Cathepsin S contributes to the pathogenesis of muscular dystrophy in mice*

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Running title: Cathepsin S in muscular dystrophy
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ABSTRACT

Duchenne muscular dystrophy is an X-linked recessive disease caused by mutations in the gene encoding dystrophin. Loss of dystrophin protein compromises the stability of the sarcolemma membrane surrounding each muscle cell fiber, leading to membrane ruptures and leakiness that induces myofiber necrosis, a subsequent inflammatory response and progressive tissue fibrosis with loss of functional capacity. Cathepsin S (Ctss) is a cysteine protease that is actively secreted in areas of tissue injury and ongoing inflammation, where it participates in extracellular matrix remodeling and healing. Here, we show significant induction of Ctss expression and proteolytic activity following acute muscle injury or in muscle from mdx mice, a model of Duchenne muscular dystrophy. To examine the functional ramifications associated with greater Ctss expression, the Ctss gene was deleted in the mdx genetic background, resulting in protection from muscular dystrophy pathogenesis that included reduced myofiber turnover and histopathology, reduced fibrosis and improved running capacity. Mechanistically, deletion of the Ctss gene in the mdx background significantly increased myofiber sarcolemmal membrane stability with greater expression and membrane localization of utrophin, integrins and β-dystroglycan, which anchor the membrane to the basal lamina and underlying contractile proteins. Consistent with these results, skeletal muscle-specific transgenic mice over-expressing Ctss showed increased myofiber necrosis, muscle histopathology and a functional deficit reminiscent of muscular dystrophy. Hence, Ctss induction during muscular dystrophy is a pathologic event that partially underlies disease pathogenesis, and its inhibition might serve as a new therapeutic strategy in DMD.

Duchenne muscular dystrophy (DMD)2 is an X-linked inherited neuromuscular disease due to mutations in the gene encoding dystrophin, which affects 1 of 5000 males (1). DMD pathogenesis is characterized by the appearance of muscle weakness and fatigue in juveniles that progresses to muscle-based paralysis in young adult patients with eventual death due to respiratory failure or cardiomyopathy (2,3). Dystrophin is a component of the membrane anchored dystrophin-glycoprotein complex (DGC) whose primary function is to link the intracellular contractile myofilaments to laminin and the extracellular matrix (ECM), thus providing structural support to the sarcolemma (4,5). Loss of dystrophin protein results in degradation of the greater DGC and sarcolemmal fragility that leads to the unrestrained influx of calcium, causing myofiber necrosis and the induction of tissue inflammation, fibrosis and a progressive loss of muscle contractile performance (4,5). Currently, there is no cure for DMD and although gene therapy to restore dystrophin expression is a promising concept, it remains in the distant future (6). Thus, it will be critical to develop novel pharmacologic therapies that address key aspects of disease pathogenesis, such as increased protease activity (7,8).

Cathepsin S (Ctss) belongs to the family of lysosomal cysteine proteases. Ctss was first identified in macrophages and dendritic cells as a major endoprotease cleaving the invariant chain from the major histocompatibility class II complex prior to antigen presentation (9-11). Genetic deletion of the Ctss gene in the mouse does not affect viability although these animals have altered antigen processing that causes a mild immune-compromised phenotype (9). In addition to its role in antigen processing, Ctss can remodel the ECM in various tissues when secreted. Unlike others acidic lysosomal
proteases, CtsS remains catalytically active and stable in a neutral pH environment allowing for its proteolytic activity when secreted. Collagen, elastin and fibronectin are some of the ECM proteins cleaved by CtsS (12,13).

Numerous studies have demonstrated that increased activity of select proteases in the skeletal muscle of mouse models of muscular dystrophy, such as the calpains and matrix metalloproteinases, contributes to tissue histopathology (7,14). For example, genetic inhibition of a broad class of serine proteases achieved by overexpressing the inhibitor Serpin3n in skeletal muscle attenuated dystrophic disease in two mouse models of muscular dystrophy (15). Furthermore, skeletal muscle function in various muscular dystrophy models was improved following application of broad serine protease inhibitors or anti-fibrotic drugs known to modulate matrix metalloproteinases activity (14,16). Such studies led us to examine proteases that are both induced in dystrophic skeletal muscle and amenable to pharmacologic inhibition, which might represent a new therapeutic approach in DMD. One of these proteases, CtsS, was previously identified as an mRNA species upregulated in both muscle from DMD patients and mdx mice, although the functional effects of this increase was not investigated (17-19).

Here we observed that CtsS expression and activity are upregulated in the skeletal muscle of mdx mice. Deletion of the CtsS gene in the mdx background resulted in healthier skeletal muscle with reduced myofiber degeneration and fibrosis, and improved running performance. Mechanistically, loss of CtsS in mdx mice stabilized membrane adhesion complexes and levels of associated proteins, such as utrophin, β-dystroglycan and the integrins, thereby increasing sarcolemma membrane stability and protecting from injury. We also generated a skeletal muscle-specific transgenic mouse overexpressing CtsS (CtsS TG). These mice had greater muscle CtsS proteolytic activity that was associated with induction of muscle histopathology reminiscent of muscular dystrophy. Hence, increased CtsS expression and activity in skeletal muscle is detrimental to DMD pathology and its inhibition represents a novel therapeutic vantage point for this disease.

