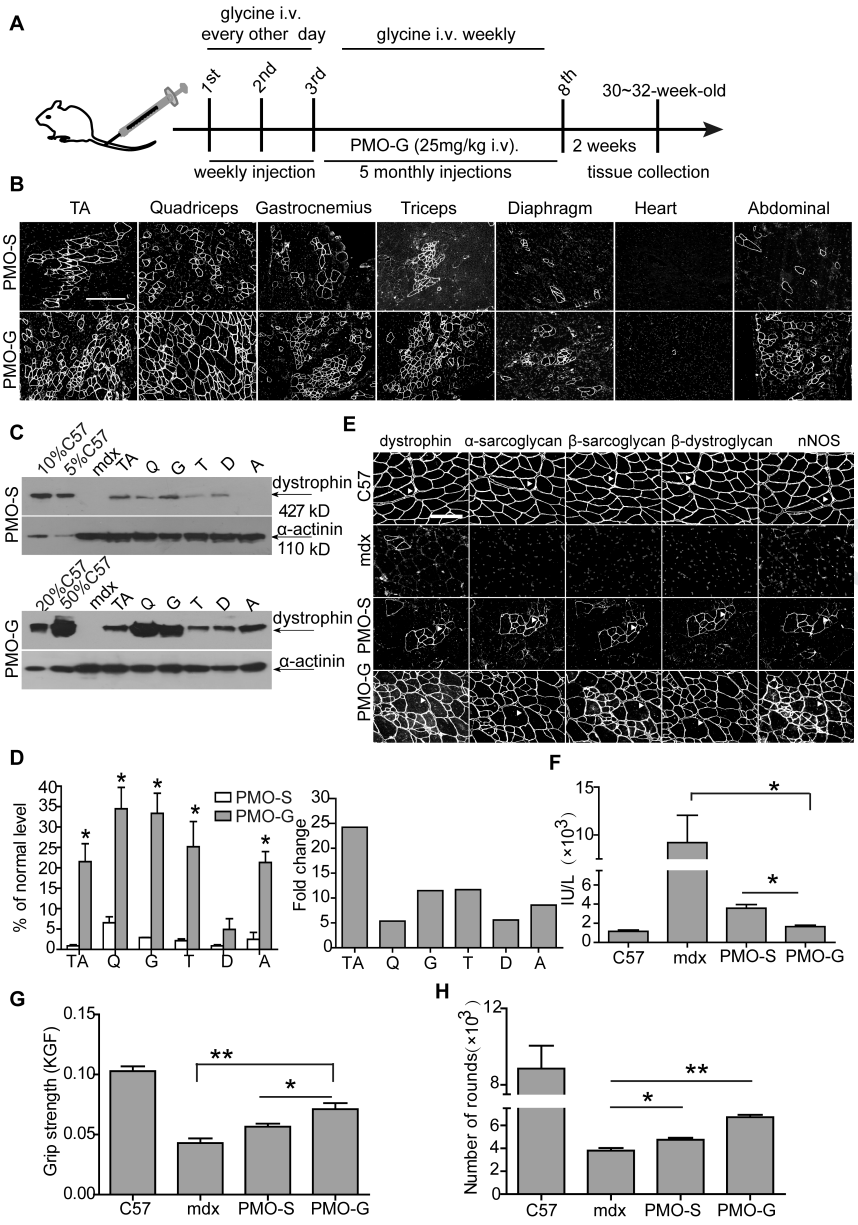
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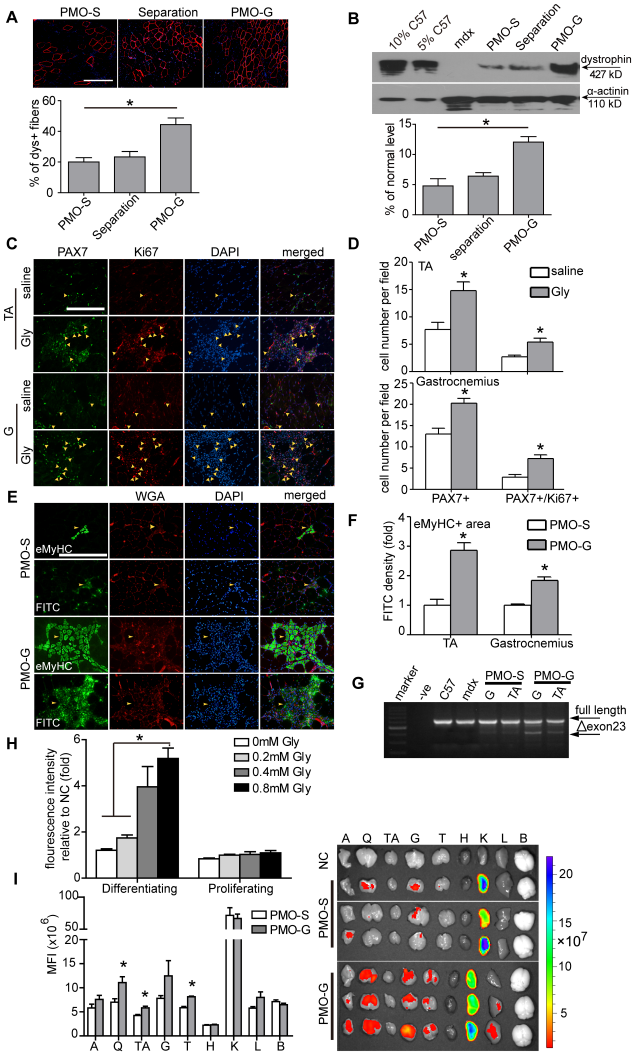
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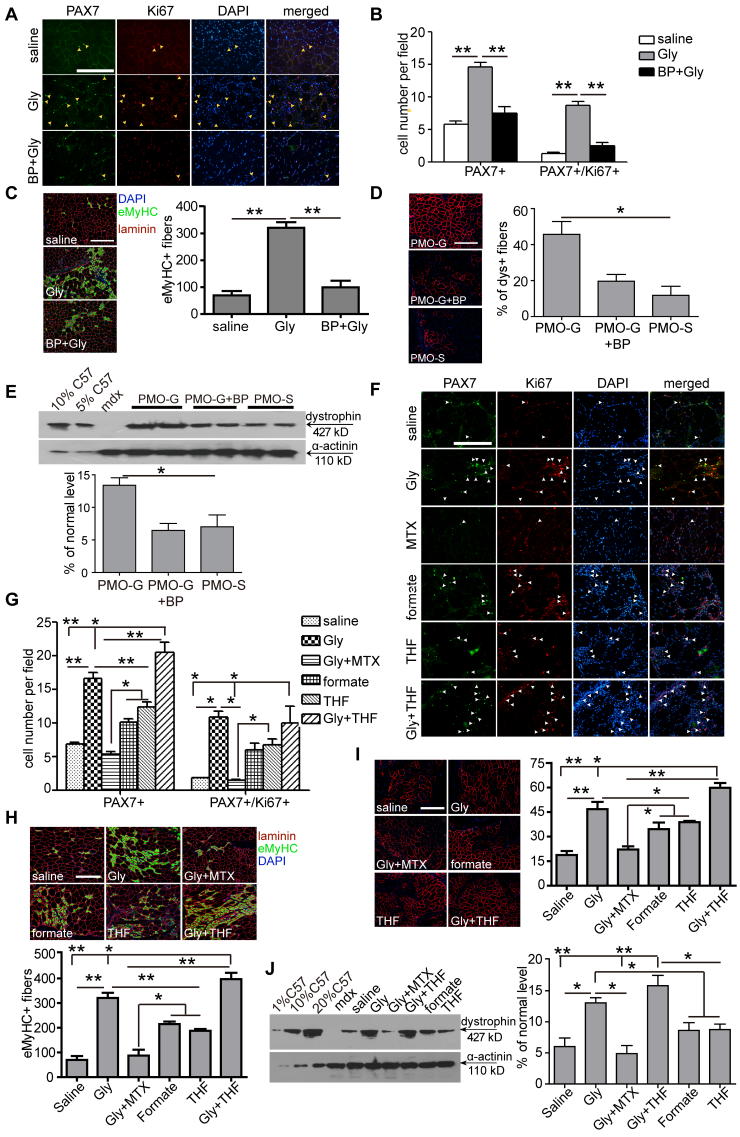
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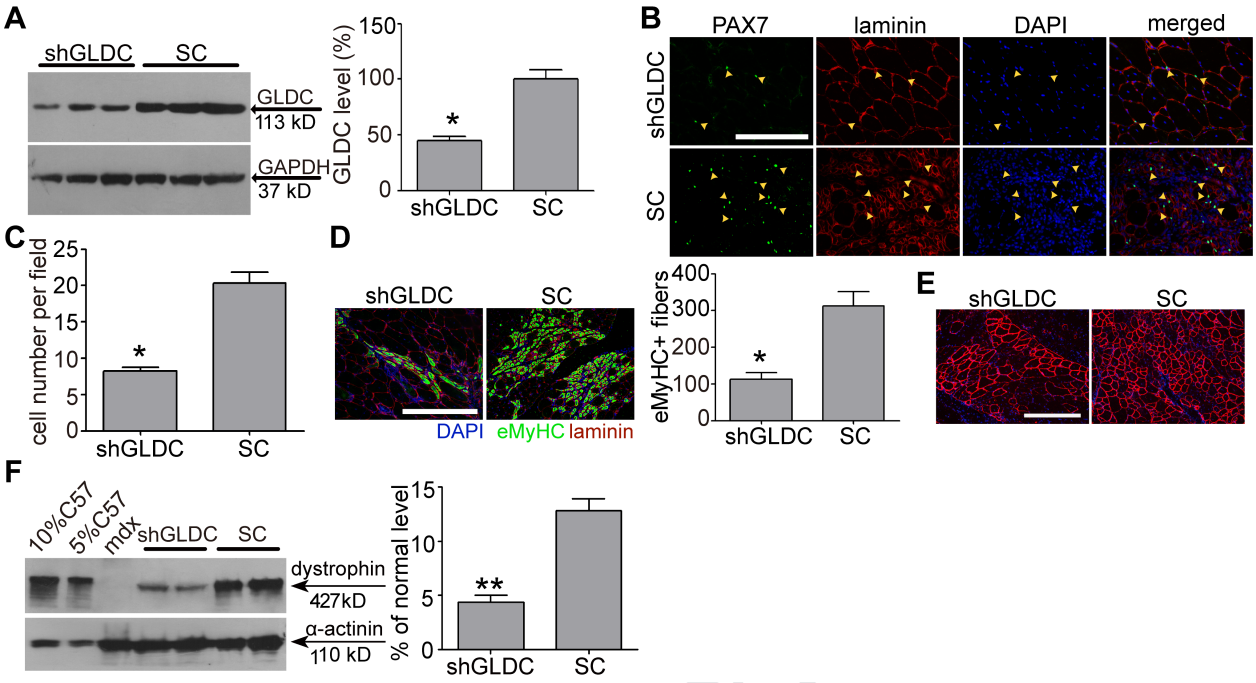
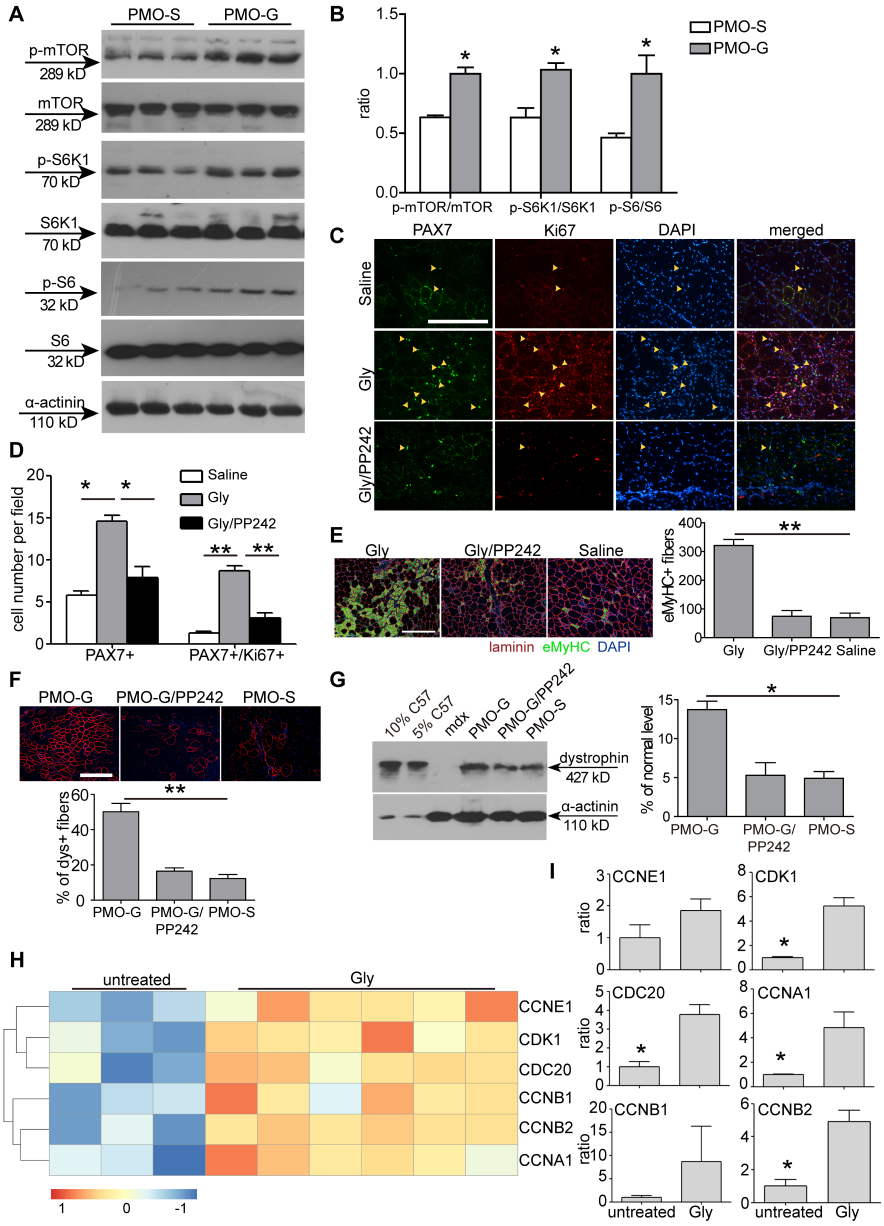
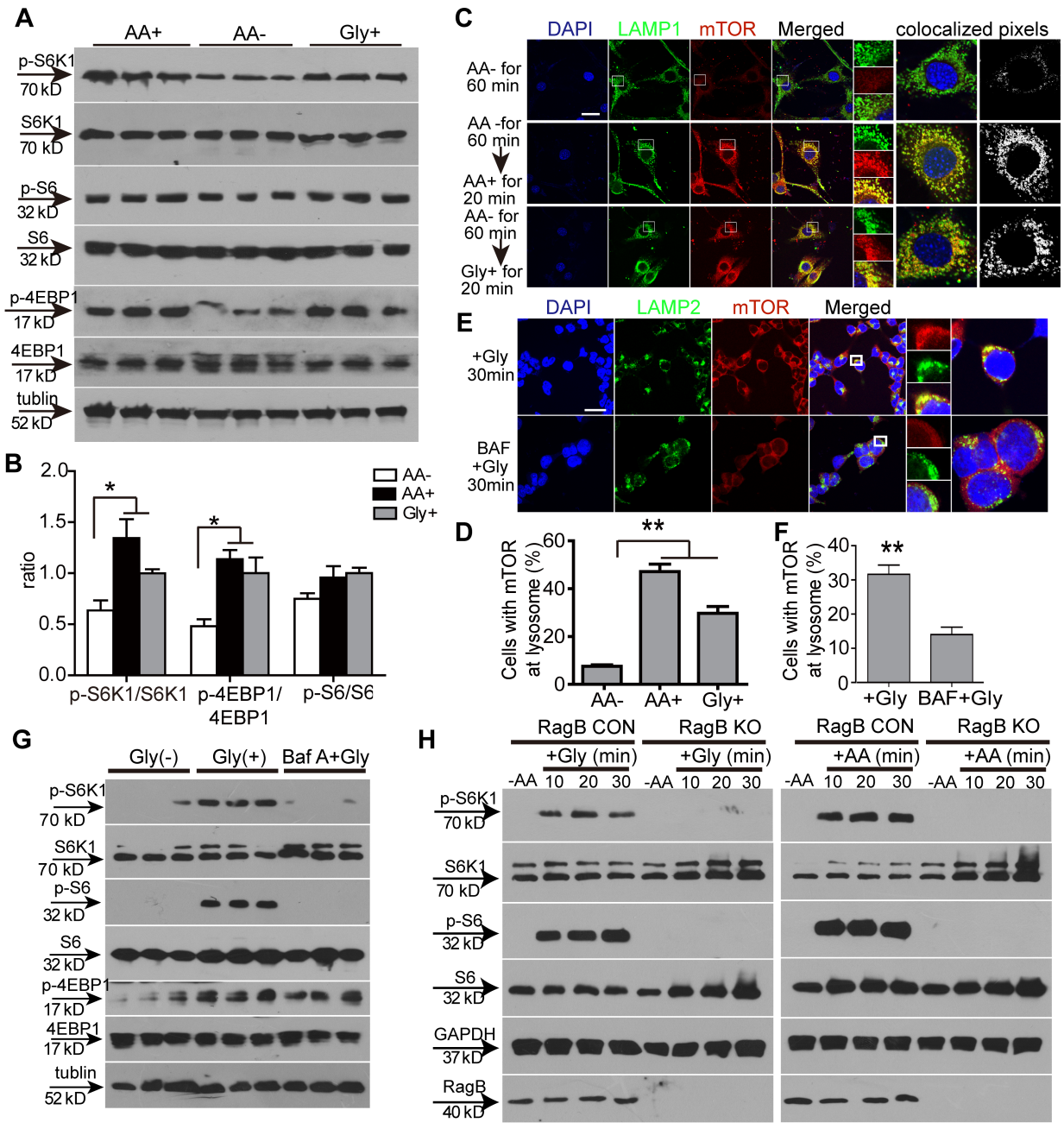
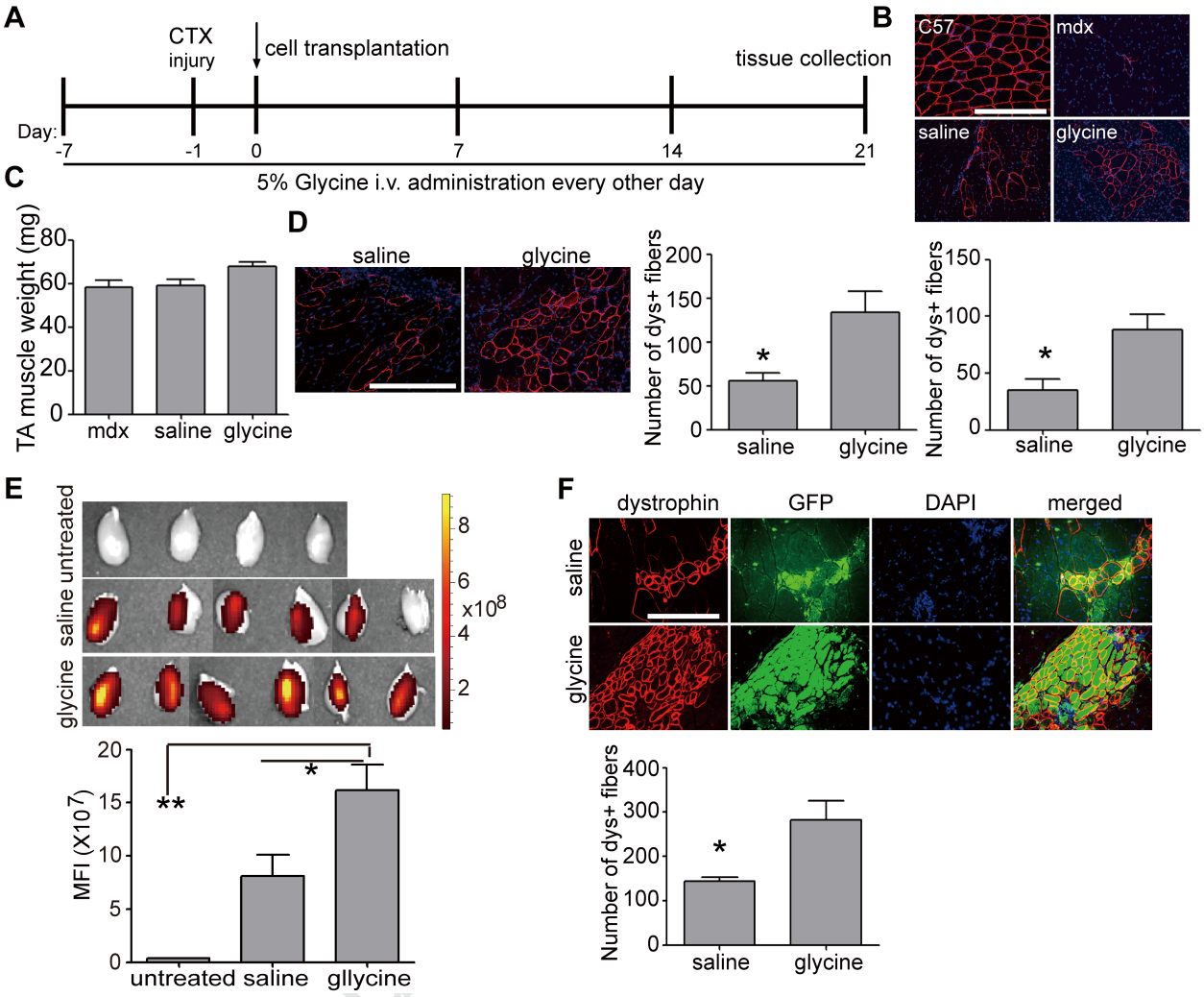
