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The p97/VCP ATPase is critical in muscle atrophy and the accelerated degradation of muscle proteins

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1st Editorial Decision 06 April 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the reports of three expert reviewers, which you will find copied below. Unfortunately, these reports offer insufficient support for publication of the study in The EMBO Journal. While it is clear that the topic of your investigation is of importance and your initial observations potentially interesting, all referees share the opinion that the study is at this stage not sufficiently developed for publication in a broad general journal. I will not repeat all their individual concerns here, but the main problems lie with inconsistencies and major discrepancies within the present set of data, as well as with incomplete and inconclusive analysis on a number of issues. As a result, the reviewers consider the main conclusions currently insufficiently supported and in many cases overinterpreted.

Given these critical opinions and shared major concerns, I have to conclude that your study remains currently too preliminary to be a good candidate for publication in The EMBO Journal, and that we cannot invite a revision at this point. As we receive a high number of submissions, we can only consider those manuscripts further that receive elevated enthusiasm from at least a majority of referees already upon initial review, and that appear to be sufficiently close to becoming publishable during a limited revision period. I am sorry we cannot be more positive on this occasion, but in any case hope that you will find our referees' detailed comments helpful for your further work on this topic.

Yours sincerely,
Editor

The EMBO Journal

Referee reports

Referee #1 (Remarks to the Author):

The authors investigated the role of p97/VCP (Valosin Containing Protein) in muscle wasting since this ATPase complex facilitates the proteasome-dependent breakdown of some peptides. In addition p97 mutation results in inclusion body myopathy. It is shown that the muscle protein content of p97 and of some of its cofactors (Ufd1 and p47 but not Ufd2) increased following denervation atrophy, but not in fasting. By contrast, electroporation of a dominant negative p97 (DNp97) into adult mouse muscles reduced fiber atrophy in both catabolic conditions. The data suggest that DNp97 associated with myofibrils and caused accumulation of polyubiquitinated components of both thick and thin filaments. In C2C12 myotubes DNp97 blocked enhanced proteasome-dependent and lysosomal proteolysis induced by FoxO3. This effect resulted in increased myotube protein content, and in adult muscles in increased muscle mass. It is concluded that p97 promotes muscle proteolysis and more particularly the breakdown of contractile proteins. The data are new and potentially interesting, but there are several major problems with the data and their interpretation.

Major points

1. The effect of DNp97 on cross-sectional areas of muscle fibers shown in Fig. 2 in either denervation or fasting is very convincing. Unfortunately this beneficial effect is not reflected by parallel changes in mRNA and protein levels of p97 and cofactors in both catabolic conditions. Furthermore, small changes in mRNA levels of both p97 and Ufd1 in denervated muscle (Fig. S1C) result in large increases in the corresponding protein levels (Fig. 1A). By contrast, a very large increase in mRNA level of p97 in starvation (Fig. S1B) results in no significant effect on p97 protein level (Fig. S1A). Therefore the precise significance of these variations remains entirely elusive.

2. Since discrepant data were observed with fasting and denervation atrophy (see above), additional experiments must be performed in at least another catabolic state (i.e. in cachectic muscles from cancer mice).

3. The experiments are incomplete. The effects of caFoxO3 and WT or DNp97 on the expression of genes of both the ubiquitin (i.e. MuRF-1 and atrogin-1) and the lysosomal (i.e. LC3 and Gararapl1) pathways must be provided.

4. All quantifications of protein levels should be corrected by an internal standard. The authors state that 'equal amounts of protein were loaded per lane' (Fig. S1 legend). However, when a loading control is provided there are obvious variations in protein loading (see Figs. 3E, 5C, 9B, S2B). Also, the Western Blot for GAPDH shown in Fig. S2A is overexposed, not interpretable and must be reperformed.

5. The authors used a *FK2* antibody to reveal polyubiquitin conjugates (Figs. 9B, 10A,B). These experiments must be re-performed with a *FK1* antibody that does not reveal monoubiquitinated species

6. The data are largely over-interpreted. For example the authors state (bottom of page 7) that 'Because these proteins are only found in intact proteasomes in stochiometric amounts, these data indicate a 2-fold rise in proteasome content in the denervated muscle'. It is totally unclear how the authors can deduce that 'these changes in muscle weight must underestimate by 25-66% the actual increase in mass of the fibers where p97 is inhibited or depleted' (bottom of page 10). 'MyLC2 and actin appear to be novel p97 substrates' (bottom of page 15). The data only show an association between p97 and these contractile proteins. Finally, the paper does not provide evidence for a role of p97 in reducing myofibrillar disassembly.

Referee #2 (Remarks to the Author):

The authors investigated the function of p97 and its cofactors during muscle atrophy when highly organized myofibrils are rapidly degraded. This study shows that p97 plays a critical role during atrophy in degrading muscle proteins via both the proteasomal and autophagic pathways, as well as in the disassembly of the myofibrillar apparatus. Thus in normal adult muscles and myotubes, p97 functions by promoting muscle protein degradation to limit muscle growth.

