Reducing sarcolipin expression mitigates Duchenne muscular dystrophy and associated cardiomyopathy in mice

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Sarcolipin (SLN) is an inhibitor of the sarco/endoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA) and is abnormally elevated in the muscle of Duchenne muscular dystrophy (DMD) patients and animal models. Here we show that reducing SLN levels ameliorates dystrophic pathology in the severe dystrophin/utrophin double mutant (mdx:utr−/−) mouse model of DMD. Germline inactivation of one allele of the SLN gene normalizes SLN expression, restores SERCA function, mitigates skeletal muscle and cardiac pathology, improves muscle regeneration, and extends the lifespan. To translate our findings into a therapeutic strategy, we knock down SLN expression in 1-month old mdx:utr−/− mice via adeno-associated virus (AAV) 9-mediated RNA interference. The AAV treatment markedly reduces SLN expression, attenuates muscle pathology and improves diaphragm, skeletal muscle and cardiac function. Taken together, our findings suggest that SLN reduction is a promising therapeutic approach for DMD.
uchenne muscular dystrophy (DMD), the most common childhood, severe form of muscular dystrophy, is an X-linked disease caused by deficiency of dystrophin protein in muscle. DMD patients experience gradual muscle weakness in early childhood and are non-ambulant by 12 years of age. The progressive nature of this disease leads to restrictive pulmonary function, diaphragm dysfunction and cardiomyopathy for which there is no effective treatment. The current therapeutic strategies aimed to either replace or compensate for the lack of dystrophin also face major challenges such as targeting cardiac and respiratory tissues and fibrosis. Therefore, identification of new therapeutic targets based on disease mechanism can complement the existing strategies and enhance the effectiveness of treatment for this lethal disease.

Accumulating evidence suggests that abnormal elevation of intracellular Ca^{2+} (Ca^{2+}) is an important, early pathogenic event that initiates and perpetuates disease progression in DMD. Among the several mechanisms that cause Ca^{2+} overload, decreased sarco/endoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA) activity has been considered as a primary cause of Ca^{2+}i overload in DMD. However, the rate of Ca^{2+} dependent Ca^{2+} uptake was not significantly different between the non-DMD and DMD dog tissues indicating that the Ca^{2+} affinity of the SERCA pump was not altered. Similar to animal models, SLN levels were increased both in the quadriceps (Supplementary Fig. 3a, b) and in the ventricles (Supplementary Fig. 3c, d) of DMD patients. These findings revealed SLN upregulation as a common molecular change in dystrophin-deficient skeletal and cardiac muscles of both DMD patients and DMD models.

### Results

**SLN is upregulated in cardiac and skeletal muscles of DMD.**

We recently reported that SLN is abnormally high in the diaphragm and slow- and fast-twitch skeletal muscles of dystrophin mutant (mdx) and dystrophin/utrophin double mutant (mdx:utr) mouse models of DMD. Nevertheless, it is not clear whether SLN upregulation underlies SERCA dysfunction and contributes to the muscle pathogenesis in DMD. In the present study, we sought to determine the physiological relevance of SLN upregulation in DMD following loss-of-function approach by germline gene deletion and by adeno-associated virus (AAV) mediated gene silencing in mdx:utr−/− mice. Our results show that reduction in SLN expression is sufficient to improve the SERCA function and ameliorate the features of muscular dystrophy and cardiomyopathy in mdx:utr−/− mice. Moreover, reducing SLN expression extends the lifespan of mdx:utr−/− mice. These findings provide the first direct evidence on the critical role of SLN upregulation in DMD pathogenesis and identified SLN as a new potential therapeutic target for the treatment of DMD.

**Ablation of SLN extends the lifespan of mdx:utr−/− mice.**

To determine the role of SLN upregulation in DMD, we generated SLN haploinsufficient mdx:utr−/− knockout (mdx:utr−/−:sln+/−) and SLN deficient mdx:utr−/− (mdx:utr−/−:sln−/−) triple knockout (tKO) mice. The mdx:utr−/−:sln+/− and tKO pups were delivered normally. The body weight of mdx:utr−/−:sln+/− and tKO mice were normalized (Fig. 1a) and their lifespan was significantly extended (Fig. 1b; p < 0.0001 by the nonparametric log-rank test). The median survival was increased by 446 and 358 days, respectively for mdx:utr−/−:sln+/− and tKO mice compared to that of mdx:utr−/− littermates (73 days). These findings suggest that SLN reduction or ablation markedly improves survival to mdx:utr−/− mice.

