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14. ABSTRACT

In Duchenne muscular dystrophy (DMD), loss-of-function mutations in the gene encoding dystrophin trigger instability of the plasma membrane in skeletal muscle, causing membrane damage during muscle contraction. This leads to progressive muscle weakness and dramatic muscle degeneration that results in early mortality in affected teenagers. Most of the patients die from respiratory failure and heart failure. A primary strategy in treating DMD is to reverse the instability of muscle membranes by increasing dystrophin levels. However, there is a major obstacle in that the dystrophin gene is too large to be packaged into current gene therapy vectors. Lipin1 is a phosphatidic acid (PA) phosphatase (PAP) that catalyzes the conversion of PA to diacylglycerol, a critical step in the synthesis of glycerophospholipids. We found that lipin1 overexpression attenuated inflammation infiltration and fibrosis in both gastrocnemius and diaphragm of mdx mice. It also strengthened membrane integrity, and resulted in markedly improved muscle contractile and eccentric force in gastrocnemius of mdx:lipin1 transgenic (mdx:lipin1^{Tg}) mice. These data suggest that lipin1 could be a potential therapeutic target for the treatment of DMD.

15. SUBJECT TERMS
None listed.

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1. Introduction

Duchenne muscular dystrophy (DMD) is a progressive and devastating muscle disease, resulting from the absence of dystrophin. This leads to cell membrane instability, susceptibility to contraction-induced muscle damage, muscle cell death, and disability and early death of patients. Currently, there is no cure for DMD. Lipin1 has dual functions acting as a phosphatidic acid (PA) phosphatase (PAP) enzyme that catalyzes diacylglycerol (DAG) biosynthesis and as a transcriptional coactivator/corepressor for metabolic nuclear receptors. Our recent publication reveals that lipin1 is critical for maintenance of membrane integrity and lipin1 deficiency alone leads to compromised muscle membrane integrity. As such, we **hypothesize** that lipin1 overexpression in mdx mice is effective in suppressing the dystrophic muscle phenotype and leads to a functional improvement in skeletal muscle. The **goal** of aim 1 in our State of Work (SOW) is to assess the pathological and functional benefit of lipin1 overexpression in mdx:lipin1 transgenic mice, which is the main task for the first-year period.

2. Keywords

Duchenne muscular dystrophy, lipin1, phosphatidic phosphatase, diacylglycerol, membrane integrity, muscle damage

3. Accomplishments

Major goals: In the approved SOW, the **major goals** of this project for the year 1 is to assess the pathological and functional benefit of lipin1 overexpression in mdx:lipin1 transgenic mice. We have completed (100%) experiments proposed in the Aim 1 by Aug 30, 2022. In addition, based on some of the data we generated, we submitted a manuscript to *Journal of Physiology* on Sep 5, 2022. Currently, we are working on another manuscript and hope to send it out within a month.

Major accomplishments:

Task 1 (Aim 1) is to assess the pathological and functional benefit of lipin1 overexpression in mdx:lipin1 transgenic mice.

Subtask 1: Determine the effect of lipin1 overexpression on dystrophic muscle morphology, membrane integrity and the suppression of necroptosis

In this subtask**,** we investigated the effect of lipin1 overexpression on dystrophic muscle morphology, membrane integrity, and the suppression of necroptosis in 6-month-old mdx:lipin1 transgenic (mdx:lipin1^{Tg}) mice. Age and gender-matched WT B10 mice and mdx mice with no lipin1 transgene expression (mdx:lipin1^{Stop} littermates, indicated as mdx in this annual report) were used as control groups.

As shown in Fig.1, we found that overexpression of lipin1 in gastrocnemius muscle of mdx:lipin1^{Tg} led to reduced inflammation infiltration and improved muscle morphology compared to mdx mice (Fig 1A). The widespread central nucleation of skeletal muscle cells are the main features of skeletal muscle pathology in DMD and is an indication of continuous cycles of muscle fiber death and regeneration. The number of muscle fibers with central nuclei expressed as the percentage of total number of myofibers was significantly reduced in gastrocnemius muscle of mdx:lipin1^{Tg} compared to mdx mice (Fig 1B). As shown in Fig 1C, the average cross-sectional area was significantly increased in gastrocnemius muscle of mdx:lipin1^{Tg} compared to mdx mice suggesting an overall improved muscle morphology and condition due to lipin1 overexpression.