Authors asked; 1) how the expression of p97 and its cofactors may change in atrophying muscles, 2) whether overproduction of WTp97 or an ATPase deficient-p97 mutant influences the progression of the atrophy process and the size of normal muscles (through effects on overall rates of protein synthesis, protein degradation or myofibrillar disassembly) and 3) whether accelerated breakdown of myofibrillar proteins is dependent on p97. The answers are appropriately discussed in the text, however the overall findings are not novel and additional experiments have to be done to clarify the authors interpretation.

Comments:

1) The muscle content of p97 and two of its cofactors increase after denervation
- To test if induction of these proteins is a general feature of atrophying muscles, the authors examined levels of p97, its cofactors and proteasomal subunits during rapid weight loss caused to fasting. However, in contrast to denervation atrophy, no increase in the amount of p97, Ufd1, p47 and Rpt1 was seen 1 or 2 d after food deprivation (Fig. S1A), despite the loss of about 10% of muscle mass.- But the level of p97, its cofactors and proteasomal subunit changed strongly by 10 d after denervation when muscle weight decreases by 40%, so it was too early to speculate about their induction during fasting. Moreover, in the next paragraph the authors claimed that 'DNp97 also caused a clear inhibition of fasting-induced atrophy'. This should be clarified. "Because, the increase in expression of p97, its cofactors and proteasomes occurs with disuse, but not during the rapid atrophy with fasting, these genes cannot be classified as atrogenes"- this conclusion needs a proper confirmation.

2) Inhibition of p97 decreases muscle atrophy

"In order to establish whether p97 function is essential for these rapid types of atrophy, we tested whether inhibition of p97 can reduce muscle wasting by comparing the effects of expression of WTp97 and the p97K524A mutant, a dominant negative inhibitor (DNp97). Mutation of lysine 524 to alanine in the D2 ATPase domain of p97 allows the binding of p97 to protein substrates while decreasing substantially p97 ATPase activity, and thus preventing substrate degradation by the ubiquitin proteasome pathway. " - It is not mentioned that the p97K524A mutation also weakens the interaction with p97 cofactors like p47. What's missing here is showing that DNp97 binds its cofactors in muscle cells. This experiment is important for further conclusions.

3) p97 inhibition decreases FoxO3-stimulated proteasomal and lysosomal proteolysis

Could be interesting to verify if inhibition of the proteasome (1 M Bortezomib/Velcade) or lysosomal acidification (10mM NH4Cl) could increase muscle fiber size similar or even more than DNp97?

4) p97 inhibition causes muscle growth independently of the myosin-chaperone UNC45B

"We did not observe any change in the content of UNC45B either during atrophy induced by denervation or food deprivation (Fig. 8A)." Loading control is missing. Level of UNC-45B varies in samples after food deprivation, day2.

"When GFP or UNC45B were expressed for 7 d, the fibers overexpressing UNC45B were reproducibly only about 10% larger in median area than control fibers (Fig. 8B), and no difference was found in the weights of these muscles (Fig. 8C)."- That's an interesting observation. Might be that UNC45B is able to increase muscle fiber size, whereas its overexpression induces degradation of muscle components (myosin) via p97?

5) p97 associates with myofibrillar proteins and promotes actin and MyLC2 degradation

"We also found that expression of the DNp97 in myotubes, but not GFP, resulted in an accumulation of ubiquitinated MyLC2 and actin (Fig. 9B)." This is not clear. There is also no signal of MyLC2 in lane DNp97-72h.

"Interestingly, other components of the myofibrils, like Myosin Heavy Chain (MyHC) and Myosin-binding protein-C (MyBP-C) (Fig. 9B), did not show any clear electrophoretic change even though these proteins are degraded at similar times and are ubiquitinated by the same E3, MuRF1, as MyLC2 (8). "- On the figure 9b it looks like MyHC and MyBP-C are less stable in the DNp97 background. How could this be explained? Is the increase in muscle content caused by DNp97 based on stabilization of thin filaments? What is the ratio of myosin-actin in these muscles? Staining of muscle myosin and actin should be done.

Referee #3 (Remarks to the Author):

The manuscript by Piccirillo and Goldberg identifies a novel role for VCP in muscle atrophy and muscle protein degradation. These findings are important to understanding the regulation of muscle atrophy, muscle disease in particular VCP related disease and developing therapeutics aimed at muscle atrophy. Many of studies are solid especially the initial observation however this reviewer has some concerns with the proposed mechanism. In particular statements in the abstract are not justified by the experiments "DNp97 becomes associated with myofibrils and caused accumulation of ubiquitinated components..." And "thus p97 appears to extract ubiquitinated proteins from myofibrils." Many of these concerns can be addressed with additional confirmatory experiments.