**Reducing SLN expression restores SERCA function in DMD.**

Deletion of one allele of the SLN gene improved the rate (Fig. 1c) and V_{max} (Fig. 1d) of SR Ca^{2+} uptake in the diaphragm of mdx:utr−/− mice close to that of WT mice. Complete loss of SLN resulted in a further increase in the rate of Ca^{2+} uptake in the tKO diaphragm; however, the V_{max} of Ca^{2+} uptake was not statistically different from the WT controls (Fig. 1d). Furthermore, the EC_{50} for Ca^{2+} activation was significantly decreased in the diaphragm of both mdx:utr−/−:sln+/− and tKO mice (Fig. 1e), indicating an increase in the apparent affinity of SERCA pump for Ca^{2+} ions in SLN deficient dystrophic muscles. Together, these findings suggest that SLN reduction or ablation markedly improves survival to mdx:utr−/− mice.

**Reducing SLN expression ameliorates muscle pathophysiology.**

We next determined whether the improvement in Ca^{2+} cycling via enhanced SERCA function reduced the Ca^{2+} dependent protease activity and prevented muscle damage. Reduction or ablation of SLN expression attenuated the calpain activity in pectoral muscle, a representative and severely affected dystrophic muscle (Fig. 2a). Histopathological analysis (Fig. 2b) and
Fig. 1 Reduction in SLN expression prolongs the lifespan of mdxutr−/− mice. a The body weight of mdxutr−/−; sln+/− and tKO mice in comparison to that of age- and sex- matched WT and mdxutr−/− control mice. We used 3–4 month old mice for this experiment. The n number for each group is shown within the bar. Data are presented as mean ± SEM. **significantly different from other groups (p < 0.005, t-test with Welch’s correction). b Kaplan–Meier survival curves indicate that the mdxutr−/−; sln+/− and tKO mice has increased lifespan in comparison to that of mdxutr−/− controls as determined by the nonparametric log-rank test; p < 0.0001. c Increased Ca2+ dependent SR Ca2+ uptake, d increased Vmax of Ca2+ uptake and e decreased EC50 values in the diaphragm of mdxutr−/−; sln+/− and tKO mice indicates improved SERCA function. The n number for each group is shown within the bar. Data are presented as mean ± SEM. **significantly different from other groups (p < 0.005, t-test with Welch’s correction). f–h Representative western blot analysis and quantitation of SLN, SERCA1, SERCA2a and CSQ proteins in the diaphragm, pectoral and quadriceps muscles respectively. Tissues are from 3 to 4 month old mice. Uncropped scans of western blots are shown in Supplementary Fig. 8a–c. Data are represented as mean ± SEM. The n number for each group is shown within the bar diagram. *significantly different from other groups, p < 0.05, t-test with Welch’s correction.
quantitation show significant reduction in necrosis and fibrosis in both diaphragm (Fig. 2c, d) and quadriceps (Fig. 2e, f) of mdx:utr−/−:sln+/− mice and in the quadriceps of tKO mice. These improvements were less prominent in the tKO diaphragm and were not statistically different from the mdx:utr−/− controls. We next determined the muscle fiber size in quadriceps by measuring the minimal Feret’s diameter variance coefficient (VC) following wheat germ agglutinin (WGA) staining (Fig. 3a). The VC was significantly increased in the muscles of mdx:utr−/− mice indicating heterogeneity in fiber size. On the other hand, in the muscles of mdx:utr−/−:sln+/− and tKO mice, the VC was significantly reduced indicating reduction in small regenerating split fibers and hypertrophic fibers. These findings prompted us to determine whether SLN ablation has any effect on the muscle regeneration process as well as fiber-type transition. Immunostaining and quantitation showed that fibers expressing embryonic myosin heavy chain (eMyHC) or Type I MyHC were significantly higher in the pectoral muscles of mdx:utr−/− mice (Fig. 3b) and was consistent with our previous findings on the dystrophic quadriceps.  

In contrast, the number of fibers expressing these proteins were significantly decreased in the muscles of mdx:utr−/−:sln+/− and tKO mice (Fig. 3b). These findings suggest that reduction in SLN expression can improve the muscle regeneration process as well as prevent the fiber-type transition in dystrophic muscles.