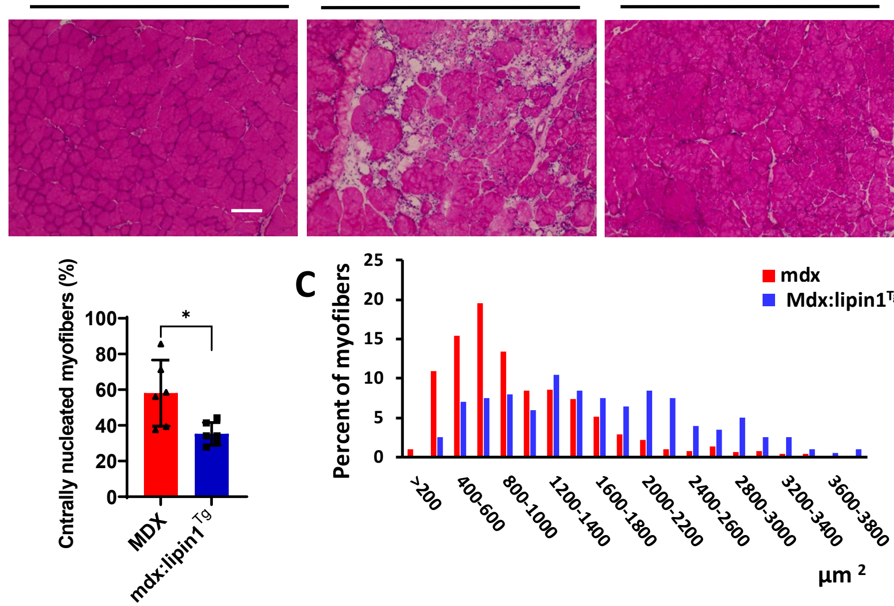

Fig 1 Mdx:lipin1 transgenic mice had improved gastrocnemius muscle morphology. (A) H&E staining of gastrocnemius muscle of 6-month-old B10, MDX and Mdx:lipin1 transgenic mice. **(B)** Quantification analysis of centrally nucleated myofibers and **(C)** the distribution of myofiber sizes in gastrocnemius muscle of 6-month-old MDX and Mdx:lipin1 transgenic mice. Scale bar = 200µm.

Patients with DMD mainly die from respiratory failure. The diaphragm is the main respiratory muscle responsible for normal ventilatory behaviors. Therefore, we also evaluated whether lipin1 restoration could inhibit diaphragm pathology development. As shown in Fig 2, the diaphragm of mdx mice had significantly elevated inflammation and necrosis. Consistent with what we observed in gastrocnemius muscle, overexpression of lipin1 in diaphragm of mdx:lipin1^{Tg} mice exhibited reduced inflammation infiltration and necrosis. In addition, comparing the cross-sectional areas of the muscle cells from WT mice, mdx and mdx:lipin1^{Tg} mouse diaphragms revealed that the mdx diaphragm was characterized by an abundance of small muscle fibers, while overexpression of lipin1 in the mdx:lipin1^{Tg} attenuated the decrease in muscle fiber size of mdx mice.

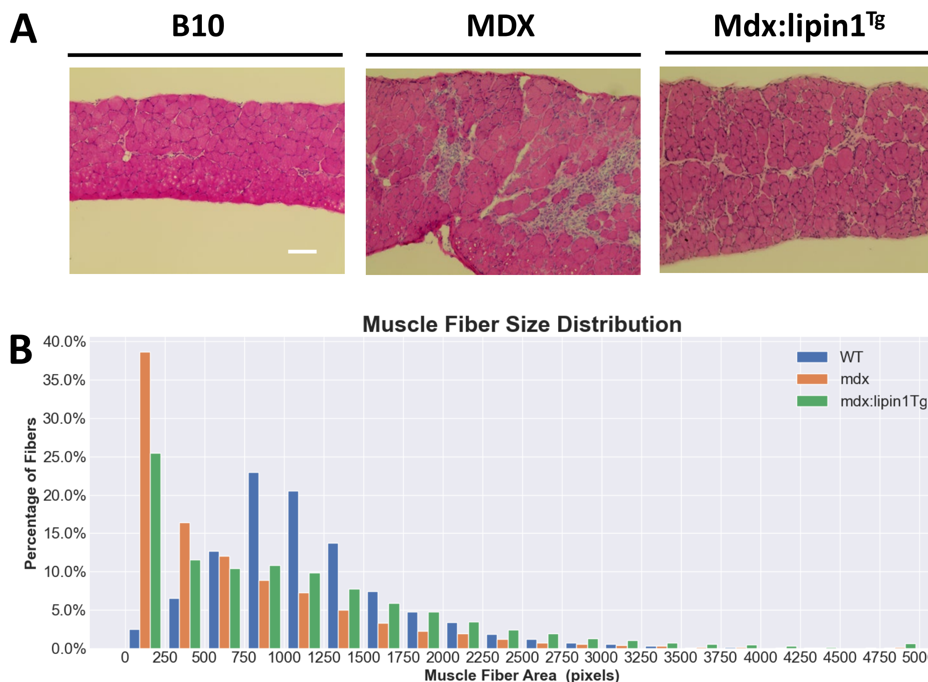

Fig 2. Overexpression of lipin1 improved muscle morphology and increased muscle fiber size in diaphragm of mdx:lipin1^{Tg} mice compared to mdx mice. (A) H&E staining and **(B)** the distribution of myofiber sizes in diaphragm muscle of 6-month-old B10, MDX and Mdx:lipin1 transgenic mice. (N = 5 mice/group). Scale bar = 200µm.