Major Concerns:
1) The use of the p97-K524A is problematic for Figure 9 and 10. The KA mutant abolishes the Walker A motif. This mutant serves as potent dominant negative. This is in contrast to the EQ mutant in the Walker B motif. This mutant can bind ATP and not hydrolyze ATP. Typically EQ mutants are used as "substrate trap" mutants. While it is true that in the citation used (27), it would be more convincing to repeat these experiments using a p97-E578Q mutant. Other problems with experiment 9 and 10 are discussed later.
2) Figure 9A suggests that the DNp97 is bound to myofibrillar proteins. How pure is the myofibrillar prep? Are aggregate proteins present? Are membranous components present? Are nuclear components present. VCP could be binding to any substrate. In addition, VCP itself could merely be misfolded and aggregated as well becoming insoluble. The conclusion for Figure 9A does not support that VCP is "bound to myofibrils". To do this immunopreciptations or at the least co-localization experiments need to be done. This experiment should also be done with EQ mutant that has less of a tendency to misfold since ATP binding at the D2 domain may help stabilize oligomeric structure.
3) Figure 9B is not convincing. The levels of actin and MyLC2 go up only modestly. I am unclear how they know that they are "ubiquitinated" since all we see is a higher molecular weight smear. In addition, a WT control is needed not just a GFP control.
4) In Figure 10A, why is there less MyLC2 in the p97DN expressing myotubes already at baseline. Doesn't this go against the hypothesis that it impedes degradation. In addition, why do I not see any MyLC2 when it is IP'ed in the presence of DNp97 (lane 6 of 10A). At best you can say that it IPs ubiquitin conjugates. Figure 10B shows an IP of actin but again, I am not certain that you can say it is ubiquitinated. These experiments all need controls with VCP-WT and the VCP-EQ mutants. In addition, since the DN increases total ubiquitin conjugates, these experiments should be done in the presence of proteasome inhibition.
5) Figure S2 I need to see quantitation of the degree of silencing. From my eyes it less than 10%. In addition, confirmation of the decrease in VCP needs to be shown in vivo. It seems to me that this should be possible with large effect seen in vivo with the shRNA constructs.
6) the increase in VCP/UFD1 protein was only performed at day 3 and day10 it is not fair to say that the increase in VCP/UFD1 occurred "exactly when the components of the thick filament are lost most rapidly" since the levels were not checked at days 4-9.
Minor Concerns:
1) The manuscript uses the word disuse atrophy (in several places) when actually they have only

looked at dennervation atrophy. For example page 9 "thus inhibition of p97 prevented completely disuse atrophy." No experiments were done using disuse models.
2) The increase in ubiquitinated conjugates in figure 5c is not very convincing. A positive control with proteasome inhibitors would be helpful.
3) In the abstract: VCP mutations more correctly cause "an inclusion body myopathy phenotype" within the context of IBMPFD.

Overall the authors have the beginnings of a good story but do not have the data to support their conclusions.

Resubmission 13 November 2011

Response to the comments of the reviewers:

The referees' critiques clearly indicated ways for us to strengthen the manuscript and our data presentation. The revised version includes appreciable new data and shows clearer and more dramatic changes than the prior version. These additions should resolve the various concerns of the referees and also provide further mechanistic insights into how p97 complex influences muscle fiber size. We are convinced that this version is much improved by the changes made.

Referee #1:

The authors investigated the role of p97/VCP (Valosin Containing Protein) in muscle wasting since this ATPase complex facilitates the proteasome-dependent breakdown of some peptides. In addition p97 mutation results in inclusion body myopathy. It is shown that the muscle protein content of p97 and of some of its cofactors (Ufd1 and p47 but not Ufd2) increased following denervation atrophy, but not in fasting. By contrast, electroporation of a dominant negative p97 (DNp97) into adult mouse muscles reduced fiber atrophy in both catabolic conditions. The data suggest that DNp97 associated with myofibrils and caused accumulation of polyubiquitinated components of both thick and thin filaments. In C2C12 myotubes DNp97 blocked enhanced proteasome-dependent and lysosomal proteolysis induced by FoxO3. This effect resulted in increased myotube protein content, and in adult muscles in increased muscle mass. It is concluded that p97 promotes muscle proteolysis and more particularly the breakdown of contractile proteins. The data are new and potentially interesting, but there are several major problems with the data and their interpretation.

Major points

1. The effect of DNp97 on cross-sectional areas of muscle fibers shown in Fig. 2 in either denervation or fasting is very convincing. Unfortunately this beneficial effect is not reflected by parallel changes in mRNA and protein levels of p97 and cofactors in both catabolic conditions. Furthermore, small changes in mRNA levels of both p97 and Ufd1 in denervated muscle (Fig. S1C) result in large increases in the corresponding protein levels (Fig. 1A). By contrast, a very large increase in mRNA level of p97 in starvation (Fig. S1B) results in no significant effect on p97 protein level (Fig. S1A). Therefore the precise significance of these variations remains entirely elusive.

We are pleased that the referee found the changes in muscle mass with DNp97 to be very convincing, but the referee's comments seem to suggest some misunderstanding of our experiments. We have never studied the mRNA and protein content during these "beneficial effects", since our data indicate them to be primarily through reduced proteolysis. We have studied the changes in expression of p97, its cofactors and proteasomes during the two extreme types of atrophy but do not find any surprising discrepancies. Since both denervation and fasting involve marked acceleration of protein degradation, it is not surprising to find increases in mRNA or even protein synthesis without a corresponding enhancement of protein accumulation. (In fact, we speculated in the text that the increased in mRNA in fasting might simply serve to maintain high levels of specific proteins in the face of accelerated proteolysis). Necessarily, mRNA accumulates before the corresponding protein, as we observe for the changes in p97 and cofactors during denervation (Fig. 10A and S1C of the revised version). Thus, it is possible that the increased p97 mRNA in fasting would have led to an increase in this protein, if the duration of fasting lasted for 3 d (which was avoided for ethical reasons). We have confirmed our original data with further samples, and have reorganized the paper, because these measurements of protein and mRNA expression are clearly not the focus of our study, and seemed to have distracted the reader from our main points, p97's role in atrophy and myofibrillar turnover.