We next investigated the effect of SLN ablation on muscle function. The forelimb muscle grip strength was significantly increased in the mdx:utr−/−:sln+/− and tKO mice compared to that of mdx:utr−/− littermates (Fig. 4a). We extended these studies by measuring the isometric contractile properties of the dystrophic extensor digitorum longus (EDL; a less severely affected) and diaphragm (a more severely affected) muscles. The twitch-tension at 2 Hz (Supplementary Fig. 4a, b) and force-frequency curves (Fig. 4d) were significantly increased in the EDL muscle of mdx:utr−/−:sln+/− mice. These contractile parameters were also increased in the EDL of tKO mice but not at a statistically significant level. The half-maximal force stimulation frequency for EDL remained unaltered among the experimental groups (WT = 27 ± 3 (n = 7), mdx:utr−/− = 28 ± 3 (n = 5), mdx:utr−/−:sln+/− = 26 ± 2 (n = 8) and tKO = 28 ± 1 (n = 5) Hz; unpaired t-test with Welch’s correction). The effect of SLN ablation on diaphragm function in the mdx:utr−/− mice was less prominent. The twitch-tension at 2 Hz was significantly increased in the hemidiaphragm of mdx:utr−/−:sln+/− but not in the tKO mice (Fig. 4e, f). The 10–90% rising slope and 90–10% decay slope of twitch-tension at 2 Hz showed a slightly increasing trend but were not significantly different from that of mdx:utr−/− controls (Supplementary Fig. 4c, d). Similarly, the force-frequency curves for the hemidiaphragm from mdx:utr−/−:sln+/− was shifted upwards but to a smaller extent in the tKO mice (Fig. 4g). The half-maximal force stimulation frequency for hemidiaphragm remained unaltered among all four mice groups (WT = 11 ± 0.3 (n = 6), mdx:utr−/− = 13 ± 0.8 (n = 5), mdx:utr−/−:sln+/− = 12 ± 0.5 (n = 6) and tKO = 14 ± 1.2 (n = 5) Hz; unpaired t-test with Welch’s correction). These functional data corroborated the differences and structural improvements seen in the diaphragm of mdx:utr−/−:sln+/− and tKO mice (Fig. 2b–f). These findings suggest that reduction in SLN expression is sufficient to improve the functional properties of dystrophic skeletal muscles.

Ablation of SLN ameliorates dystrophic cardiomyopathy. We next determined whether SLN ablation ameliorates cardiac pathology in the mdx:utr−/− mice. Western blot analysis (Supplementary Fig. 5a) and quantitation showed that loss of one SLN allele was sufficient to reduce SLN protein expression in atria (mdx:utr−/− = 1.7 ± 0.2 vs. mdx:utr−/−:sln+/− = 0.9 ± 0.1 fold; n = 4; p < 0.05, t-test with Welch’s correction) and in the ventricles of mdx:utr−/− mice near to WT levels. SLN ablation had no effect on the expression levels of SERCA2a, PLN, ryanodine receptor (RYR), and CSQ (Supplementary Fig. 5a). H&E and trichrome staining and quantitation showed that mononuclear infiltration and fibrosis were significantly reduced in the mdx:utr−/−:sln+/− and tKO ventricles compared to that of mdx:utr−/− controls (Fig. 5a). Cardiac function evaluated by echocardiography (Supplementary Fig. 5b) showed a marked improvement in left ventricular (LV) function as evident from the increased LV ejection fraction (EF; Fig. 5b) and fractional shortening (FS; Fig. 5c) in the mdx:utr−/−:sln+/− and tKO mice. There was an increase in interventricular septal end systole (IVSs) and posterior wall thickness along with significant reduction in LV internal diameter end diastole (LVIDd) in the mdx:utr−/−:sln+/− and tKO mice (Table 1). These findings suggested that hearts from these mice undergo specific concentric remodeling that contributes to the improved cardiac function. These findings indicate that normalizing SLN level is sufficient to preserve cardiac function and mitigate dystrophic cardiomyopathy in mice.

Postnatal AAV9 SLN shRNA gene therapy mitigates DMD. Findings from the above studies suggest that normalizing SLN expression is sufficient to mitigate the severe DMD phenotype including muscle pathophysiology, diaphragm function and cardiomyopathy. To translate these findings into a therapeutic strategy, we knocked down SLN expression postnatally in 1-month old mdx:utr−/− mice via AAV9 mediated expression of SLN specific short-hairpin RNA (shSLN). The AAV9.shSLN treatment for 12 weeks significantly reduced SLN expression in both skeletal muscle (0.22-fold vs. saline treated controls; p < 0.05) and LV (similar to WT) of mdx:utr−/− mice (Fig. 6a, b) and Supplementary Fig. 6a, b). In the mdx:utr−/− myocardium, AAV treatment had no effect on the protein expression of SERCA2a, PLN, CSQ and RYR (Fig. 6b). On the other hand, AAV treatment reduced SERCA2a (p < 0.05) and CSQ (p < 0.07) protein levels in the pectoral muscles of mdx:utr−/− mice (Fig. 6a and Supplementary Fig. 6a). These data are consistent with the findings on the mdx:utr−/−:sln+/− mice (Fig. 1g) and suggest that postnatal reduction in SLN expression can also restore the SLN in the mdx:utr−/− mice.