Fibrosis is a hallmark of DMD, leading to muscle wasting and impaired muscle functions. We evaluated muscle fibrosis by Picrosirius red staining in B10, mdx, and mdx:lipin1^{Tg} mice (Fig. 3A and 3B). Picrosirius red staining revealed a significantly increased fibrosis in gastrocnemius of 6-month-old mdx mice. Most importantly, collagen deposition was substantially reduced in age-matched mdx:lipin1^{Tg} mice. These data suggest that lipin1 overexpression in dystrophic gastrocnemius muscles clearly exhibited suppression of histological lesions, including centrally-nucleated fiber, fibrosis and inflammation infiltration.

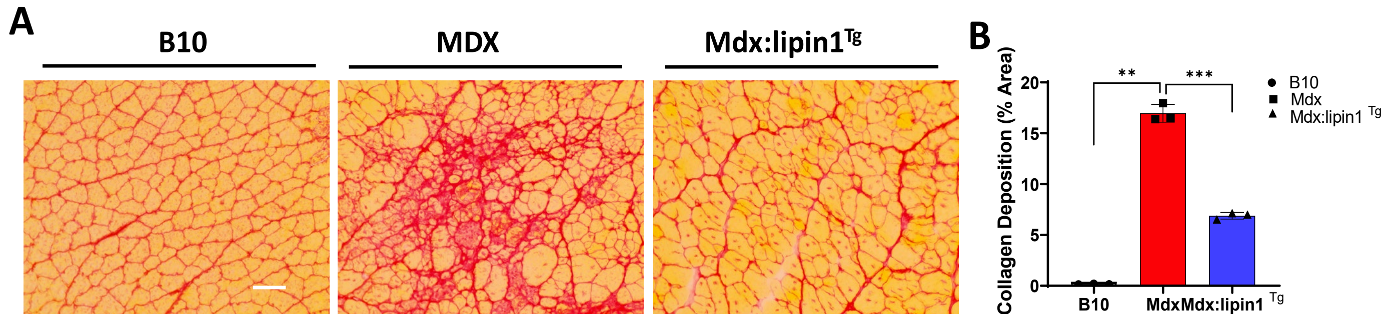

Fig 3 Gastrocnemius of mdx:lipin1 transgenic mice had less fibrosis compared to mdx mice. Picro Sirius red staining **(A)** and quantification analysis of collagen deposition **(B)** of gastrocnemius muscle of 6-month-old B10, MDX and Mdx:lipin1^{Tg} mice. Scale bar = 200μm.

Diaphragm fibrosis is associated with increased muscle stiffness, and prevents the diaphragm from achieving the excursion lengths required for respiratory. We also evaluated the collagen deposition in the diaphragm of mdx:lipin1^{Tg} mice (Fig 4). Collagen deposition was substantially attenuated by 1.3-fold in transgenic mice compared to mdx mice.

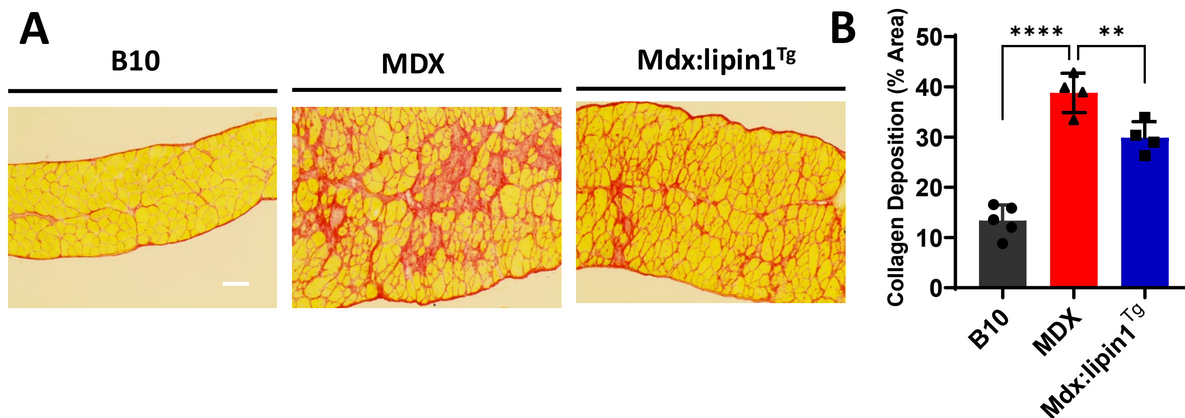

Fig 4 Overexpression of lipin1 reduced collagen deposition in diaphragm of mdx:lipin1^{Tg} mice compared to mdx mice. Picro Sirius red staining **(A)** and quantification analysis of collagen deposition **(B)** in diaphragm muscle of 6-month-old B10, MDX and Mdx:lipin1 transgenic mice. (N = 4-5 mice/group). Scale bar = 200μm.