2. Since discrepant data were observed with fasting and denervation atrophy (see above), additional experiments must be performed in at least another catabolic state (i.e. in cachectic muscles from cancer mice).

We do not understand how data on a third catabolic state would clarify an apparent difference between atrophy induced by fasting and denervation (especially when our main point is that p97 is essential for both). Furthermore, this apparent discrepancy may not exist, since mRNA for p97

rises in both conditions (Fig. S1B and C), and the protein levels for p97 may therefore rise subsequently in the fasted group, i.e. at times we could not assay (for ethical reasons). Our earlier version of the data and discussion may have been confusing. The present findings would suggest that p97 actually might be classified as an "atrogene" based on the increased mRNA levels in both conditions. Apparently, the referee failed to note that we actually did measure another catabolic state. As mentioned in the discussion, in muscle wasting in aged animal (sarcopenia), we also found increases in p97 and p47 protein and mRNA, which was published elsewhere (Altun et al, 2010).

3. The experiments are incomplete. The effects of caFoxO3 and WT or DNp97 on the expression of genes of both the ubiquitin (i.e. MuRF-1 and atrogin-1) and the lysosomal (i.e. LC3 and Gabarapl1) pathways must be provided.

We agree with the referee and have added the requested experimental data, showing that the DNp97 did not affect the expression of these atrogenes upon overexpression of caFoxO3 (Fig. 5C). Thus, p97 is affecting the proteolysis catalyzed by these ubiquitin ligases and autophagic proteins, not their induction by FoxO3.

4. All quantifications of protein levels should be corrected by an internal standard. The authors state that 'equal amounts of protein were loaded per lane' (Fig. S1 legend). However, when a loading control is provided there are obvious variations in protein loading (see Figs. 3E, 5C, 9B, S2B). Also, the Western Blot for GAPDH shown in Fig. S2A is overexposed, not interpretable and must be reperformed.

The reviewer has raised a major challenge that has complicated the study of atrophy. Since the levels of nearly all proteins change during atrophy, it is very difficult to normalize to an internal control. (None of the typical internal standard proteins are constant in the various types of atrophy.) Therefore, we currently study 5-7 animals for each condition, measure protein

concentrations with the BCA or Bradford methods on each, load equal amounts of proteins (e.g. see (Brault et al, 2010)), and average results. Nonetheless, because of the referee's concern with variability in loading equal amount of proteins (GAPDH), in the experiments on normal muscles and cells, we provide data with another loading control in Fig. 2E (for muscle electroporated with WT and DNp97, Rpl26, which is not altered by these treatments) and replaced the overexposed GAPDH with tubulin in Fig. S2A.

*5. The authors used a *FK2* antibody to reveal polyubiquitin conjugates (Figs. 9B, 10A,B). These experiments must be re-performed with a *FK1* antibody that does not reveal monoubiquitinated species*

We fully agree with the referee on this point and repeated all the blots with FK1 and described the antibody in the Results section to avoid possible misunderstandings. In the revised version, Fig. 4C, 5C, 8E, 9A-D and S4 contain blots that shows polyubiquitinated conjugates detected with FK1 (which does not recognize monoubiquitinated proteins).

6. The data are largely over-interpreted. For example the authors state (bottom of page 7) that 'Because these proteins are only found in intact proteasomes in stochiometric amounts, these data indicate a 2-fold rise in proteasome content in the denervated muscle'.

We do not understand why the referee thinks that our statements are over-interpreted. Perhaps, we failed to explain our logic sufficiently. A variety of prior studies have indicated that these proteasome subunits (Rpt1, the ATPase subunit, as well as the various 20S subunits that react with the polyclonal antibody) were present in cells overwhelmingly in the 19S and 20S complexes. (Precursors do exist, but their levels are minor.) Thus, levels of these two proteins can be used as a pretty good approximate measure of proteasome content, as we and others have found (e.g. (Altun et al, 2010)).

It is totally unclear how the authors can deduce that 'these changes in muscle weight must underestimate by 25-66% the actual increase in mass of the fibers where p97 is inhibited or depleted' (bottom of page 10).

In retrospect, we agree with the referee that this statement was not clear and deleted this confusing sentence from the text. However, the logic was not invalid. We had calculated the degree of underestimation of fiber hypertrophy by dividing the mean change in weight by the percent of electroporated fibers. Such calculations were unnecessary and were dropped in this revised version.

'MyLC2 and actin appear to be novel p97 substrates' (bottom of page 15). The data only show an association between p97 and these contractile proteins. Finally, the paper does not provide evidence for a role of p97 in reducing myofibrillar disassembly.