We next determined, whether AAV gene therapy mitigates cardiac and skeletal muscle pathophysiology in the mdx:utr−/− mice. The outcome of these studies mimics the data from the mdx:utr−/−:sln+/− mice. H&E staining (Supplementary Fig. 6c) and quantitation showed that the invasion of mononuclear cells and cell necrosis were significantly reduced both in the skeletal muscle (Fig. 6c) and in the ventricles (Fig. 6d) of AAV treated mdx:utr−/− mice. AAV treatment also improved the LV systolic function and cardiac remodeling (Fig. 6e and Table 2) in mdx:utr−/− mice. Forelimb muscle strength was significantly improved in AAV treated mdx:utr−/− mice (Fig. 6f). Moreover, the twitch-tension (Fig. 6g–h), 10–90% rising slope and 90–10% decay slope at 2 Hz and force–frequency relationships, (Supplementary Fig. 7a–f) were significantly increased in both EDL and hemidiaphragm of AAV treated groups indicating improved muscle mechanics. Altogether these findings suggest that AAV mediated postnatal reduction in SLN expression is sufficient to mitigate the severe muscular dystrophy and associated cardiomyopathy in mdx:utr−/− mice.
Fig. 2 Reduction in SLN expression ameliorates muscle pathology. a Calpain activity is restored to normal levels in the pectoral muscles of mdx:utr−/−:sln+/− and tKO mice. Data are presented as mean ± SEM (t-test with Welch’s correction) of five independent experiments performed in duplicates. The n number for each group is shown within the bar. b Representative H&E and Masson’s trichrome stained quadriceps and diaphragm muscles. Arrow indicates increased mononuclear infiltration (indicative of necrosis) and collagen (blue) accumulation (indicative of fibrosis) in mdx:utr−/− mice. Original magnification is ×20. Scale bar=100 μm. c–f Quantitation show that the necrotic and fibrotic areas were significantly reduced in both diaphragm and quadriceps of mdx:utr−/−:sln+/− mice and in the quadriceps of tKO mice in comparison to that of mdx:utr−/− controls. The n number for each group and the p values (t-test with Welch’s correction) are shown within the graph. Data are presented as mean ± SEM. Tissues from 3 to 4 month old mice are used for all the above experiments.
Discussion

We have previously shown that SLN protein levels are abnormally elevated in the diaphragm and slow- and fast-twitch skeletal muscles of mouse models of DMD. Here, we further show that SLN upregulation is a common molecular change in both skeletal muscle and heart in murine and canine DMD models and human patients. Furthermore, the SERCA function as measured by SR Ca\(^{2+}\) uptake is also significantly decreased in both skeletal and cardiac muscles of DMD models. These findings led to the hypothesis that increased SLN protein expression could chronically inhibit SERCA pump and cause sustained elevation of Ca\(^{2+}\), levels, which subsequently contribute for the activation of Ca\(^{2+}\) dependent proteases and tissue remodeling, and muscle pathogenesis in DMD. Accordingly, reducing SLN protein levels...
is anticipated to improve SERCA function and mitigate DMD. This hypothesis was tested following a loss of function approach in mdxutr<sup>−/−</sup>, a severe and lethal mouse model of DMD. As predicted, our findings suggest that reduction in SLN expression is sufficient to improve the SERCA function in dystrophic muscles. Furthermore, reduction in SLN protein expression ameliorated the severe muscular dystrophy phenotype, and extended the lifespan of mdxutr<sup>−/−</sup> mice.

SERCA function in muscle is regulated by three small molecular weight SR membrane proteins: PLN, SLN, and myoregulin.

**Fig. 4** Reduction in SLN expression improves skeletal muscle function in mdxutr<sup>−/−</sup> mice. a Forelimb strength measured using a grip strength meter shows improved muscle strength in the mdxutr<sup>−/−</sup>:sln<sup>+/−</sup> and tKO mice. We used 3-4 month old male and female mice for this study. The n number for each group is shown within the bar. Data are presented as mean ± SEM (t-test with Welch’s correction). # p < 0.0001 vs. mdxutr<sup>−/−</sup>; *p < 0.05 vs. mdxutr<sup>−/−</sup>:sln<sup>+/−</sup> and tKO mice. b, e Representative traces of twitch force at 2 Hz for EDL and hemidiaphragm respectively. c, f The maximum force generated by the EDL and hemidiaphragm at 2 Hz are significantly increased in the mdxutr<sup>−/−</sup>:sln<sup>+/−</sup> mice compared to that of mdxutr<sup>−/−</sup> mice. The n number for each group is shown within the bar. Data are presented as mean ± SEM. d, g Force-frequency curves indicating that force generated by EDL and hemidiaphragm in the mdxutr<sup>−/−</sup>:sln<sup>+/−</sup> mice are significantly increased at all frequencies. EDL and hemidiaphragm are from 3 to 4 month old mice.
In rodents, MLN is primarily expressed in skeletal muscles\textsuperscript{31, 32}; whereas SLN is predominantly expressed in the tongue followed by diaphragm and slow-skeletal muscles but not expressed in the fast-twitch skeletal muscles\textsuperscript{32, 33}. In contrast, in larger mammals SLN is expressed in all skeletal muscle tissues that have been evaluated\textsuperscript{33}. In the heart, PLN expression is high in the ventricles\textsuperscript{32, 33}, while SLN expression is very high in atria and very low in the ventricles of both rodents and larger mammals\textsuperscript{33}. Findings from the present study along with our earlier report demonstrate that in DMD, SLN but not PLN is elevated in the diaphragm, and slow- (soleus) and fast-twitch (quadriceps and pectoral muscle) skeletal muscles, as well in atria and in the ventricles. Our findings are also consistent with the microarray and semi-quantitative RT-PCR data, which show SLN mRNA upregulation in the medial gastrocnemius of \textit{mdx} mice\textsuperscript{34}. Altogether these findings suggest that SLN upregulation is a common molecular change in all skeletal muscle tissues and in the heart in DMD.