Embryonic myosin heavy chain (eMyHC) was activated in response to muscle injury. Immunofluorescence staining for laminin and eMyHC in the diaphragms of B10 WT, mdx, and mdx:lipin1^{Tg} mice revealed an apparent reduction of eMyHC positive fibers in the mdx:lipin1^{Tg} mice, compared to the mdx mice (Fig 5A and 5B). Quantification analysis showed that approximately 16% of the muscle fibers in the mdx diaphragm stained positive for eMyHC, while less than 5% of muscle fibers in the mdx:lipin1^{Tg} diaphragm stained positive for eMyHC (Fig 5B).

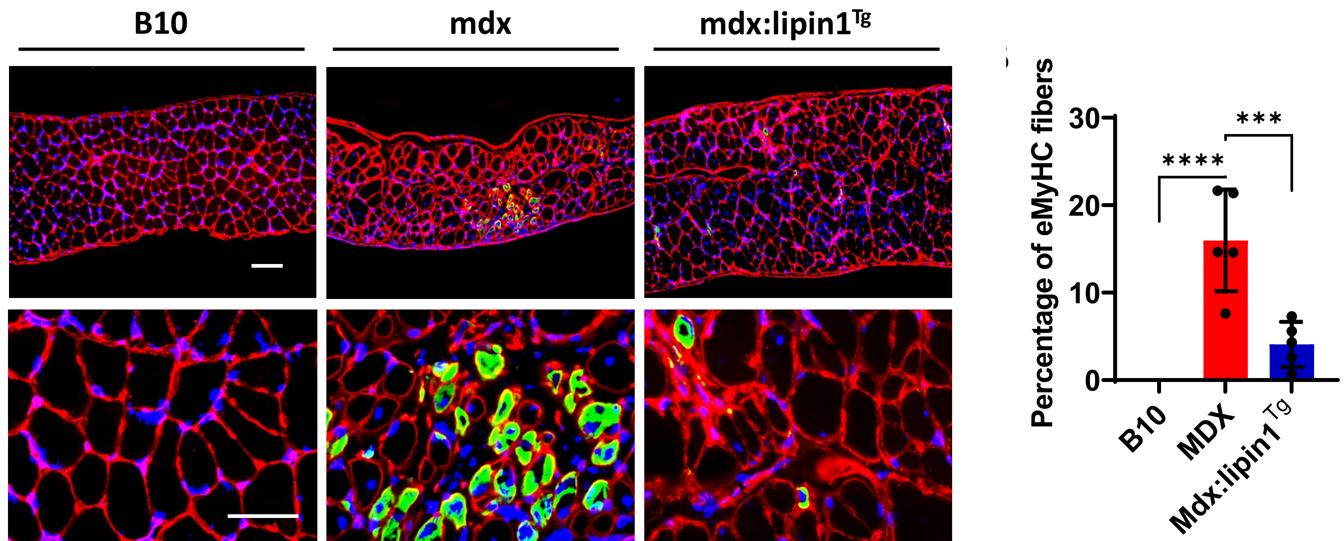

Fig 5 Overexpression of lipin1 reduced newly formed muscle fibers in diaphragm of mdx:lipin1^{Tg} mice compared to mdx mice. (A) eMyHC staining and **(B)** quantification analysis of newly formed muscle fibers in diaphragm muscle of 6-month-old B10, MDX and Mdx:lipin1 transgenic mice. (N = 5 mice/group).

Our recent publication suggested that lipin1 deficiency alone led to a compromise in muscle membrane integrity and function in skeletal muscle-specific lipin1 deficient lipin1^{Myf5cKO} mice[20]. We also evaluated whether lipin1 restoration could reduce membrane damage in dystrophic muscle. EBD, a membrane-permeable marker, has been widely used in an *in vivo* tracer technique which detect membrane damage of skeletal muscle fibers[31, 32]. B10 control mice did not show dye uptake into their skeletal muscles by visual inspection. In mdx mice, we found that gastrocnemius muscles of mdx mice had 7.4% EBD-positive muscle fibers which presumably underwent a stage of segmental necrosis and regeneration (Fig 6A and 6B). The area percentage of EBD uptake in Mdx:lipin1^{Tg} mice was dramatically reduced to 3.37% (n = 5 mice/group). These data show that lipin1 plays a critical and complementary role to dystrophin in maintaining membrane integrity and that overexpression of lipin1 leads to an ameliorated disease pathology.