We do not agree with the reviewer's final statement and believe our evidence is quite strong. Previously, this mutation in p97 (K524A) was shown to bind substrates in an ATP-dependent manner and not to release them (Ye et al, 2003). Consequently, this DN mutant has been used previously as a "substrate trap mutant", as we do here. Therefore, the binding of these contractile proteins (specifically, after ubiquitination) to DNp97 with specific p97 cofactors and also their accumulation as ubiquitinated species (i.e. intermediates in the ubiquitin-proteasome pathway) indicate that they are substrates (Fig. 9). Furthermore, we show that the association between these contractile proteins and p97 requires ATP (as we mention in the Results section in this new version). Finally, we are surprised that the referee questions whether DNp97 blocks myofibrillar disassembly. As we showed, inhibiting p97 reduces atrophy and causes increases in total muscle proteins (Table I). Consequently, there is a sparing of myofibrillar proteins and as we show, simultaneously the non-dissociating p97 accumulates on the myofibrils especially during

denervation atrophy (Fig. 8B and C). In summary, MyLCs and actin behave as substrates because they associate with p97 in ubiquitinated form and in an ATP-dependent fashion with specific cofactors. Together, these various findings indicate a role of p97 in protein release from myofibrils. In this revised version, we have included a careful presentation of the different observations, indicating a role of p97 in myofibrillar disassembly (see page 16 and 23).

Referee #2:

The authors investigated the function of p97 and its cofactors during muscle atrophy when highly organized myofibrils are rapidly degraded. This study shows that p97 plays a critical role during atrophy in degrading muscle proteins via both the proteasomal and autophagic pathways, as well as in the disassembly of the myofibrillar apparatus. Thus in normal adult muscles and myotubes, p97 functions by promoting muscle protein degradation to limit muscle growth.

Authors asked; 1) how the expression of p97 and its cofactors may change in atrophying muscles, 2) whether overproduction of WTp97 or an ATPase deficient-p97 mutant influences the progression of the atrophy process and the size of normal muscles (through effects on overall rates of protein synthesis, protein degradation or myofibrillar disassembly) and 3) whether accelerated breakdown of myofibrillar proteins is dependent on p97. The answers are appropriately discussed in the text, however the overall findings are not novel and additional experiments have to be done to clarify the authors interpretation.

In the referee's summary, there is the very surprising comment that "the overall findings are not novel". We completely disagree with this statement and cannot understand its basis. To our knowledge, our conclusions and approach are highly novel. In fact, we know of no prior publication that has investigated any of the questions addressed here concerning the role of p97 on myofibrillar turnover, muscle atrophy, hypertrophy, or p97 expression during atrophy.

1) The muscle content of p97 and two of its cofactors increase after denervation
- To test if induction of these proteins is a general feature of atrophying muscles, the authors examined levels of p97, its cofactors and proteasomal subunits during rapid weight loss caused to fasting. However, in contrast to denervation atrophy, no increase in the amount of p97, Ufd1, p47 and Rpt1 was seen 1 or 2 d after food deprivation (Fig. S1A), despite the loss of about 10% of muscle mass.- But the level of p97, its cofactors and proteasomal subunit changed strongly by 10 d after denervation when muscle weight decreases by 40%, so it was too early to speculate about their induction during fasting. Moreover, in the next paragraph the authors claimed that 'DNp97 also caused a clear inhibition of fasting-induced atrophy'. This should be clarified. "Because, the increase in expression of p97, its cofactors and proteasomes occurs with disuse, but not during the rapid atrophy with fasting, these genes cannot be classified as atrogenes"- this conclusion needs a proper confirmation.

The referee's comments suggest some confusion, which may have been generated by a typographical error in the prior version. That version may have also been unclear in not

distinguishing sufficiently strongly between responses one or two days after food deprivation and between changes in mRNA and protein. In the revised version, we make the simple point that p97 function is absolutely essential for atrophy upon fasting and denervation, although increases in p97 content were not evident in fasting for 2 days. Secondly, the increase in p97 mRNA seen at late times after denervation and fasting could lead to its being viewed as an atrogene (Fig. S1B and C).

Because the prior version was somewhat confusing, the revised version has been extensively reorganized to emphasize the main findings and novel conclusions. Therefore, the text now starts with the point that the referee wanted to have clarified—that the DNp97 caused a clear inhibition of atrophy due to fasting as well as denervation atrophy (Fig. 1). In the new version, we present these findings on atrophy initially and also show new, more compelling data than was shown previously. This major conclusion probably was obscured by the less important data on changes in expression of p97 and cofactors. We certainly agree with the reviewer's insight that the failure to see increases in p97, Ufd1 and proteasome subunit levels in fasting may be deceptive, and only results from the brief duration of the fasting (which has to be terminated at 2 days for ethical reasons). The possibility noted by the referee is now mentioned in the text and Discussion.

2) Inhibition of p97 decreases muscle atrophy

"In order to establish whether p97 function is essential for these rapid types of atrophy, we tested whether inhibition of p97 can reduce muscle wasting by comparing the effects of expression of WTp97 and the p97K524A mutant, a dominant negative inhibitor (DNp97). Mutation of lysine 524 to alanine in the D2 ATPase domain of p97 allows the binding of p97 to protein substrates while decreasing substantially p97 ATPase activity, and thus preventing substrate degradation by the ubiquitin proteasome pathway. " - It is not mentioned that the p97K524A mutation also

weakens the interaction with p97 cofactors like p47. What's missing here is showing that DNp97 binds its cofactors in muscle cells. This experiment is important for further conclusions.