In this study, we did not investigate whether MLN expression is altered and it has a role in SERCA function in DMD. We were

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**Fig. 5** Ablation of SLN expression prevents cardiomyopathy in \textit{mdx:utr}−/− mice. All cardiac studies are performed in 3–4 month old mice.\textbf{a} Representative H&E and Masson’s trichrome stained ventricular tissue sections. Original magnification is ×10. Scale bar=100 μm. Bar graph indicate that reduction or ablation of SLN significantly reduced fibrosis and necrosis in the \textit{mdx:utr}−/− hearts. The n number for each group and the p values (t-test with Welch’s correction) are shown within the graph. Data are presented as mean ± SEM.\textbf{b, c} Echocardiographic measurements demonstrate that the LV ejection fraction (EF) and fractional shortening (FS) are restored to normal values in the \textit{mdx:utr}−/−;\textit{sln}+/− and tKO mice. **p < 0.0001 vs. other groups; n = 6 per group. Data are presented as mean ± SEM (t-test with Welch’s correction).
also unable to demonstrate the absolute stoichiometry of SLN/ SERCA, and its relation to SR Ca\(^{2+}\) uptake in various dystrophic muscles. These studies are necessary to validate the direct role of SLN in SERCA function in various dystrophic muscles. Nevertheless, the current study suggests that SLN upregulation could be a major cause of SERCA dysfunction and elevation of Ca\(^{2+}\), in DMD. The increased \(V_{\text{max}}\) of Ca\(^{2+}\) uptake and the increased apparent binding affinity of SERCA pump for Ca\(^{2+}\) (as shown by decreased \(EC_{50}\)) in the \(mdx:\text{utr}^-\text{sln}^-\) and tKO muscles supports this view. The possible indirect effect of improved Ca\(^{2+}\) cycling, including reduced Ca\(^{2+}\) dependent protease activity and decreased fibrosis and necrosis in mice of \(mdx:\text{utr}^-\text{sln}^+\) and tKO mice further supports our hypothesis.

Studies from Stariyasm's laboratory have demonstrated that uncoupling of SERCA by SLN is an important regulator of mitochondrial function in various dystrophic muscles. Nevertheless, the current study suggests that SLN upregulation could be a major cause of SERCA dysfunction and elevation of Ca\(^{2+}\), in DMD. The increased \(V_{\text{max}}\) of Ca\(^{2+}\) uptake and the increased apparent binding affinity of SERCA pump for Ca\(^{2+}\) (as shown by decreased \(EC_{50}\)) in the \(mdx:\text{utr}^-\text{sln}^-\) and tKO muscles supports this view. The possible indirect effect of improved Ca\(^{2+}\) cycling, including reduced Ca\(^{2+}\) dependent protease activity and decreased fibrosis and necrosis in mice of \(mdx:\text{utr}^-\text{sln}^+\) and tKO mice further supports our hypothesis.

### Table 1 Baseline echocardiographic data of 3–4 month old mice

|                | WT         | \(mdx:\text{utr}^-\) | \(mdx:\text{utr}^-\text{sln}^-\) | tKO        |
|----------------|------------|------------------------|-------------------------------|------------|
| IVSd (mm)      | 0.74 ± 0.05| 0.83 ± 0.07            | 0.96 ± 0.05\(^s\)             | 0.85 ± 0.07|
| IVSs (mm)      | 1.28 ± 0.06| 0.99 ± 0.05*            | 1.39 ± 0.09                   | 1.45 ± 0.08|
| LVIDd (mm)     | 4.2 ± 0.13 | 3.5 ± 0.24$             | 3.34 ± 0.13$                  | 3.25 ± 0.06$|
| LVIDs (mm)     | 2.68 ± 0.07| 2.81 ± 0.20             | 2.215 ± 0.10**                | 1.91 ± 0.03**|
| LVPWd (mm)     | 0.63 ± 0.05| 0.76 ± 0.07             | 0.78 ± 0.03                   | 0.83 ± 0.09|
| LVPWs (mm)     | 1.13 ± 0.03| 0.83 ± 0.06*            | 1.07 ± 0.05                   | 1.21 ± 0.09|
| FS (%)         | 36 ± 1.0   | 20 ± 0.8**              | 36 ± 1.0                      | 41 ± 0.4$   |
| EF (%)         | 69 ± 1.5   | 44 ± 1.8**              | 68 ± 0.6                      | 73 ± 0.4$   |
| HR (bpm)       | 466 ± 16   | 498 ± 52                | 531 ± 47                      | 529 ± 25    |