**EXPERIMENTAL PROCEDURES**

**Animals**—All animal procedures were approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Hospital Medical Center, protocol IACUC2013-0013. The exact number of mice used was given in the figure legends or within the figures and the numbers used reflects the minimum needed to achieve statistical significance (see Statistics section below). Animals used in the study were not randomized or handled in a blinded manner, as this was not commensurate with the experimental design. No data collected from animals was excluded. No human subjects were used. CtsS−/− mice were kindly provided by Dr. Guo-Ping Shi (9). mdx mice were obtained from Jackson Labs (Stock No: 001801). To generate, mdx/CtsS−/− mice, mdx female mice in the C57Bl/10 background were mated with male CtsS−/− mice in the related C57Bl/6 background. The subsequent litters were backcrossed 3 times to generate mdx/CtsS−/− mice, and only male mdx/CtsS−/− and mdx littermates were used. WT C57Bl/6 male mice were used as controls. The following primers were used for CtsS genotyping: Neo cassette (F: 5’TGA ATG AAC TGC AGG AGC AG and R: 5’AAT ATC ACG GGT AGC CAA CG); KO (F: same primer as WT Forward sequence and R: 5’ACT TGT TGT GCA AAA AGC TTT AAC). Genotyping for mdx was performed as previously shown (20).

The mouse CtsS cDNA was cloned in a modified human skeletal α-actin promoter (21) to create skeletal muscle-specific TG mice (FVBN strain). Two TG lines were generated by DNA injection into newly fertilized mouse oocytes. TG males were compared to NTG littermate males. The following primers were used for genotyping of the CtsS transgene: F: 5’CGA GAG TAG CAG TTG TAG CTA and R: 5’CAT TGT GAG AGC TAA TCA TCT (22).

**Protease activity assay**—A CtsS activity assay (Abcam, ab65307) was used to measure the cysteine proteolytic activity in either the dystrophic or BaCl2 injured skeletal muscle. Other cysteine protease activities measured include cathepsin B (CtsB, Abcam, ab65300) and cathepsin L (CtsL, Abcam, ab65306). Briefly, isolated muscles were frozen, ground into a fine powder using a mortar and pestle and sonicated before the ice cold buffer from the assay kit was added to the samples. The enzymatic assay itself was conducted according to the manufacturer’s instructions.

**Western blotting and protein extraction from skeletal muscle**—Isolated muscles were frozen and ground to a fine powder using a mortar and pestle, sonicated and then added to ice-cold RIPA buffer. CtsS was detected using rabbit polyclonal antibody from EMD Millipore, catalog# 219384 (1:1000,
against human and mouse epitope) by western blotting as previously described (15).

Skeletal muscle membrane enrichment protein fractions were prepared following a previous protocol (22). Standard western blotting was then performed with the membrane-enriched protein extracts as previously shown (15). Primary antibodies used were α5-integrin (EMD Millipore AB1928; 1:1000, polyclonal rabbit against mouse) and fibronectin (Abcam ab2413 1:1000, rabbit polyclonal against mouse). The other antibodies used are listed in a previous published study (15).

ECM protein fractionation from quadriceps muscle was generated as described previously (23). Primary antibodies used included: collagen IV (Abcam ab19808 1:1000, rabbit polyclonal against mouse) and fibronectin (Abcam ab2413 1:1000, rabbit polyclonal against mouse). These extracts were then subjected to western blotting as previously described (15).

RNA expression analysis—Total RNA was extracted from muscles using the RNeasy Kit according to manufacturer’s instructions (Qiagen) and reverse transcribed using SuperScript® III Reverse Transcriptase (Invitrogen 18080-044). Transcription differences were analyzed by real-time qPCR using SYBR green (Applied Biosystems). Primer sequences for Ctss were (F: 5’TGC GTC ACT GAG GTG AAA TAC C and R: 5’ CTT CAT TTG AGT CAT TTG AGC AGT CCA CCA G). The primer sequences for other genes are listed in a previous study (15).