We are surprised by the referee's assertion that Kobayashi et al. (Kobayashi et al, 2002) showed that the interaction with cofactors including p47 is lower for the DNp97. In this new version, we include Western Blots and show that the interaction with p47 is not markedly decreased in the K524A mutant (Fig. 9D), when equal amounts of p47 are pulled down with WT and the DNp97. In the revised version, we provide the information desired by the referee, i.e. in Fig. 9C and 9D, we show that immunoprecipitation of Ufd1 or p47 brought down WT and the DNp97.

3) p97 inhibition decreases FoxO3-stimulated proteasomal and lysosomal proteolysis

Could be interesting to verify if inhibition of the proteasome (1 μM Bortezomib/Velcade) or lysosomal acidification (10mM NH4Cl) could increase muscle fiber size similar or even more than DNp97

We agree with the referee that such *in vivo* experiments are potentially interesting, and we had previously carried out some, but the results are not interpretable for several reasons. In unpublished studies, we have observed an inhibition of muscle atrophy with Bortezomib treatment of mice, and similar data have been published by one group (Beehler et al, 2006). It is noteworthy that the inhibition observed is smaller than that seen here with DNp97. However, it is impossible to make any rigorous conclusions because only low doses of Bortezomib (that inhibit total protein breakdown by 30%) are possible for long periods without killing the mice. Similarly, NH_4Cl and chloroquine cannot be used *in vivo* at concentrations that markedly inhibit protein degradation. Also, experiments with these inhibitors in many cultured cells cause toxicity by 10 hours at doses that block lysosomal function and 12-18 hours with complete blockage of

proteasome function. (In light of the toxicity of these inhibitors, the effects of p97 prolonged inhibition in myotubes and muscle are particularly surprising.)

4) p97 inhibition causes muscle growth independently of the myosin-chaperone UNC45B

"We did not observe any change in the content of UNC45B either during atrophy induced by denervation or food deprivation (Fig. 8A)." Loading control is missing. Level of UNC-45B varies in samples after food deprivation, day2.

The reviewer has raised a major challenge that has complicated the study of atrophy. Since the levels of nearly all proteins change during atrophy, it is very difficult to normalize to an internal control. (None of the typical internal standard proteins are constant in the various types of atrophy.) Therefore, we currently study 5-7 animals for each condition, measure protein concentrations with the BCA or Bradford methods on each, load equal amounts of proteins (e.g. see (Brault et al, 2010)), and average results.

"When GFP or UNC45B were expressed for 7 d, the fibers overexpressing UNC45B were reproducibly only about 10% larger in median area than control fibers (Fig. 8B), and no difference was found in the weights of these muscles (Fig. 8C)."- That's an interesting observation. Might be that UNC45B is able to increase muscle fiber size, whereas its overexpression induces degradation of muscle components (myosin) via p97?

The referee may have misunderstood our findings. We found that UNC45B overexpression caused a small, but significant increase in fiber diameter (Fig. 7B). Consequently, we agree with the referee that UNC45B can promote growth (although much less than the DNp97) and may even affect myosin degradation. However, we do not see any rationale for the referee's hypothesis that it promotes myosin degradation. Since we also found that downregulation of UNC45B has similar effects as overexpression for reasons that are not clear (Fig. 7D), it seems

premature to propose a model or hypothesize about UNC45B's effects on synthesis and degradation.

5) p97 associates with myofibrillar proteins and promotes actin and MyLC2 degradation

"We also found that expression of the DNp97 in myotubes, but not GFP, resulted in an accumulation of ubiquitinated MyLC2 and actin (Fig. 9B)." This is not clear. There is also no signal of MyLC2 in lane DNp97-72h.

"Interestingly, other components of the myofibrils, like Myosin Heavy Chain (MyHC) and Myosin-binding protein-C (MyBP-C) (Fig. 9B), did not show any clear electrophoretic change even though these proteins are degraded at similar times and are ubiquitinated by the same E3, MuRF1, as MyLC2 (8). "- On the figure 9b it looks like MyHC and MyBP-C are less stable in the DNp97 background. How could this be explained? Is the increase in muscle content caused by DNp97 based on stabilization of thin filaments? What is the ratio of myosin-actin in these muscles? Staining of muscle myosin and actin should be done.

We apologize for the lack of clarity in the prior version, which we have clarified this time. As shown in Fig. 8E, unmodified MyLC2 was missing at 72h of DNp97 expression, because at this time, the protein is present almost completely in higher molecular weight forms due to ubiquitination, as we show in Fig. 8E and S4B of the new version. In Fig. S4B, the IP of MyLC2 showed a smear corresponding to the ubiquitinated species. Therefore, the amount of unmodified MyLC2 immunoprecipitated was much less than in GFP infected cells. However, the IP of MyLC2 in the new Fig. 9B has been repeated at 48h to avoid confusing the reader.