F
PAX7
Ki67
DAPI
merged
saline
Gly
MTX
formate
THF
Gly+THF
G
saline
Gly
Gly+MTX
formate
THF
Gly+THF
cell number per field
PAX7+
PAX7+/Ki67+
H
laminin
eMyHC
DAPI
saline
Gly
Gly+MTX
formate
THF
Gly+THF
eMyHC+ fibers
Saline
Gly
Gly+MTX
Formate
THF
Gly+THF
I
saline
Gly
Gly+MTX
formate
THF
Gly+THF
J
1%C57
10%C57
20%C57
mdx
saline
Gly
Gly+MTX
Gly+THF
formate
THF
dystrophin
427 kD
α-actinin
110 kD
% of normal level




A
PMO-S
PMO-G
p-mTOR
289 kD
mTOR
289 kD
p-S6K1
70 kD
S6K1
70 kD
p-S6
32 kD
S6
32 kD
α-actinin
110 kD
B
PMO-S
PMO-G
ratio
p-mTOR/mTOR
p-S6K1/S6K1
p-S6/S6
C
PAX7
Ki67
DAPI
merged
Saline
Gly
Gly/PP242
D
Saline
Gly
Gly/PP242
cell number per field
PAX7+
PAX7+/Ki67+
E
Gly
Gly/PP242
Saline
laminin eMyHC DAPI
eMyHC+ fibers
Gly
Gly/PP242
Saline
F
PMO-G
PMO-G/PP242
PMO-S
% of dys+ fibers
PMO-G
PMO-G/ PP242
PMO-S
G
10% C57
5% C57
mdx
PMO-G
