[redacted]:

From: [redacted]
Sent:
To:
Subject:

Dear Dr [redacted]

I am writing to you on behalf of the Journal of Clinical Investigation's Editorial Board to thank you for submitting your manuscript, "[redacted]ves the muscle phenotype of laminin alpha 2 chain deficient mice [redacted]G-1). After careful examination of your manuscript by both expert outside reviewers as well as the Editors, the Board has determined that your manuscript, in its current form, does not achieve the priority necessary for publication in the Journal of Clinical Investigation. Although all reviewers agree that your study is of potential interest and importance, a number of substantive concerns were raised upon review. If you believe that you can respond to the issues raised in the review process, we would be pleased to consider a revised version of this manuscript. Given the scope and time frame required to address some of the reviewers comments, we reserve the right to send a revised manuscript to an additional reviewer for comment s.

We hope that you will find the reviewers' comments helpful as you revise your manuscript. Following receipt of a revised manuscript, your paper will be re-evaluated and assigned a new priority ranking. Please be aware that current editorial policy permits only one revision of this manuscript and that we can offer no assurances that the work will ultimately be accepted. Also bear in mind that we will be reluctant to approach the original referees again in the absence of appropriate changes to address their concerns.

PLEASE PAY SPECIAL ATTENTION TO THE ITEMS IN THE CHECKLIST BELOW AND SPECIFICALLY ACKNOWLEDGE THE CHANGES MADE IN YOUR POINT BY POINT RESPONSE.

If you choose to resubmit, please enclose a point-by-point response to the reviewers' comments and a list of the incorporated changes, including the location in the manuscript of those changes.

Thank you for giving us the opportunity to review this work, and we look forward to seeing the revised manuscript.

Sincerely,

[redacted] M.D.

REVISION CHECKLIST ==========

In addition to addressing items noted in the decision above, please ensure that your manuscript adheres to the following guidelines before submitting your revision:

PDF + Points

FILES REQUIRED FOR REVISED SUBMISSIONS
_ PDF of the revised manuscript that includes a preface of point-by-point responses to reviewer comments.
_ Text document (formatted as Word or RTF) of the revised manuscript (do not include point-by-point responses).

TITLE PAGE (required components)
_ Title: does not exceed 15 words; contains no subtitles, colons, periods, or nonstandard abbreviations; and mentions the species in which the study was performed, for example, "in humans and mice"; "in a mouse model of cystic fibrosis"; "in rhesus macaques."

June 9, 2010

The Journal of Clinical Investigation
15 Research Drive
Ann Arbor
Michigan 48103
USA

Dear Dr. [redacted]

Thank you very much for considering our manuscript entitled "[redacted] the muscle phenotype of laminin α2 chai[redacted] (Manuscript No. 43064-RG-1). We have revised the manuscript to address each of the reviewer's specific comments and believe that their input has substantially improved the manuscript. Our response to the reviewer's comments can be found on the following pages.

Thank you again for your interest in our work. We hope that the revisions have made the manuscript acceptable for publication.

Sincerely,

[redacted]

Itemized response to previous reviews

[redacted]

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Reviewer A

1. The authors comment that muscular atrophy results from an imbalance of muscle protein degradation vs. synthesis. While this may be true in the case of normal muscle atrophy, in muscular dystrophy, this scenario may not be the case. In addition, in a muscular dystrophy context, if the primary cause of the muscle disease is not addressed, then long term blockade of muscle protein degradation is not likely a viable long term solution for treatment. Furthermore, the referee wonders what will happen to the proteins that the cell normally degrade but instead accumulate in the cell.

We basically agree with the reviewer. Indeed, previous therapies for MDC1A have focused on replacing the defective laminin α2 chain with similar proteins (Kuang et al., 1998, J Clin Invest; Moll et al., 2001, Nature; Gawlik et al 2004, Hum Mol Genet; Meinen et al., 2007, J Cell Biol). However, such gene therapy presents significant challenges before it becomes a practical approach to treating disease. Therefore, we believe that potential novel therapeutic approaches (such as our study) should also be explored. Finally, a long-term study of proteasome inhibition with a more clinically relevant drug (Velcade) is in progress in our lab.

2. Much of the manuscript is not tied together in a manner that relates to one major question or hypothesis.

We have rewritten the results section in order to increase the cohesion of the various results.

3. Was the shift in fiber size distribution following the local and systemic injection found to be statistically significant?

We now show in Fig. 6D (previously 7D) and Supplemental Fig. 1B that the shift in fiber size distribution is statistically significant between all genotypes. In both figures, fiber size distribution is significantly different between dy^{3K}/dy^{3K} and injected dy^{3K}/dy^{3K} animals, but fiber size distribution is also significantly different between injected and wild-type animals.