Histological analysis and immunohistochemistry—Muscles were paraffin-embedded and 6-μm histological sections were cut at the center of the muscle and stained with H&E or Masson’s trichrome. Pictures were generated at a magnification of 200x, and fibers of the quadriceps muscle were counted for central nucleation using ImageJ software (NIH). Interstitial fibrotic regions were quantified using MetaMorph 7.1 (Molecular Devices) as percentage of blue area stained with Masson’s trichrome. Immunohistochemistry was performed on muscle histological cryosections from optimal cutting temperature compound (OCT)-embedded muscles using the following antibodies: Ctss (Abcam ab96788, 1:100, rabbit polyclonal against human epitope), Laminin α-2 chain (Sigma-Aldrich L0663, 1:200, rat monoclonal against mouse and human) and lysosome-associated membrane protein-2 (Lamp-2, Abcam ab13524, 1:100, rat monoclonal against mouse and rabbit). Nuclei were stained blue with 1 mg/ml 4,6-diamidino-2-phenylindole (Dapi) diluted to 1:5000 in saline. Wheat germ agglutinin (WGA)-FITC (Sigma-Aldrich; L4895) was used at 50 μg/mL to outline membranes in the Evan’s blue dye (EBD) experiments. Antibodies used for immunohistochemistry of sarcolemma adhesion components were previously described (15).

EBD uptake and involuntary running—Mice were injected with Evan’s blue dye (intraperitoneally, 10 mg/ml, 0.1 ml/10 g body weight). Twenty-four hours later, mice were subjected to forced treadmill running for 30 minutes, and sacrificed. The tibialis anterior (TA) muscles were removed, embedded in OCT and snap-frozen in liquid nitrogen as previously described (15). For the treadmill running protocol, approximately 2-4 month-old mice were acclimatized to the treadmill (Omni-Pacer LC4/M; Columbus Instruments International) for 10 min at a speed of 6 m/min prior to shock grid activation. The speed was then changed to 10 m/min for 30 minutes. Running capacity was assessed by the maximum running endurance time before exhaustion. Exhaustion was determined by the animal remaining on the shock grid for more than 10 consecutive seconds.

BaCl2 injury—Sixty microliters of an aqueous 1.2% mass/volume (m/w) BaCl2 (Sigma-Aldrich 202-7385g) solution was injected along the whole length of the left tibialis anterior (TA) muscle to induce injury and degeneration (24).

Enzymatic dissociation of myofibers and laser sarcolemma membrane stimulation—Myofiber isolation from flexor digitorum brevis (FDB) was performed as previously described (15). Enzymatically dissociated FDB fibers were plated onto Mattek glass-bottomed dishes containing 1.25 mM Ca2+ isotonic Tyrode solution. Damage to the myofibers was induced by irradiating a 5 × 5 pixel area on the sarcolemma membrane with 80 milliwatts at 351/364 nm wavelength for 10 s using a Nikon A1 confocal microscope equipped with a 60x water immersion lens, in the presence of 2.5 μM FM1-43 dye (Molecular Probes) in the extracellular Tyrodes solution. Images were captured at 5 s intervals following irradiation with the laser. The mean fluorescence intensity in the damaged area was quantified by ImageJ software. Calcium-free analysis was performed by replacing the 1.25 mM Ca2+ with isotonic Tyrode solution lacking this divalent cation, which was also supplemented with 0.5 mM EGTA.

Statistics—All results are presented as mean ± S.E.M. Statistical analysis was performed with unpaired two-tailed t-test (for two groups) and one-way ANOVA with Bonferroni correction (for groups of three or more). p-values of <0.05 were considered
significant. Animal numbers reflected the minimal number needed for statistical significance based on power analysis and prior experience. No data was excluded from the animal experiments, and blinding was not performed.

RESULTS

Ctss is upregulated in injured skeletal muscle—mRNA profiling in humans with DMD and the mdx mouse showed an increase in Ctss expression (17-19), which we also observed in Affymetrix arrays from the muscle of δ-sarcoglycan deficient mice (Sgcd−/−), a model of limb-girdle muscular dystrophy, and in transgenic mice overexpressing the calcium/sodium influx channel Trpc3 that also develop a muscular dystrophy phenotype (25). To validate these prior observations from various gene arrays, we performed quantitative PCR for Ctss, which revealed a 6-fold upregulation of this mRNA in the skeletal muscle of mdx mice (Fig. 1A). More importantly, Ctss activity assays and western blotting from skeletal muscle of mdx mice demonstrated Ctss upregulation, this time at 6 weeks and 10 months of age (Fig. 1B and C). Immunohistochemistry showed essentially no Ctss expression (green) in normal skeletal muscle, yet skeletal muscle from mdx mice had abundant Ctss protein co-localized with laminin within the ECM region between myofibers, but not within lysosomes as co-stained with lysosome-associated membrane protein-2 (Lamp-2, Fig. 1D). This suggested that in dystrophic muscle, Ctss was upregulated and primarily localized to the extracellular compartment.

To assess whether upregulation of Ctss is induced following acute muscle injury, 60 μl of 1.2% BaCl$_2$ solution was injected into the tibialis anterior (TA) muscle of WT mice (24). Significant induction of Ctss proteolytic activity and protein expression was observed with initial expression at 3 days post injury that was sustained through day 6 (Fig. 1E and F). Myogenin was used to show myofibers undergoing regeneration due to a successful injury response. Collectively, these results demonstrate that endogenous Ctss is upregulated following acute injury to skeletal muscle and during chronic muscular dystrophy.