\(IVSd\) interventricular septal end diastole, \(IVSs\) interventricular septal end systole, \(LVIDd\) left ventricular internal diameter end diastole, \(LVIDs\) left ventricular internal diameter end systole, \(LVPWd\) left ventricular posterior wall end diastole, \(LVPWs\) left ventricular posterior wall end systole, \(FS\) fractional shortening, \(EF\) ejection fraction, \(HR\) heart rate

\(*: p < 0.005\) vs. WT, \(\#: p < 0.05\) vs. other groups, \(\#: p < 0.0001\) vs. other groups. \(\pi: p < 0.05\) vs. WT & \(mdx:\text{utr}^-\text{sln}^-\); \(\pi: p < 0.05\) vs. WT & \(mdx:\text{utr}^-\). \(n = 6\) per group. Data are presented as mean ± SEM.
Postnatal AAV9.shSLN treatment ameliorates DMD and associated cardiomyopathy in mdx<sup>−/−</sup> mice. 1-month old male and female mice are injected with AAV or saline and experiments are performed 12 weeks post-injection. 

**a** Representative western blots show that AAV9.shSLN treatment effectively reduces SLN expression in skeletal (pectoral muscle) and cardiac (ventricle) muscles. Uncropped scans of western blots are shown in Supplementary Fig. 8d, e. The AAV treatment reduced SERCA2a and CSQ levels in the pectoral muscles of mdx<sup>−/−</sup> mice. 

**c, d** Quantitation of areas with mononuclear infiltration in the H&E stained tissue sections show that cell necrosis is significantly reduced in both quadriceps and ventricles of AAV treated mdx<sup>−/−</sup> mice compared to that of saline injected mdx<sup>−/−</sup> controls. Data are presented as mean ± SEM. The n number for each group and the p values (t-test with Welch’s correction) are shown within the graph. 

**b** Representative western blots of cardiac (ventricle) muscles show that AAV9.shSLN treatment ameliorates DMD and associated cardiomyopathy in mdx<sup>−/−</sup> mice. Uncropped scans of western blots are shown in Supplementary Fig. 8d, e. The AAV treatment reduced SERCA2a and CSQ levels in the pectoral muscles of mdx<sup>−/−</sup> mice. 

**e** LV EF, **f** forelimb muscle strength, and **g, h** the maximum twitch force generated by EDL and hemidiaphragm at 2 Hz are significantly improved in the AAV treated mice compared to that of saline injected mdx<sup>−/−</sup> controls. **p < 0.0001 vs. other groups.** Data are presented as mean ± SEM. The n number for each group and the p values (t-test with Welch’s correction) are shown within the graph.
to Dr. Dongsheng Duan. Animal studies were performed at the University of Missouri by artistic means and were performed in accordance with NIH guidelines. All the experimental dogs were on a mixed genetic background consisting of golden retriever, labrador retriever, Corgi and beagle and generated in house at the University of Missouri by artificial insemination. Affected dogs carry various mutations in the dystrophin gene that abort dystrophin expression. The genotype of the NIH NeuroBioBank network were used for this study. All samples were derived and obtained from Drs. Mark Grady and Joshua Sanes (Washington University) and performed in accordance with relevant guidelines and regulations. The research use of these samples was approved by the Institutional Review Board (IRB) at Rutgers New Jersey Medical School (to Dr. Diego Fraidenraich).

The research use of the human quadriceps tissues was approved by the IRB at the Ohio State University/Nationwide Children’s Hospital (to Dr. Jerry R. Mendell, MD) and performed in accordance with relevant guidelines and regulations. The two non-DMD human quadriceps were from a 6-year-old and 8-year-old normal children. The two quadriceps samples were from 11 to 15-year-old DMD patients. Informed consent was obtained from all subjects from whom tissues were analyzed.