PMO-G/PP242
PMO-S
dystrophin
427 kD
α-actinin
110 kD
% of normal level
PMO-G
PMO-G/ PP242
PMO-S
H
untreated
Gly
CCNE1
CDK1
CDC20
CCNB1
CCNB2
CCNA1
1
0
-1
I
CCNE1
CDK1
CDC20
CCNA1
CCNB1
CCNB2
ratio
untreated
Gly


A
AA+
AA-
Gly+
p-S6K1
70 kD
S6K1
70 kD
p-S6
32 kD
S6
32 kD
p-4EBP1
17 kD
4EBP1
17 kD
tublin
52 kD
B
ratio
AA-
AA+
Gly+
p-S6K1/S6K1
p-4EBP1/
4EBP1
p-S6/S6
C
DAPI
LAMP1
mTOR
Merged
colocalized pixels
AA- for
60 min
AA -for
60 min
AA+ for
20 min
AA- for
60 min
Gly+ for
20 min
E
DAPI
LAMP2
mTOR
Merged
+Gly
30min
BAF
+Gly
30min
D
Cells with mTOR
at lysosome (%)
AA-
AA+
Gly+
F
Cells with mTOR
at lysosome (%)
+Gly
BAF+Gly
G
Gly(-)
Gly(+)
Baf A+Gly
p-S6K1
70 kD
S6K1
70 kD
p-S6
32 kD
S6
32 kD
p-4EBP1
17 kD
4EBP1
17 kD
tublin
52 kD
H
RagB CON
RagB KO
+Gly (min)
+AA (min)
-AA
10
20
30
p-S6K1
70 kD
S6K1
70 kD
p-S6
32 kD
S6
32 kD
GAPDH
37 kD
RagB
40 kD


A
CTX injury
cell transplantation
tissue collection
Day:
-7
-1
0
7
14
21
5% Glycine i.v. administration every other day
B
C57
mdx
saline
glycine
C
TA muscle weight (mg)
mdx
saline
glycine
D
saline
glycine
Number of dys+ fibers
saline
glycine
Number of dys+ fibers
saline
glycine
E
saline
untreated
glycine
x10^8
MFI (X10^7)
untreated
saline
glycine
F
dystrophin
GFP
DAPI
merged
saline
glycine
Number of dys+ fibers
saline
glycine