Loss of Ctss expression in mdx mice mitigates dystrophic disease—Genetic deletion of Ctss in an atherosclerotic mouse model background resulted in reduced disease progression, either through modulation of the immune response or stabilization of ECM components (26,27). To determine whether Ctss is an effector of chronic muscle disease, we crossed the viable and fertile Ctss+/- mouse with the mdx model. Basal characterization of skeletal muscle from Ctss+/- mice showed no morphology signs of disease or any observable alterations (data not shown). Western blot analysis and Ctss activity assays confirmed the loss of Ctss protein expression and proteolytic activity in the skeletal muscle of mdx/Ctss+/- mice (Fig. 2A and B). Genetic deletion of Ctss in mdx mice also blunted the increase in activity of the cysteine protease Ctsb, but not the increase in Ctsl, both of which are also upregulated in dystrophic skeletal muscle (Fig. 2B).

Histological assessment of skeletal muscle from 2 month-old mdx/Ctss+/- mice showed a noticeable reduction in myofiber necrosis, fibrosis and other aspects of muscle histopathology compared to mdx mice (Fig. 2C). This early time-point (2 months) is especially important as it corresponds to the first few cycles of myofiber regeneration-degeneration during DMD pathogenesis in mice. Improvements in muscle histopathology were also observed in older (10 month-old) mdx/Ctss+/- mice (Fig. 2C). Furthermore, histological analysis of the diaphragms of mdx/Ctss+/- mice showed noticeably healthier tissue at both 2 and 10 months of age compared with mdx alone (data not shown). Quantitative assessment of the tissue histopathology and general muscle disease features also showed significant improvements in mdx mice with deletion of the Ctss gene. For example, central nucleation, tissue fibrosis and serum creatine kinase (CK) levels were all significantly reduced in mdx/Ctss+/- mice compared with mdx alone (Fig. 2D-F). This improvement in muscle histopathology and reduction in serum CK levels also correlated with improved muscle function, as mdx/Ctss+/- mice performed significantly better than mdx mice when subjected to forced treadmill running at 3 months of age (Fig. 2G). Collectively, these results indicate that deletion of the Ctss gene improves muscle structure and function in dystrophic mdx mice.

To examine the potential mechanisms whereby loss of the Ctss gene might protect skeletal muscle in the mdx genetic background we further interrogated sarcolemmal stability given the significant reduction in total serum CK levels observed in the double genetically modified mice, which typically indicates levels of ongoing membrane rupture. Here we injected Evan’s blue dye (EBD) intraperitoneally and subjected the mice to downhill running on a treadmill to increase the stress on myofiber membranes. Remarkably, significantly fewer EBD positive myofibers (autofluoresces red) were observed in the
skeletal muscle of mdx/Ctss−/− mice compared to mdx, suggesting decreased membrane rupture and increased membrane stability with Ctss deletion (Fig. 3A and B).

To more directly evaluate sarcolemma membrane integrity at the single myofiber level, we measured the capacity of myofibers to be injured and/or reseal following direct laser injury to the sarcolemma, measured by influx of the membrane impermeant dye FM1-43. Consistent with the known decrease in membrane stability of mdx myofibers, significantly greater FM1-43 fluorescence (green) was observed following acute laser membrane injury in myofibers from these mice (Fig. 3C and D). However, less FM1-43 dye entry was observed in the myofibers isolated from the mdx/Ctss−/− skeletal muscle, further confirming that loss of Ctss protects against sarcolemma membrane damage in the dystrophic background (Fig. 3C and D). To dissect whether deletion of Ctss primarily affects sarcolemma membrane stability or its resealing capacity, we repeated the assay in the absence of extracellular calcium (membrane resealing is a calcium-dependent process). Removal of calcium resulted in greater FM1-43 dye entry in the myofibers from all genotypes (Fig. 3C and E), but the level of influx in the mdx background was still much greater and this greater value was significantly reduced by deletion of the Ctss gene (Fig. 3E). Hence, loss of Ctss does not directly affect membrane resealing, but instead regulates the inherent stability of the membrane.

Increased protection against sarcolemma damage in mdx/Ctss−/− myofibers could be associated with restoration or increased abundance of sarcolemma adhesion protein components. More importantly, proteases are known to target and cleave individual proteins within the DGC and utrophin/integrin complexes (28-31). Analysis of these component proteins that stabilize the membrane in membrane-enriched protein preparations showed that mdx muscle had reduced levels of β-dystroglycan, α5-integrin, α7-integrin and β1-integrin, with a mild compensatory increase in utrophin (Fig. 4A and B). Deletion of the Ctss gene in the mdx background restored the levels of each of these proteins within the sarcolemma, and even promoted a greater increase in utrophin levels (Fig. 4A and B). Confocal analysis of histological sections from skeletal muscle for membrane localization of each of these proteins also showed a noticeable increase in the levels of β-dystroglycan, α5-integrin, α7-integrin, β1-integrin and utrophin in mdx/Ctss−/− mice versus mdx (Fig. 4C). Upregulation of utrophin partially compensates for the loss of dystrophin in the mdx mouse to augment sarcolemma stability (32). To show that the observed changes were due to alterations in protein stability we also measured mRNA levels for these genes from skeletal muscle. The results show that while mRNA levels for utrophin and the dystroglycan gene were unchanged, α7-integrin and β1-integrin were actually increased in muscle of mdx mice (Fig. 4D), although this increase is opposite of the observed decrease in protein. This result suggests that Ctss is primarily affecting protein levels and stability and not gene transcription or mRNA stability of the key membrane stabilizing complexes. Collectively, these data indicate that genetic deletion of Ctss reduces the degradation of the sarcolemma adhesion components in skeletal muscle of mdx mice, thereby increasing membrane stability.