AAV9.slnLN generation and delivery into mdx<sup>cu</sup>−/− mice. The shSLN cassette (ACTTCAACAGTGTCCTCATACGAGTGAAGAGCACACTTG) was cloned into plasmid pds-PLB<sup>fi</sup> using HindIII and BamHI sites to replace the shPLB<sup>fi</sup> promoter in pFastBacDual. The entire 975 bp AAV cassette (ITR-U6-promoter-shSLN-5'-IPTG) and also some plasmid backbone regions flanking the ITRs were digested out with BspHI and inserted into pFastBacDual at the NcoI site. The viral titers were determined by qPCR using primers and probes binding in the bGH region (forward: 5'-TGCCTTCCTTGACCCT; reverse: 5'-CAGAAGTGGAAGTCGCTGAACTT). After 3 days post-infection, cells were collected and the AAV was purified by iodixanol gradient ultracentrifugation and dialyzed into Lactated Ringer’s solution for transduction. The titer of the shSLN virus was determined by qPCR using primers and probes binding in the bGH region (forward: 5'-TGCCTTCCTTGACCCT; reverse: 5'-CAGAAGTGGAAGTCGCTGAACTT). All samples were tested for contaminating adventitious agents by PCR analysis using previously published sequences<sup>[58, 59]</sup>. Animal numbers were determined based on pilot studies and sample sizes were similar to generally employed in the field. No samples, mice or data points were excluded from the data analysis. Animals were not randomized except for the genotypes. For

### Table 2 Baseline echocardiographic data of AAV treated mdx<sup>cu</sup>−/− mice

| Group | LVIDd (mm) | Pp (%) | LVPWs (mm) | HR (bpm) | FS (%) |
|-------|------------|--------|------------|----------|--------|
| WT    | 0.90 ± 0.04| 37 ± 0.11| 1.12 + 0.02| 499 ± 49| 36 ± 0.11|
| mdx<sup>cu</sup>−/− | 0.95 ± 0.14| 38 ± 0.11| 0.89 ± 0.01 | 488 ± 49| 28 ± 0.08 |
| Saline | 1.31 ± 0.04| 39 ± 0.08| 1.27 ± 0.09 | 548 ± 49| 27 ± 0.06 |
| AAV9.slnLN | 0.78 ± 0.08| 39 ± 0.09| 1.02 ± 0.02 | 499 ± 49| 40 ± 1.4 |

### Table 3 Tissues used for various biochemical, histopathological and functional analyses

| Name of the assay | Tissues used |
|-------------------|--------------|
| Western blot analysis | Atria, ventricles, diaphragm, quadriceps and pectoral muscle |
| Histopathology | Quadriceps, diaphragm and ventricles |
| Immunostaining | Pectoral muscle |
| Calpain assay | Pectoral muscle |
| Muscle physiology | EDL and diaphragm |

**Table 2 Baseline echocardiographic data of AAV treated mdx<sup>cu</sup>−/− mice**

| Group | LVIDd (mm) | Pp (%) | LVPWs (mm) | HR (bpm) | FS (%) |
|-------|------------|--------|------------|----------|--------|
| IVSd (mm) | 0.90 ± 0.04| 37 ± 0.11| 1.12 + 0.02| 499 ± 49| 36 ± 0.11|
| IVSs (mm) | 0.95 ± 0.14| 38 ± 0.11| 0.89 ± 0.01 | 488 ± 49| 28 ± 0.08 |
| LVIDd (mm) | 1.31 ± 0.04| 39 ± 0.08| 1.27 ± 0.09 | 548 ± 49| 27 ± 0.06 |
| LVIDs (mm) | 0.78 ± 0.08| 39 ± 0.09| 1.02 ± 0.02 | 499 ± 49| 40 ± 1.4 |
| LVPWd (mm) | 0.77 ± 0.08| 0.72 ± 0.06| 0.9 ± 0.09 | 499 ± 49| 40 ± 1.4 |
| LVPWs (mm) | 1.12 ± 0.06| 0.89 ± 0.05 | 1.02 ± 0.02 | 499 ± 49| 40 ± 1.4 |
| FS (%) | 37 ± 0.6 | 22 ± 1.0 | 40 ± 1.4 |
| EF (%) | 71 ± 2.3 | 44 ± 1.8 | 71 ± 1.8 |
| R (mm) | 449 ± 27 | 585 ± 75 | 559 ± 58 |

**Table 3 Tissues used for various biochemical, histopathological and functional analyses**

| Name of the assay | Tissues used |
|-------------------|--------------|
| Western blot analysis | Atria, ventricles, diaphragm, quadriceps and pectoral muscle |
| Histopathology | Quadriceps, diaphragm and ventricles |
| Immunostaining | Pectoral muscle |
| Calpain assay | Pectoral muscle |
| Muscle physiology | EDL and diaphragm |
For AAV9.shSLN injection studies, we used 1-month-old male and female mdx/cut−/− mice. Mice were divided into two groups: the AAV-treated group and the saline treated group. We used 6 mice per group. The AAV9.shSLN vector (1 × 1011 genome) was delivered to the mice via a single bolus tail vein injection. The mice were sacrificed 16 weeks after measuring the forelimb strength and cardiac function by M-mode echocardiography and the tissues were used for functional and biochemical studies.