In contrast to the referee's impression, based on Fig. 8E (new version), we did not find any consistent changes in the levels of MyHC and MyBP-C in DNp97 samples, even after band quantitation using a densitometric analysis. We have quantified the bands of Fig. 8E, normalized to GAPDH and found no accumulation over time of MyHC or MyBP-C. In the legend of Fig. 8, we added further data to answer the reviewer's other concern: No appreciable difference of the

MyHC/actin ratio in the Coomassie Blue-stained myofibrils from muscles expressing WTp97 or DNp97 was found, suggesting that both thin and thick filaments are degraded by p97-dependent mechanisms. As requested, we did measure the ratio myosin/actin in muscle electroporated with WT or DN and that does not change. We mention this result and provide values in the text (see aforementioned legend of Fig. 8). These points should be clearer in the revised version.

We therefore see no reason for staining of muscle sections, which would be less precise than the accurate quantification of myofibrillar protein (i.e. the ratio of myosin/actin) that we performed.

Referee #3:

The manuscript by Piccirillo and Goldberg identifies a novel role for VCP in muscle atrophy and muscle protein degradation. These findings are important to understanding the regulation of muscle atrophy, muscle disease in particular VCP related disease and developing therapeutics aimed at muscle atrophy. Many of studies are solid especially the initial observation however this reviewer has some concerns with the proposed mechanism. In particular statements in the abstract are not justified by the experiments "DNp97 becomes associated with myofibrils and caused accumulation of ubiquitinated components..." And "thus p97 appears to extract ubiquitinated proteins from myofibrils." Many of these concerns can be addressed with additional confirmatory experiments.

We are pleased that the referee found our findings important for understanding muscle atrophy and disease. We are surprised that the referee questions whether DNp97 blocks myofibrillar disassembly. As we showed, inhibiting p97 reduces atrophy and causes increases in total muscle proteins (Table I). Consequently, there is a sparing of myofibrillar proteins and as we show, simultaneously the non-dissociating p97 accumulates on the myofibrils especially during denervation atrophy (Fig. 8B and C). In this revised version, we have included a careful presentation of the different observations, indicating a role of p97 in myofibrillar disassembly (see page 16 and 23).

Major Concerns:

1) The use of the p97-K524A is problematic for Figure 9 and 10. The KA mutant abolishes the Walker A motif. This mutant serves as potent dominant negative. This is in contrast to the EQ mutant in the Walker B motif. This mutant can bind ATP and not hydrolyze ATP. Typically EQ mutants are used as "substrate trap" mutants. While it is true that in the citation used (27), it would be more convincing to repeat these experiments using a p97-E578Q mutant. Other problems with experiment 9 and 10 are discussed later.

We do not see why the p97K524A mutation is problematic, especially since these results have been confirmed by another approach. Although we agree with the referee that the E578Q mutant might have some advantages to confirm our observations, we have confirmed these results in a

totally independent way, by inhibiting p97 function with a shRNA for p97 (Fig. 2D, F and G). We do not see any disadvantage in our using the ATP-binding mutant in the D2 domain, since it still forms a hexamer, binds to Ufd1 or p47 equally (as we show in Fig. 9C and D of this revised manuscript) and was shown to trap substrates (Ye et al, 2003). Furthermore, the association with substrates requires ATP binding to the D1 domain (Ye et al, 2003), which is intact in p97K524A mutant. Accordingly, without the addition of ATP to the IP, we could not see binding to the myofibrillar proteins (as mentioned in the Results of the revised paper). Therefore, the trap DNp97 is acting as it should in substrate binding and as a dominant-negative inhibitor. Finally, the referee may not appreciate that in these experiments, we are probably studying complexes containing WTp97 plus some mutant subunits.

2) Figure 9A suggests that the DNp97 is bound to myofibrillar proteins. How pure is the myofibrillar prep? Are aggregate proteins present? Are membranous components present? Are nuclear components present. VCP could be binding to any substrate. In addition, VCP itself could merely be misfolded and aggregated as well becoming insoluble. The conclusion for Figure 9A does not support that VCP is "bound to myofibrils".

The referee raises important questions about the quality of myofibrillar preps, which we failed to document in the prior version. Data have been added to provide the key information requested by the referee. In Fig. 8A we provide clear evidence of the purity of the myofibrillar preps by blotting for different subcellular markers, requested by the referee (HDAC1, VDAC1 and MyLC2). Also, we have followed the protocol we described previously in *J Cell Biol* (Cohen et al, 2009) where the purity of these preparations was documented. Even when we performed only a gentle washing method to isolate myofibrils (see Materials and Method) in Fig. 8C and D, we still did not see measurable contamination of the myofibrillar fraction by other subcellular fractions (e.g. using the nuclear marker, HDAC1) (see Fig. 8D). Importantly, we also show in

this version that a proportion of endogenous p97 is actually bound to myofibrillar proteins, since KI extraction, which solubilizes the contractile proteins, partially solubilizes p97 (Fig. 8D). The DNp97 used is unlikely to be misfolded since it still fluoresces like the WT, and its association with the myofibrils is ATP-dependent (see Fig. 8B and text in the Results section). The DNp97 does not seem to be aggregated or associated with large protein aggregates, since we could not see inclusions by immunofluorescence in sections of electroporated muscles (Fig. 1C). Moreover, in Fig. S3 we showed that DNp97 is present in the soluble fraction of fully differentiated myotubes even if overexpressed. In the revised (and improved) version, we also were able to show a relocalization of DNp97 to myofibrils during denervation by fluorescence measurements (Fig. 8C).