Ctss overexpression induces a dystrophy-like phenotype in skeletal muscle of mice—Since Ctss is induced in both human DMD and mouse models of muscular dystrophy, we attempted to model this increase by creating skeletal muscle-specific transgenic mice with increased Ctss expression. Ctss TG mice were generated in the FVBN genetic background using a modified human skeletal α-actin promoter (Fig. 5A). Western blotting analysis for Ctss protein expression in skeletal muscle defined two different TG lines with higher or lower protein expression (Fig. 5B). All of experiments presented here were performed in the higher TG-line1, although similar phenotypes with less pathology were observed in TG-line2 (data not shown). Ctss transgene expression was uniform in multiple skeletal muscles that are predominantly fast-twitch, although lower levels of expression were observed in the soleus with its greater slow-fiber content, while the heart showed no expression (Fig. 5C). Ctss activity assays also showed significantly higher levels in skeletal muscle from Ctss TG mice (Fig. 5D), which was similar to the observed increase in Ctss activity in mdx skeletal muscle (Fig. 1B). Immunohistochemistry also showed that the majority of overexpressed Ctss localized to the edges of the myofibers in the ECM region (Fig. 5E), similar to what was observed for endogenous Ctss in muscle from mdx mice (Fig 1D). However, Ctss TG mice also showed some protein expression within the myofibers, likely as part of the secretory pathway within vesicles, which we did not readily observe in mdx muscle (Fig. 5E, see discussion).

Histological analysis of skeletal muscle from Ctss TG mice showed disease at 2 months of age
characterized by accumulation of central nuclei in presumed regenerating myofibers, fatty tissue replacement and fibrosis, all features of muscular dystrophy (Fig. 5F-H). Similar tissue histopathology with increased quantitative indexes of disease was observed in skeletal muscle from Ctsss TG mice at 10 months of age (Fig. 5F-H). These pathogenic changes in skeletal muscle also correlated with a loss of skeletal muscle functional performance assessed by treadmill running at both 2 and 4 months of age (Fig. 5J). Finally, consistent with the proposed mechanism whereby increased Ctsss expression and activity in dystrophic muscle might be degrading DGC or ECM components, we observed increased degradation of collagen IV and fibronectin in ECM protein preparations from skeletal muscle of Ctsss TG mice (Fig. 5J). Taken together, these results suggest that increased Ctsss expression and activity in skeletal muscle is pathologic and hence could be the target for inhibition during muscular dystrophy as a novel therapeutic approach.

DISCUSSION

Our study provides the first genetic evidence that elevated Ctsss proteolytic activity in skeletal muscle is pathological and contributes to muscular dystrophy. Indeed, genetic deletion of Ctsss in the skeletal muscle of mdx mice protected muscle from dystrophic disease as shown by increased running endurance and blunted muscle histopathology, as well as reduced serum CK levels. Broadly acting protease inhibition in muscular dystrophy achieved with either genetic modification or pharmacological inhibition has reduced muscular dystrophy manifestations in mouse models of this disease (15,16,33). However, an early pilot study testing the efficacy of Bestatin and Loxistatin, both of which are general inhibitors for multiple cathepsin family members, showed improved muscle function in infant DMD patients, but the effects were not reproduced in juvenile patients (34). This negative result might be explained by the lack of specificity and harmful off-target effects of these agents (35,36).

A small compound N-morpholinoureao-leucine-homophenylalanine-vinyl-sulphonephenol (LHVS) is a more specific Ctsss inhibitor with an IC₅₀ of 5 nM (10,11). In vitro and in vivo studies using LHVS have shown promising therapeutic effects such as decreased ECM remodeling in hypertension induced heart failure (37) and increased neuroprotection following traumatic brain injury (38) or peripheral nerve trauma (39,40). These results confirm the specificity and efficacy of LHVS in targeting Ctsss for a biologic effect in vivo that is consistent with its presumed function. Our results in Ctsss⁻/⁻ mice also suggest that a Ctsss specific inhibitor could be an interesting new strategy to treat DMD with potentially less side-effects or undesirable off-target effects. Another interesting concept is that Ctsss is somewhat unique in its ability to function outside the lysosome, at neutral pH, unlike other cathepsins. Hence Ctsss might preferentially contribute to pathogenic events in muscular dystrophy compared with other proteases.