Isometric force measurements. Isometric force in isolated muscle tissues was determined as described67. Briefly, hemidiaphragm and EDL were harvested immediately following euthanasia and kept in cold oxygenated Ringer’s solution (in mmol/l, 135 NaCl, 5 KCl, 2 MgCl2, 1 Na2HPO4, 15 NaHCO3, and 5.5 glucose). Hemidiaphragm and EDL preparations were mounted in a Rodnoti chamber (In, Inc., CA, USA) containing oxygen (95% O2–5% CO2) Ringer’s solution at room temperature (~22 °C). One tendon was attached to the bottom of the Rodnoti chamber while the other tendon was attached to a Grass force transducer. For direct stimulation of muscles, two parallel plate (1 × 1 cm2) silver electrodes that were attached to the inner wall of Rodnoti chamber were used. Tissues were attached to a Grass force transducer. The force that was measured using grip strength meter (Columbus Instruments). The grip strength meter was attached to the bottom of the Rodnoti chamber while the other tendon was

Grip-strength measurements. An assessment of muscle function was recorded using grip strength meter (Columbus Instruments). The grip strength meter was positioned horizontally and the mouse was held by its tail and allowed to securely grip the triangular pull bar. After the mouse obtained a solid grasp of the triangular pull bar, the mouse was pulled backward parallel to the device. The force that was applied to the bar at the time of release was recorded as the peak grip strength (Newton). This was repeated three times and an average force was determined for each mouse. Grip strength values were normalized by the weight (g) of each animal to get the grip strength (N/g) ratio.

SR Ca2+ uptake. SR Ca2+ uptake was measured following the Millipore filtration technique, as previously described89. Briefly, about 150 µg of the total protein extract was incubated at 37 °C in 1.5 ml of Ca2+ uptake medium (in mmol/l, 40 imidazole, pH 7.0, 100 KCl, 5 MgCl2, 5Na2ATP, 5 potassium oxalate, and 0.5 EGTA) and various concentrations of CaCl2 to yield 0.03–3 mM free Ca2+ (containing 1 µCi/mmol[35S]CaCl2). To obtain the maximal stimulation of SR Ca2+ uptake, ruthenium red was added to a final concentration of 1 µM immediately prior to the addition of the substrates to begin the Ca2+ uptake. The reaction was initiated by the addition of ATP to a final concentration of 5 mM and terminated at 1 min after filtration. Each assay was performed in duplicate. The rate of SR Ca2+ uptake and the Ca2+ concentration required for EC50 were determined by non-linear curve fitting analysis using GraphPad Prism v6.01 software.

Histological analysis. Five micron paraffin sections of various skeletal and cardiac tissues from WT, mdx/cut−/−, mdx−/−sn−/− and tKO mice were stained with Hematoxylin and Eosin (H&E) and Masson’s trichome following standard procedures. The red stained collagen areas by trichome staining indicated fibrosis and necrotic areas containing mononuclear cells stained by H&E were calculated using NIH Image 1.43u program.

Immunofluorescence. The mouse monoclonal antibodies specific for eMiHC (BF45) and type 1 MyHC (BA88) were purchased from Developmental Studies Hybridoma Bank. Tissues were cryo-sectioned at 10 µm and immunostained using antibodies specific for eMiHC (1:10) or type 1 MyHC (1:3) overnight at 4 °C and processed as before69. For fiber size measurements, the tissue sections were stained with WGA, (fluorophore conjugated, 1:100, Cat.# W11262, Life Technologies). Images were obtained using a Zeiss LSM 510 on Zeiss Axiovert 100 M Base and processed using ITK-ITGS Elements. The minimal “Ferrer’s” diameter variance coefficients of the muscle fiber size was calculated on the WGA stained sections using the ImageJ 1.43u program.

Statistical analysis. We followed the established DMD-standard operating procedures for outcome measurements for fiber-size, quantitation of fibrosis and necrosis and muscle mechanics (http://www.treat-nmd.eu/research/preclinical/dmd-sops/). All statistical analyses were performed using GraphPad Prism v6.01 software. Results are presented as the mean ± SEM. Differences were determined using a two-tailed, unpaired Student’s “t” test with Welch’s correction. Two-way analysis of variance (ANOVA) with post-hoc Bonferroni correction were used for multi-group comparison when necessary. A value of p < 0.05 was considered as significant. The survival curve was generated using Kaplan–Meier survival analysis and data was analyzed using log-rank (Mantel–Cox) test.

Data availability. The data reported in this study are available from the corresponding author upon reasonable request.

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Author contributions

G.J.B. conceived the study. A.V. and G.J.B. designed experiments and wrote the manuscript. A.V., V.P., R.P., V.S., M.B., E.K., and G.J.B. performed experiments; A.V., V.P., R.P., J.J.M., J.J.D., J.R.M., L.-H.X., R.J.H., D.D., D.F., and G.J.B. analyzed and critically discussed the data. All authors discussed the results and implications and commented on the manuscript at all stages.

Additional information

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