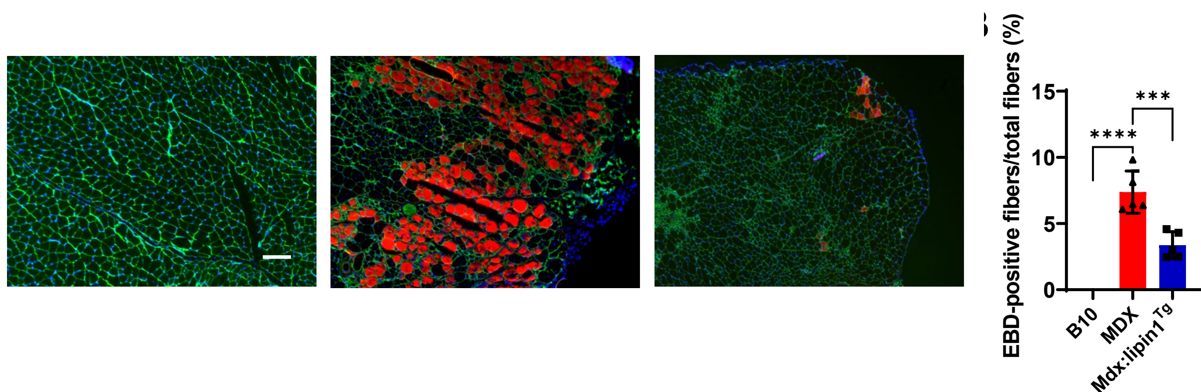

Fig 6 Lipin1 plays a critical and complementary role to dystrophin in maintaining membrane integrity. (A) EBD uptake of gastrocnemius muscle sections from 6-month-old B10, MDX and Mdx:lipin1 transgenic mice. Scar bar = 200um. **(B)** Quantification analysis of EBD-positive muscle fiber expressed as the percentage of the total number of muscle fibers in each mouse. N = 5 mice/group. *p<0.05.

To evaluate how overexpression of lipin1 impacts dystrophic pathology, we measured the changes in the expression levels of proteins involved in necroptosis, apoptosis and inflammation in gastrocnemius muscle of B10, MDX and Mdx:lipin1^{Tg} mice by Western blotting (Fig. 7). Upregulation of necroptotic markers is an indication of enhanced necroptosis. As shown in Fig. 7, mdx muscle had 7.7-fold, 7-fold, 5-fold and 2-fold increase in the protein expression levels of RIPK1, RIPK3, MLKL and p-MLKL, respectively. In the gastrocnemius muscle of mdx:lipin1^{Tg} mice, RIPK1 was significantly reduced by 316%, MLKL reduced by 190% and p-MLKL decreased by 132% compared to mdx mice. The protein expression levels of apoptotic marker, cleaved caspase 3, was reduced by 196% in mdx:lipin1^{Tg} mice compared to mdx mice suggesting that overexpression of lipin1 in dystrophic muscle suppressed both apoptosis and necroptosis which are the major contributors for the pathology of mdx mice.

DMD is characterized by chronic inflammation. NF-κB transcription factor is the master regulator of the inflammatory response. We observed increased phosphorylated NF-kB at serine 468 (3.9-fold) and serine 536 (4.6-fold) residues, indicating that NF-kB were activated in mdx mice. Most importantly, p-NFkB serine 468 and p-NFkB were reduced by 226% and 177%, respectively, in mdx:lipin1^{Tg} muscles compared to mdx muscles, suggesting lipin1 overexpression prevented deleterious inflammation. Taken together, these results suggest there was an reduced necroptosis, inflammation and fibrosis in gastrocnemius of mdx:lipin1^{Tg} mice compared to mdx:lipin1^{Stop} mice which could be due to inhibition of muscle membrane damage.