Human genetic studies have shown that the primary cause of muscular dystrophy is a loss in one of a myriad of genes that can directly or indirectly affect the structural integrity of the sarcolemma (4,5). Activation of proteases in damaged muscle further diminishes the stability of the sarcolemma due to aberrant cleavage of other membrane adhesion complex proteins such as β-dystroglycan and integrins (28-31). For example, upregulation of integrin RNA expression in muscle in the mdx background (Fig. 4D) did not produce increased protein expression and localization in the sarcolemma membrane (Fig. 4A-C), suggesting a continuous proteolysis of the integrin subunits in damaged mdx muscle, of which Ctsss may contribute. Restoration and increased protein expression and localization of these sarcolemma adhesion components greatly enhances and protects the sarcolemma against contraction/exercise induced injury (41). Thus, the improved sarcolemma stability of myofibers from mdx/Ctsss⁻/⁻ mice is likely due to increase abundance and stability of utrophin, β-dystroglycan, and integrin subunits (α5, α7, and β1) within this membrane (Fig. 4A-C).

We also generated Ctsss TG mice that exhibited comparable expression and proteolytic activity as that normally induced in the skeletal muscle of mdx mice. Ctsss TG mice showed dystrophic-like disease pathology associated with muscle dysfunction and tissue histopathology. However, in addition of extracellular expression of Ctsss as normally observed for endogenous Ctsss in mdx muscle, we observed intracellular Ctsss protein expression within the myofibers of the TG mice, which is of unknown specificity and hence could potentially promote an artefactual mechanism whereby this protease cleaves important membrane stabilizing proteins from within the vesicular compartment and membrane trafficking network (Fig. 5E). While this is a possibility, clearly endogenous Ctsss that is induced with disease also traverses the intracellular vesicular trafficking network on its way to being secreted, where it could
also have pathologic effects on these same membrane stabilizing protein components. Hence we believe that the Ctss TG model still provides important information that is consistent with the observations in Ctss⁻/⁻ mice, as well as with previous studies that showed some degree of protection from muscular dystrophy with pharmacologic protease inhibitors. Thus, moving forward it will be important to determine how central Ctss is versus several other induced proteases annotated at the mRNA level in dystrophic skeletal muscle (although only Ctss might be secreted and active outside of the myofibers, compared with other cathepsins that only function at low pH). Such knowledge will help inform the best pharmacologic strategy and how one might want to design a drug to block Ctss versus other related and potentially pathogenic proteases of the same class, or even of other classes. Despite this argument, our results do suggest that Ctss is a critical protease and perhaps a highly specific inhibitor that blocks just this enzyme would be an ideal starting point for future studies, with possible translation to the clinic.

*This work was supported by grants from the National Institutes of Health grant P01NS072027 (J.D.M.) and the Howard Hughes Medical Institute (J.D.M.). T.G.S. is funded by the DFG - German Research Foundation (SCHI 1290/1-1) and O.K. is funded by Postdoctoral fellowship from the American Heart Association (15POST25480009). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Acknowledgements:
None

Conflict of interest:
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions:
AT conducted most of the experiments, analyzed the results, and wrote the paper with MM and JDM. TS conducted the western blotting and extraction of extracellular protein fractionation. MAS, DV, OK, VP, and S-CJL provided technical assistance. MM wrote the paper with AT and JDM. The study was conceived by JDM, who also oversaw all experimentation and interpretation of results.

REFERENCES


Footnotes:

5The abbreviations used are: BaCl2, barium chloride; CK, creatine kinase; Ctsb, Cathepsin B; Ctsl, Cathepsin L; Ctss, Cathepsin S; Ctss TG, Ctss transgenic; Dapi, 4',6-diamidino-2-phenylindole; DGC, dystrophin-glycoprotein complex; DMD, Duchenne muscular dystrophy; EBD, Evan’s blue dye; ECM, extracellular matrix; FDB, flexor digitorum brevis; H&E, Hematoxylin & Eosin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mdx, dystrophin deficient mouse model of DMD; NTG, non-transgenic; TA, tibialis anterior; TG, transgenic; WGA, wheat germ agglutinin; WT, wild-type.
FIGURE LEGENDS