4. I am bothered by the small number of animals used in many of the results. Were power analyses performed?

We have since added more numbers of animals. The number of animals per genotype used in each experiment/figure is presented in the methods section and in figure legends (along with information on which statistical test was used).

The number of mice used is also summarized below:

Fig. 1A: N=7 for WT; N=6 for dy^{3K}/dy^{3K} and N=3 for WT and dy^{3K}LNα1TG; 1B: N=6; 1C: N=3 for WT and N=5 for dy^{3K}/dy^{3K}
Fig. 2: N=7 for WT; N=6 for dy^{3K}/dy^{3K} and N=3 for WT and dy^{3K}LNα1TG
Fig. 3: N=6
Fig. 4A: N=6

Fig. 4B: N=5
Fig. 5: N=4 for WT; N=7 for dy^{3K}/dy^{3K}; N=5 for single injection; N=3 for double injection
Fig. 6A-D: N=4 for WT; N=7 for dy^{3K}/dy^{3K}; N=5 for single injection; N=3 for double injection
Fig. 6E: N=5 for WT; N=4 for dy^{3K}/dy^{3K}; N=5 for single injection
Fig. 7: N=6 for WT; N=6 for dy^{3K}/dy^{3K}; N=9 for injection 24 h and N=4 for injection 18 days
Fig. 8A: N=3 for WT; N=5 for dy^{3K}/dy^{3K}; N=5 for injected
Fig. 8B: N=6 for WT; N=4 for dy^{3K}/dy^{3K}; N=5 for injected
Fig 9: N=3 for WT; N=5 for dy^{3K}/dy^{3K}; N=5 for single injection; N=3 for double injection
Fig. 10A: N=14 for WT; N=7 for dy^{3K}/dy^{3K}; N=10 for single injection; N=9 for double injection
Fig. 10B: N=24 for dy^{3K}/dy^{3K}; N=7 for single injection; N=6 for double injection

Supp 1: N=3
Supp 2: N=4

We have since performed power analyses to estimate the number of mice needed.

5. The question of autophagy was very superficially assessed and the findings do not add anything to the manuscript.

We agree with the reviewer and have removed the autophagy related findings previously presented in Fig. 2.

6. I would like to see one more than one mouse in the movie, and treated and untreated side by side in the same movie.

We have added an additional movie. Since the non-injected mice do not live as long as injected, we show a video of age-matched 3.5-week-old mice (WT, dy^{3K}/dy^{3K} and two injected dy^{3K}/dy^{3K}) (Supplementary video 3).

7. Why did the investigators examine AKT phosphorylation in situ rather than directly assaying the muscles from treated mice?

We have examined Akt phosphorylation (and Hsp90) in injected animals. We show in a new Fig. 7A that levels of Hsp90 and pAkt308/Akt are normalized 24 hours after MG-132 injection. The level of pAkt473/Akt is also significantly higher in MG-132 treated muscle compared to dy^{3K}/dy^{3K} mice but remains reduced compared to wild-type animals.
Eighteen days after MG-132 injection, both pAkt308/Akt and pAkt473/Akt levels remain normal but Hsp90 expression is reduced (Fig. 7B).

8. The markers of congenital muscular dystrophy measured by the investigators are not the best indicators of overall muscle health. Would traditional muscle strength test and more markers of apoptosis be better? The significance of tenascin-C positive fiber as an outcome measure is not clear.

In a new Figure 9, we use the TUNEL enzymatic labeling assay to further determine the proportion of apoptosis in treated muscle. We find that the number of TUNEL positive fibers is significantly reduced in MG-132 treated dy^{3K}/dy^{3K} muscles.

The significance of tenascin-C positive fibers in laminin α2 chain deficient muscle is now described in more detail in the text (results section). It was initially reported (Ringelmann et al., 1999, Exp Cell Res) that expression of tenascin-C is upregulated and extends to the interstitium between muscle fibers, especially within focal lesions of mature *dy/dy* muscle (another mouse model of laminin α2 chain deficiency). In control muscle, tenascin-C is restricted to the myotendinous junction. We have since successfully used tenascin-C as a marker for pathological fibrosis in dy^{3K}/dy^{3K} mice (Gawlik et al., Hum Mol Gen 2004; 2006; Gawlik and Durbeej, Muscle Nerve in press) and its expression correlates well with fibrosis detected by hematoxylin and eosin. However, tenascin-C has scarcely been used as a marker in patients and as the reviewer indicates its importance in MDC1A pathogenesis remains to be defined. To further confirm the inhibition of fibrosis in MG-132 injected animals, we performed immunofluorescence staining for collagen III (Qiao et al., 2005, Proc Natl Acad Sci USA) and quantified the expression. Results presented in a new Fig. 5B corroborate our initial findings that fibrosis is significantly reduced. It should be noted however that tenascin-C positive areas are smaller compared to collagen III positive areas in both non-treated and treated muscles indicating that tenascin-C is only expressed in a subset of fibrotic tissue.