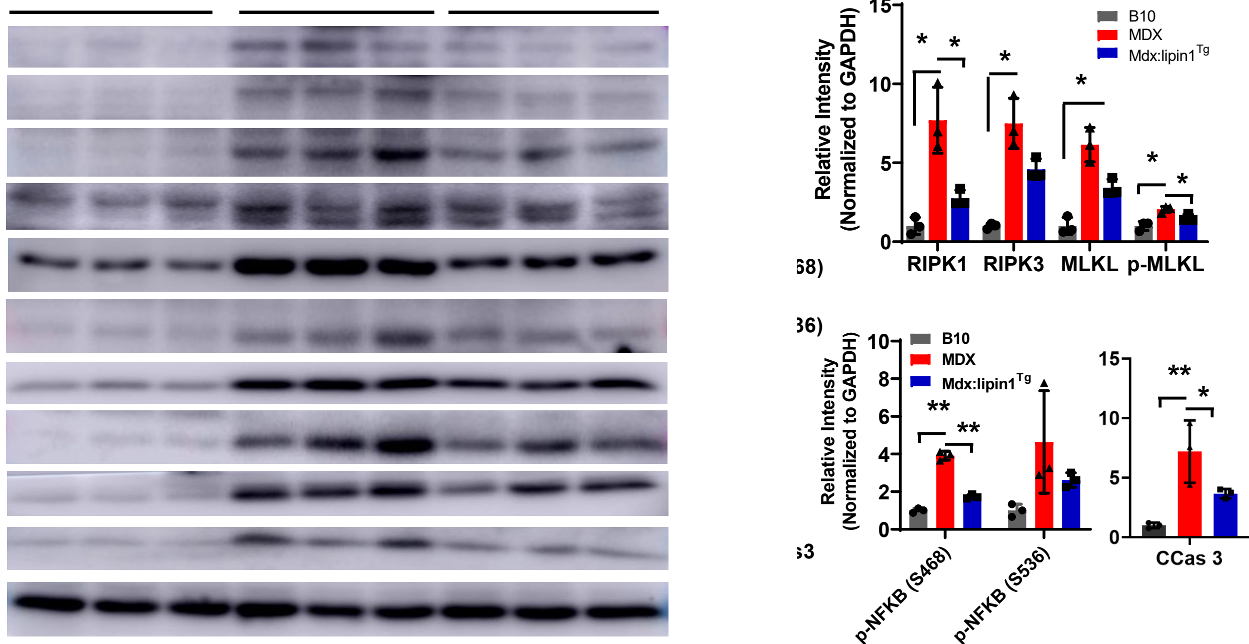

Fig 7 Overexpression of lipin1 in dystrophic gastrocnemius muscle leads to suppressed expression levels of necroptotic, apoptotic, and inflammation markers in gastrocnemius. (A) Representative immunoblots and **(B)** quantitative analysis of necroptotic (RIPK1, RIPK3, MLKL and p-MLKL), apoptotic (BAX, BAK and Bid), and inflammation (p-NFKB S468 and S536) markers in gastrocnemius muscles of indicated mice. GAPDH was used as the loading control; protein expression was normalized to GAPDH. (N = 3 mice/group. *p < 0.05; **p < 0.01.)

Subtask 2: Define the effect of lipin1 overexpression on eccentric force

Through collaboration with Dr. Andrew Voss, we found that overexpression of lipin1 improved contractile force in the mdx:lipin1^{Tg} compared to mdx mice. Further, we assessed muscle function by measuring eccentric force production in B10 control mice, mdx, and mdx:lipin1^{Tg} mice (Fig. 8). During the plateau of an isometric tetanic contraction (100 Hz stimulation), the plantar flexor muscles was stretched at 40 mm/s for 200 ms. This protocol was repeated 15 times with 20s rest periods between protocols. Stimulus amplitude and pulse width was ≥5 V at 1 ms. The muscle force output was normalized by muscle weight. As shown in Fig. 8, We found that membrane damage in mdx dystrophic muscle caused by the lengthening contractions leads to a reduction in isometric force. Most importantly, overexpression of lipin1 in mdx:lipin1^{Tg} mice exhibited significantly improved isometric force.

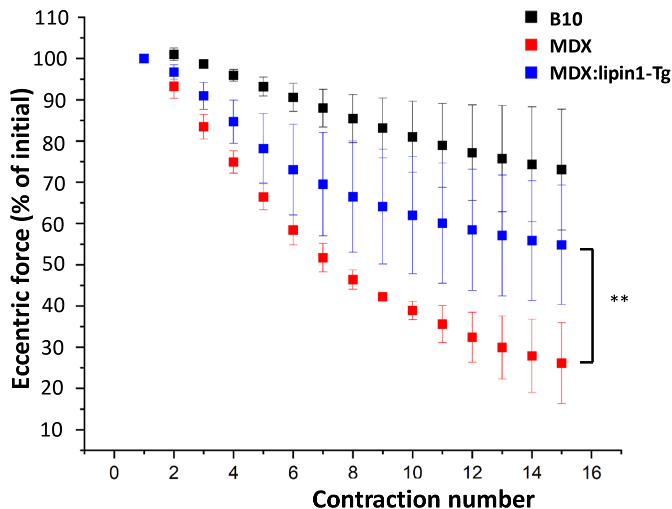

Fig 8. Overexpression of lipin1 improved eccentric force production in Mdx:lipin1^{Tg} mice (blue) compared to mdx mice (red).

Subtask 3: Evaluate motor function by the limb hanging wire test, grip force test, and treadmill

Grip strength test has been widely used method to assess skeletal muscle performance in animal. In this study, we assessed the grip strength of the forelimb of B10, mdx and mdx:lipin1^{Tg} mice (Fig 9A). For forelimb grip test, the mouse was made to grasp the grid tightly with both forepaws. The force transducer recorded the maximal force required to break the mouse's grip from the mesh surface. We found that mdx mice had reduced grip force by 41% compared to B10 WT mice. Most importantly, the grip force production was increased in mdx:lipin1^{Tg} mice by 2.2 fold.