FIGURE 1. Ctss is expressed in damaged skeletal muscle. A, Quantitative PCR for Ctss mRNA measured from the quadriceps of 2-3 month-old mdx mice compared to wildtype (WT). *p<0.05 vs WT. The number of mice analyzed is indicated in the graph. B, Increased Ctss protease activity in the quadriceps of mdx mice as compared to WT at 6 weeks and 10 month of age. *p<0.05 vs WT at the same time point. The number of mice analyzed is indicated in the graph. C, Western blot for Ctss expression in the quadriceps of 6 week and 10 month-old mdx mice compared to WT mice of the same age. GAPDH was a processing and loading control. KO represents muscle protein extracts isolated from the quadriceps of Ctss<sup>−/−</sup> mice as a negative control. D, Immunohistochemistry from the quadriceps of WT and mdx mice for Ctss expression (green) versus the myofiber outline with laminin (red) or the lysosomal marker Lamp2 (red). Nuclei are shown in blue with Dapi staining. E,F, Ctss activity assay and (F) expression in the TA muscle of WT mice at the indicated days following acute BaCl<sub>2</sub> injection injury, or from uninjured (uninj.) muscle. Myogenin (Myog) was used as a marker for muscle injury and GAPDH was a processing and loading control. *p<0.05 vs uninjured. All error bars are S.E.M. The number of mice analyzed in panel E is indicated in the graph.

FIGURE 2. Loss of Ctss in mdx mice attenuates disease pathogenesis. A,B, Western blot analysis for Ctss protein and (B) Ctss, cathepsin B (Ctsb) and cathepsin L (Ctsl) activity from the quadriceps of 2 month-old mdx/Ctss<sup>−/−</sup>, WT and mdx mice. *p<0.05 vs WT and #p<0.05 vs mdx. The number of mice analyzed is indicated in the graph. GAPDH was used as a processing and loading control in panel A. C, Representative H&E and Masson’s trichrome-stained histological sections from quadriceps of 2 and 10 month-old mice of the indicated genotypes. Magnification is 200x. Blue areas represent fibrosis in the trichrome-stained sections. D,E, Quantitation of percentage of myofibers with centrally localized nuclei and (E) fibrotic area from histological sections of the quadriceps in the genotypes shown at 2 and 10 month of age. Number of mice analyzed is shown in the graphs. *p<0.05 versus WT and #p<0.05 versus mdx. F, Serum creatine kinase (CK) levels from the (F) expression in the TA muscle of WT mice at the indicated days following acute BaCl<sub>2</sub> injection injury, or from uninjured (uninj.) muscle. Myogenin (Myog) was used as a marker for muscle injury and GAPDH was a processing and loading control. *p<0.05 vs uninjured. All error bars represent S.E.M.

FIGURE 3. Loss of Ctss in mdx muscle protects against sarcolemma membrane damage. A,B, Representative histological images and (B) quantitation of Evan’s blue dye (EBD) uptake in myofibers of the tibialis anterior (TA) muscle in the indicated genotypes of mice. Wheat germ agglutinin-FITC (WGA-FITC, green) is used as a membrane marker. EBD containing fibers fluoresce red under the imaging conditions used. Number of mice used is shown in the graph. *p<0.05 versus WT and #p<0.05 versus mdx. C, Representative images for FM1-43 dye entry (green) from individually isolated flexor digitorum brevis (FDB) myofibers from the indicated genotypes of mice. The top row of fibers were uninjured while the bottom row were all laser injured in the presence of 1.25 mM calcium (left) or absence of extracellular calcium (right). The red asterisks show the area that will be injured with the laser. D,E, Quantitative traces of FM1-43 dye entry after laser injury in the (D) presence or (E) absence of calcium as shown in “C” from n≥ 6 fibers for each mouse and a total of 5 separate animals for each genotype shown. *p<0.05 versus WT; #p<0.05 versus mdx. All error bars represent S.E.M.

FIGURE 4. Loss of Ctss in the muscle of mdx mice stabilizes and restores the abundance of sarcolemma adhesion complex proteins. A, Western blotting of membrane protein extracts isolated from the quadriceps muscle of the indicated genotypes of mice at 2-3 months of age, for the indicated proteins. Cav1.1 expression and Ponceau staining were used as loading controls. The red box shows the critical proteins that were increased in expression by loss of Ctss. B, Quantitative protein analysis for the indicated proteins in the quadriceps of the indicated genotypes of mice at 2-3 months of age, for the indicated proteins. A total of 3 separate animals and protein preparations were used for densitometric analysis. *p<0.05 versus WT; #p<0.05 versus mdx. All error bars represent S.E.M. C, Immunohistochemistry of the indicated proteins (green) from transverse histological sections of quadriceps of WT, mdx and mdx/Ctss<sup>−/−</sup> mice. The red box again shows the proteins that are stabilized and increase in expression in the absence of Ctss.
Quantitative PCR analysis for relative mRNA expression levels of the indicated genes from the quadriceps of the indicated mice at 2-3 months of age. Error bars represent S.E.M. N=3 mice each. *p<0.05 versus WT. Abbreviations: Dystroph, dystrophin; α5-Itg, α5-integrin; α7-Itg, α7-integrin; β1-Itg, β1-integrin; β-Dystrog, β-dystroglycan; α-Dystrog, α-dystroglycan; Cav1.1, L-type calcium channel.