9. Since immunohistochemistry is not quantitative, I would like to see a western blot for laminin using antibodies for different chains.

We have carried out Western blot analysis to quantify the expression of laminin α4 chain. We show in a new Fig. 8B that levels of laminin α4 chain are significantly upregulated in dy^{3K}/dy^{3K} muscle compared to wild-type muscle. In MG-132 treated muscle, the amount of laminin α4 chain is significantly reduced, although it is not decreased to wild-type levels.

Unfortunately, the antibodies used for laminin β2 immunostaining did not work well in Western blot analyses (no band of correct size was detected in wild-type muscle sample). We have also tried another polyclonal antibody, but without success in immunoblotting. However, it has previously been convincingly shown that laminin β2 chain is downregulated at the sarcolemma of both mouse and human laminin α2 chain deficient muscle (e.g. Cohn et al., 1997, Neuromusc Disord; Gawlik et al., 2004, Hum Mol Genet).

10. The statement that Velcade "is a potential curative for MDC1A" is not founded by the data.

The referee is correct and we have rewritten that statement. It now reads "Hence, MDC1A patients may benefit from this drug."

Reviewer B

1. In the introduction and results, it should be emphasized that the transgenic rescue used is actually broadly expressed.

We provide a more thorough description of the transgenic rescue animals in the results and methods sections.

2. Is Akt and Hsp90 affected in nerve tissue? Is it reversed by the transgene? Is it reversed by MG-132?

pAkt308/Akt appears to be reduced whereas pAkt473/Akt appears unchanged in laminin α2 chain deficient sciatic nerve. Furthermore, Hsp90 expression is not altered and ubiquitination is not increased in laminin α2 chain deficient sciatic nerve, as evidenced by Western blot analyses. Hence, it appears that increased proteasome activity is <u>not</u> a feature of laminin α2 chain deficient nerve. Therefore, MG-132 should not be of any apparent help in nerve tissue and many injected mice displayed hind leg paralysis (of one leg only) at day 18 after injection (thus, we did not analyze nerve tissue from rescued and MG-132 injected animals). The sciatic nerve results are presented in Supplementary Figure 2 and discussed in the results section.

3. Fig. 4 should be shown as total Akt and phosphorylated Akt (both sites) relative to each other.

In Fig. 3 (which was previously Fig. 4), we show the levels of phosphorylated Akt relative to total Akt.

4. A longer treatment with MG132 should be carried out. What happens if additional injections are given?

This is a great suggestion and such experiments were carried out while the paper was under review. Briefly, we injected animals at 2.5 weeks of age (as previously described) and gave an additional injection at 3.5 weeks of age. Interestingly, this second injection was of no further functional benefit on the main outcome measures used to assess efficacy of treatment. Data on second injections are incorporated in Figs. 5, 6, 9 and 10.

5. Is myoblast function affected by MG-132?

It has previously been demonstrated that MG-132 induces apoptosis in human myoblast cultures (Sassone et al., 2006, Eur J Histochem). However, in a similar experimental setup (in our laboratory) MG-132 did not induce any cytotoxicity, whereas a more potent proteasome inhibitor MG-262 clearly induced cell death (data not shown). Furthermore, MG-132 rather reduces apoptosis in laminin α2 chain deficient muscle *in vivo*.

6. Does the single injection of MG132 reverse the Akt and Hsp90 findings in vivo?

We have examined Akt phosphorylation and Hsp90 levels in injected animals. We show in a new Fig. 7A that levels of Hsp90 and pAkt308/Akt are normalized

24 hours after MG-132 injection. The pAkt473/Akt level is also significantly higher in MG-132 treated muscle compared to dy^{3K}/dy^{3K} mice but remains reduced compared to wild-type animals.
Eighteen days after MG-132 injection, both pAkt308/Akt and pAkt473/Akt levels remain normal but Hsp90 expression is reduced (Fig. 7B).

Reviewer C

1. A more thorough description of the dy^{3K}/dy^{3K} mouse model would benefit those not familiar with the mouse mutations or the disease.

We now present a more comprehensive depiction of the dy^{3K}/dy^{3K} mouse model in the introduction and methods sections.