Forced treadmill running was assessed to evaluate systemic muscle function (Fig 9B). B10, mdx, mdx:lipin1^{Tg} mice were placed on a treadmill to run with a downward inclination of 15°, at 5 m/min for 5 min, 10 m/min for 5 min, 15 m/min for 5 min, 20 m/min until exhaustion. The mouse was encouraged to run by using a mild electric shock grid at the end of the treadmill (0.2 mA, pulse 200 ms, 1 Hz). The experimental mouse was considered to be exhausted after their refusal to remain on the treadmill belt for more than 5 s. As shown in Fig 10, the average exhaustion time for mdx mice was reduced by 3.2 fold compared to B10 WT mice, and was improved by 231% in mdx:lipin1^{Tg} mice suggesting that overexpression of lipin1 could provide functional improvement by increasing running capability of dystrophic muscle.

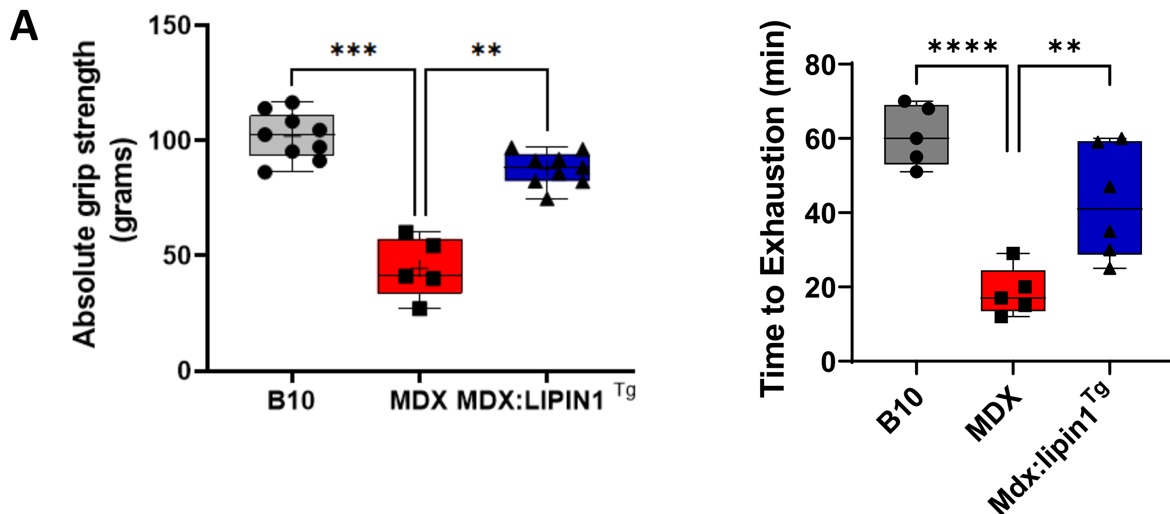

Fig 9 Overexpression of lipin1 improved grip force and running capability in Mdx:lipin1^{Tg} mice (blue) compared to mdx mice (red) by downhill treadmill.

Major Task 2: Determine the therapeutic efficacy of AAV-mediated systemic lipin1 gene delivery.
Subtask 1: Determine the optimal dosing regimen by evaluating safety, specificity and transduction efficiency in mdx mice using a dose-escalation study.

Currently, we collaborate with Dr. Scott Harper to generate lipin1-AAV virus which will be used to determine the therapeutic efficiency of AAV-mediated systemic lipin1 gene delivery. We expect to receive the virus soon.

Overall, these results suggest that overexpression of lipin1 in dystrophic skeletal muscle inhibited inflammation infiltration and necrosis, strengthened membrane integrity, and led to markedly improved muscle eccentric force in gastrocnemius of mdx:lipin1 transgenic mice. Consistently, lipin1 overexpression also suppressed inflammation, necroptosis and fibrosis in the diaphragm of mdx:lipin1 transgenic mice. These data suggest that lipin1 could be a potential therapeutic target for the treatment of DMD.

4. Impact

Our findings suggest that lipin1 is a novel regulator to prevent dystrophic pathologies. Importantly, it is expected to be useful as a novel therapeutic target for the treatment of DMD as well as related disorders. DMD is a devastating muscle disorder and incurable disease caused by mutations in the gene that encodes the 427kDa cytoskeletal protein dystrophin. In DMD, loss-of-function mutations in the gene encoding dystrophin trigger instability of the plasma membrane in skeletal muscle, causing membrane damage during muscle contraction. This leads to progressive muscle weakness and dramatic muscle degeneration that results in early mortality in affected teenagers. Ideally, targeting the primary defect aiming to restore dystrophin could be a primary strategy for the treatment of DMD. However, the dystrophin gene is the largest gene in the mammalian genome and cannot be packaged into the current viral gene delivery vectors. Currently, there is no effective curative treatment for DMD. The current gene delivery techniques, including exon skipping, are only applicable to a subset of DMD patients with corresponding targeted mutation. Glucocorticosteroids are used to slow disease progression by facilitating the maintenance of muscle strength longer; however, they have serious side effects. Cardiomyopathy is addressed with the use of angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers, and/or beta blockers. Unfortunately, these treatments are only temporarily effective. An alternative or complementary target is ***urgently needed*** to improve cardiac muscle stability and integrity so that it can withstand structural stress in the absence of dystrophin.