FIGURE 5. Overexpression of Ctss in skeletal muscle produces a dystrophy-like phenotype. A, Schematic of the Ctss transgene construct used to make mice, directed by the skeletal α-actin promoter. pA is the polyadenylation sequence. B, Western blot analysis of Ctss and GAPDH expression from the quadriceps muscle of 2 separate transgenic founder lines at 2 months of age. NTG represents non-transgenic controls. C, Western blotting for Ctss expression in the different skeletal muscles shown, as well as heart from TG line 1, compared to control non-transgenic samples between each transgenic (TG) sample. GAPDH was a loading and processing control. Abbreviations: quadriceps (quad), gastrocnemius (gastroc), TA (tibialis anterior), EDL (extensor digitorum longus), soleus (sol) and diaphragm (Diaph). D, Ctss protease activity assay from the quadriceps muscle of non-transgenic (NTG) and Ctss TG mice. *p<0.05 versus NTG. Number of mice used is shown in the graph. E, Immunohistochemistry from the quadriceps of Ctss TG mice for Ctss expression (green) versus the myofiber outline with laminin staining (red). Nuclei are shown in blue with Dapi staining. F, Representative H&E and Masson’s trichrome histological sections from quadriceps of NTG and TG mice at the indicated ages. Magnification is 200x. G,H, Quantitation of percentage of myofibers with centrally localized nuclei and fibrotic area in histological sections from quadriceps in the indicated genotypes of mice at 2 and 10 months of age. *p<0.05 versus NTG. Number of mice used is shown in the graph. I, Treadmill running time for NTG and TG mice at the indicated 2 or 4 months of age, measured at 10 m/minute speed. *p<0.05 versus NTG. J, Western blot analysis of an enriched extracellular protein fraction from the quadriceps of NTG or TG mice for the indicated proteins. Ponceau is a loading control. Error bars represent S.E.M for the entire figure.
Fig 1

(A) Relative RNA expression (AU) for WT and mdx mice.

(B) Ctss activity (AU) for WT and mdx mice at 6 wks and 10 mo.

(C) Western blot analysis of Ctss and GAPDH in WT and mdx mice at 6 wks and 10 mo.

(D) Immunofluorescence images of Ctss/Laminin/Dapi and Ctss/Lamp2/Dapi in WT and mdx mice.

(E) Ctss activity (AU) post BaCl2 injury in d1, d3, d6, and uninj.

(F) Western blot analysis of Ctss, Myog, and GAPDH post injury.

Post BaCl2 injury

Post injury

d1  d3  d6  uninj.
**Fig. 2**

**A** Western blot for Ctss and GAPDH. 

**B** Bar graphs showing Ctss activity (AU) for WT, mdx, and mdx/Ctss−/− groups. 

**C** H&E and Trichrome staining of muscle tissue for WT, mdx, and mdx/Ctss−/− groups at 2 and 10 months. 

**D** Central nuclei (%) for WT, mdx, and mdx/Ctss−/− groups at 2 and 10 months. 

**E** Fibrosis (%) for WT, mdx, and mdx/Ctss−/− groups at 2 and 10 months. 

**F** Serum CK (U/L) for WT, mdx, and mdx/Ctss−/− groups. 

**G** Running time (s) for WT, mdx, and mdx/Ctss−/− groups. 

* indicates significant difference from WT. # indicates significant difference from mdx.
Fig. 3

(A) Immunofluorescence images of WT, mdx, and mdx/Ctss−/− mouse diaphragms stained with antibodies against EBD. Scale bar: 200 µm.

(B) Bar graph showing the percentage of EBD fibers in WT, mdx, and mdx/Ctss−/− mouse diaphragms. WT: black, mdx: blue, mdx/Ctss−/−: light blue. Error bars indicate standard error (n=6 each).

(C) Confocal images of WT, mdx, and mdx/Ctss−/− mouse diaphragms stained with antibodies against the actin cytoskeleton before and after laser injury. Images are divided into three categories: Before, Laser injury, and +Ca2+. Images are labeled with asterisks to indicate significant differences.

(D) Graph showing the change in ΔF/Fo FM1-43 (AU) over time for WT, mdx, and mdx/Ctss−/− mouse diaphragms with +Ca2+. Data points are labeled with asterisks to indicate significant differences.

(E) Graph showing the change in ΔF/Fo FM1-43 (AU) over time for WT, mdx, and mdx/Ctss−/− mouse diaphragms without Ca2+. Data points are labeled with asterisks to indicate significant differences.
**A**

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**B**

- **Urophin**
- **α5-Itg**
- **α7-Itg**

**C**

**D**

- **Urophin**
- **α7-Itg**
- **β1-Itg**
- **Dystrog**
Cathepsin S contributes to the pathogenesis of muscular dystrophy in mice
Andoria Tjondrokoesoemo, Tobias G. Schips, Michelle A. Sargent, Davy Vanhoutte, Onur Kanisicak, Vikram Prasad, Suh-Chin J. Lin, Marjorie Maillet and Jeffery D. Molkentin

J. Biol. Chem. published online March 10, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.719054

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