2. A more general distinction between the "WT" associated with the dy^{3K}/dy^{3K} and the dy^{3K}LNα1TG is needed throughout the figures and results presentation.

At this point, we supply a better explanation for the wild-type animals used in the methods section. Briefly, we always compare dy^{3K}/dy^{3K} with age-matched wild-type littermates. When we analyze dy^{3K}LNα1TG mice, (a different strain) we used age-matched wild-type littermate mice from the dy^{3K}LNα1TG strain.

3. Images for immunoblots of ubiquitination (Fig.1) and Hsp90 (Fig. 5) are needed. For ubiquitination, what is being quantified? For Hsp90, the arbitrary units are so different between the graphs in panel A, that it is unclear what these values actually mean.

We provide images for immunoblots of ubiquitination (Fig. 1) and Hsp90 (previously Fig. 5; now Fig. 4). For ubiquitination, the intensity within the entire lane is quantified. Moreover, we have repeated the Hsp90 Western blot analyzes and loaded the samples on the same gel. Previously, the samples from P6, 3.5-week-old- and 5.5-week-old animals, respectively, were run on different gels and thus, the arbitrary units were different between the graphs.

4. All of the immunostaining images need to have secondary antibody controls. Alternatively, dual labeling with a control protein would help to determine if the localization is appropriate.

In a new Fig. 1C, we show both secondary antibody controls as well as dual labeling with laminin γ1 antibodies (in green), which stain the sarcolemma, and 20S α1 and 20S β1 and MuRF1 antibodies (in red), respectively. The double stainings clearly indicate that MuRF1, 20S α1 and 20S β1 are enriched inside of the laminin α2 chain deficient skeletal muscle fiber. Also, no staining was evident in dy^{3K}/dy^{3K} muscle when omitting the primary antibodies.
5. For the autophagy genes, a Western blot would be more convincing than the immunohistochemistry. Again, the staining appears non-specific (why would LC3B be localized to the sarcolemma?).

Reviewer A found the question of autophagy very superficially assessed and that the findings did not add anything to the manuscript. Therefore, we have removed the data (LC3B and Cathepsin L mRNA analyses and LC3B immunostaining) previously presented in Fig. 2.

6. The explant methodology is not appropriate for determining if MG-132 is therapeutically useful.

We agree with the reviewer and have therefore removed the results obtained with the explant methodology.

7. Where is the dye in the histological images of MG-132 injections in quadriceps? Are the units for fiber size classes pixels or microns (Supplemental figure 1)? How do these fibers sizes compare to normal mice?

The dye can actually be seen in some of the figures (as small dots present mostly within connective tissue).
The units for fiber size classes are pixels.
We have now added the distribution of fibers in wild-type animals and the fiber size distribution of MG-132 injected muscle more resembles the wild-type situation.

8. Given the small size of dy^{3K}/dy^{3K} animals, is there a change in fiber number? What are the wet weights of muscle (fiber size may not be the appropriate index).

In a new Fig. 6A, we have counted the number of muscle cells in a randomly selected area. No change in fiber number of dy^{3K}/dy^{3K} animals was detected and the number of muscle fibers was not further altered upon MG-132 injections. The average fiber diameter of dy^{3K}/dy^{3K} muscle, on the other hand, was smaller compared to wild-type muscles (presented in Fig. 6C). Upon MG-132 injection, the fiber diameter was normalized.

We also report the wet weights of quadriceps muscles. The ratio of quadriceps wet weight per body weight was significantly reduced in dy^{3K}/dy^{3K} animals, compared to age-matched wild-type littermates. However, the ratio was significantly increased in MG-132 injected dy^{3K}/dy^{3K} animals (see new Fig. 6E).

9. An antibody that detects both pro- and cleaved caspase 3 can not be utilized to determine the proportion of apoptosis in any tissue. A more appropriate antibody would be one that detected only the cleaved form, which is associated with apoptosis, or to present the data by immunoblotting.

In a new Fig. 9, we use the TUNEL enzymatic labeling assay to further determine the proportion of apoptosis in treated muscle. We find that the number of TUNEL positive fibers is significantly reduced in MG-132 treated dy^{3K}/dy^{3K} muscles.

10. A brief description of the mutations/genotype and strain of the mouse models is needed.

We have provided a short explanation of the mutations/genotype of the mouse models in the methods section.

11. What is the rationale for using all hindlimb skeletal muscle vs. quadriceps?

Since different muscles may have different architecture and fiber composition, we reasoned that the UPS should be examined both at the global and single muscle level of laminin α2 chain deficient animals. This is now better clarified in the result section.

12. Muscle morphometry. Which muscle was used?

We used the quadriceps muscle for morphometric measurements (now indicated in the methods section).