Our data suggest that increasing the expression level of lipin1 lessened skeletal muscle and diaphragm muscle degeneration and fibrosis, and strengthened membrane integrity and present promising as a complementary therapy for older DMD patients. Our study is the first to investigate the role of lipin1 in maintaining myofiber stability and integrity, especially in the context of dystrophic muscle. Lipin1 gene delivery has the potential to offer treatment to all DMD patients irrespective of their dystrophin mutation. It could be an effective therapeutic target to preserve and improve the function and quality of life, and to extend the life span of military beneficiaries, and/or the American civilian who suffer from muscular dystrophy.

5. Changes/Problems

Changes in approach and reasons for change

There is no change in approach.

Actual or anticipated problems or delays and actions or plans to resolve them

The project has been going very well. We have completed the Aim 1, and have submitted one manuscript and currently working on another manuscript. We do not anticipate problems at this stage.

Changes that had a significant impact on expenditures

Due to travel restriction last year, we haven't been able to travel to attend conference. Since the COVID situation is getting better, and the travel restrictions have been eased, I plan to attend MDA meeting in 2023.

Due to pandemic COVID, the hiring process for a research assistant position was delayed. Alexandra Brown was a graduate student and has been working on Aim 1 for this project. She was graduated in April 2022, and has been hired since September 6th, 2022 as a Research Associate to continue working on Aim 2 for this project.

6. Products

Publications, conference papers, and presentations

Manuscript in preparation or submitted

1. Abdulrahman Jama, Abdullah A. Alshudukhi, Steve Burke, Lixin Dong, John Karanja Kamau, Andrew Alvin Voss, **Hongmei Ren**. Lipin1 plays complementary roles in myofiber stability and regeneration in dystrophic muscles. (2022) Journal of Physiology. submitted.

2. Alexandra Sue Brown, Ryan Joseph Rakoczy, Christopher Wyatt, Brian Fink, **Hongmei Ren**. Lipin1 restoration improved diaphragm muscle morphology and respiratory function in mdx mice. in preparation.

Invited Talks

Lipin1 is a potential therapeutic target for Duchenne Muscular Dystrophy. (2021) Boonshoft School of Medicine Dean's town Hall meeting. Presenter: **Hongmei Ren**

Exploring the role of lipin1 in dystrophic pathology and muscle function. (2022) Department of Pharmacology and Toxicology, Wright State University. Presenter: **Hongmei Ren**

Examine how lipin1 ameliorates dystrophic phenotype using mdx:lipin1 transgenic mice. (2022) Brown bag seminar, BMB department, Wright State University. Presenter: **Jama Abdulrahman**

Examine the role of lipin1 in dystrophic muscle using mdx mice. (2022) Biomedical Sciences PhD program Student Seminar**.** Presenter: **Jama Abdulrahman**

Conferences and meeting

Due to COVID-19 pandemic, there was a travel restriction at Wright State University. I will attend the following conference in 2023:

2023 MDA Clinical & Scientific Conference

But we actively attended several meetings:

Poster presentation:

Jama A, Alshudukhi A, Voss A, **Ren H.** The role of lipin1 in skeletal muscle of mdx mice. (2022) Annual BSOM Research Symposium, Wright State University.

Brown A, Rakoczy RJ, Wyatt CN, **Ren H.** Effects of lipin1 deficiency and overexpression in the dystrophic diaphragm. (2022) Annual BSOM Research Symposium, Wright State University.

Jama A, **Ren H.** The role of lipin1 in skeletal muscle of mdx mice. (2021) COSM Festival of Research, Wright State University.

7. Participants & Other Collaborating Organizations

There will be no change in PI and co-I for this grant.

PI: Hongmei Ren (3 months effort)

Co-I: Andrew Voss (1 month effort)

Research assistant: Steve Burke (2-month effort)

Research assistant: Alexandra Brown (12-month effort)

This project is currently undertaken by our research team of graduate students and research assistants. Students have been receiving stipend/ tuition fees from by college or department or other programs. Therefore, they were not included in the grant budget. Studies proposed in Aim 1 was carried out by two graduate students, Jama Abdulrahman and Alexandra Brown. Alexandra was graduated in April 2022. She has been hired as a Research Associate to continue working on Aim 2 for this project. In addition, research assistant, Steve Burke, generated force production data of mdx:lipin1 transgenic mice proposed in Aim 1c.

8. Special Reporting Requirements

N/A

9. Appendices

N/A