To do this immunoprecipitations or at the least co-localization experiments need to be done.

Perhaps the referee failed to notice the immunoprecipitations that were included in the previous version of our paper. In this version, we repeated the additional controls requested by the referee (i.e. with proteasome inhibitors and/or in conditions of WTp97 overexpression) in Fig. 9A and B and moved the IPs from the previous version to Fig. S4. Colocalization experiments in muscle section have been done, but unfortunately, GFP showed periodic striations by itself that is indistinguishable from DNp97. Also, muscle self-fluoresces a lot showing a very high green background. However, our selective extraction of p97 by KI together with the contractile proteins supports nicely our conclusions (Fig. 8D).

This experiment should also be done with EQ mutant that has less of a tendency to misfold since ATP binding at the D2 domain may help stabilize oligomeric structure.

For the reasons explained above, we do not believe that there is any reason to conclude that the DNp97 used in our study is misfolded *in vivo*. As discussed above, the p97 mutant studied here is associated with cofactors, and it binds proposed substrates in an ATP-dependent manner, in accord with prior studies (Kobayashi et al, 2002; Ye et al, 2003).

3) Figure 9B is not convincing. The levels of actin and MyLC2 go up only modestly. I am unclear how they know that they are "ubiquitinated" since all we see is a higher molecular weight smear. In addition, a WT control is needed not just a GFP control.

It is not surprising that these contractile proteins rise only modestly upon DNp97 overexpression, since these are very long-lived proteins and we are inhibiting proteolysis only partially (Fig. 6). Accordingly, their levels do not increase when proteasomes are inhibited for 3h by Bortezomib (which in this time period causes an accumulation only of ubiquitinated short-lived proteins) (Fig. 9). Moreover, although the unmodified versions of these proteins increase modestly with DNp97, they also accumulated as a higher molecular weight smear, implying ubiquitination (Fig. 8E). Furthermore, the immunoprecipitation in the following Figure showed that these higher molecular weight species were ubiquitinated proteins (Fig. 9). In addition, because of the referee's question, we have added a blot using FK1 in Fig. 8E, showing an accumulation of the polyubiquitinated species with time, only with DNp97. We have also repeated the IP using the WT as control (Fig. 9 of new version). Therefore, these arguments and observations should be much clearer in this version.

4) In Figure 10A, why is there less MyLC2 in the p97DN expressing myotubes already at baseline. Doesn't this go against the hypothesis that it impedes degradation. In addition, why do I not see any MyLC2 when it is IP'ed in the presence of DNp97 (lane 6 of 10A). At best you can say that it IPs ubiquitin conjugates. Figure 10B shows an IP of actin but again, I am not certain that you can say it is ubiquitinated. These experiments all need controls with VCP-WT and the VCP-EQ mutants. In addition, since the DN increases total ubiquitin conjugates, these experiments should be done in the presence of proteasome inhibition.

We apologize for the lack of clarity in the prior version, which we have clarified this time. Unmodified MyLC2 was missing at 72h of DNp97 expression, because at this time, the protein is present almost completely in higher molecular weight forms due to ubiquitination, as we show in Fig. 8E and S4B of the new version. In Fig. S4B, the IP of MyLC2 showed a smear corresponding to the ubiquitinated species. Therefore, the amount of unmodified MyLC2 immunoprecipitated was much less than in GFP infected cells. However, the IP of MyLC2 in the new Fig. 9B has been repeated at 48h to avoid confusing the reader.

As we discussed above, we see no advantages for these studies to repeat them with the mutant. We agree with the reviewer that WTp97 and WTp97 plus a proteasome inhibitor are better controls, and we repeated all the immunoprecipitations with these controls in Fig. 9 of this new version. The conclusions are similar (though stronger).

5) Figure S2 I need to see quantitation of the degree of silencing. From my eyes it less than 10%. In addition, confirmation of the decrease in VCP needs to be shown in vivo. It seems to me that this should be possible with large effect seen in vivo with the shRNA constructs.

The referee's visual impression is misleading. We did quantify the effect of the shRNA constructs used, by densitometric analysis of the bands shown in Fig. S2 and found a 40% decrease in the ratio of p97/tubulin or UNC45B/GAPDH. In this newer version, we did provide such quantitation in the legend of Fig. S2.

An actual measurement of the decrease in VCP *in vivo* would be misleading and not valid if done in whole muscles, since with this large plasmid (about 11 Kb), most of the fibers are untransfected.

6) The increase in VCP/UFD1 protein was only performed at day 3 and day10 it is not fair to say that the increase in VCP/UFD1 occurred "exactly when the components of the thick filament are lost most rapidly" since the levels were not checked at days 4-9.

We agree with the reviewer and have been more careful in the revised text. We simply meant to indicate that p97/UFD1 levels were high at the time when myofibrillar degradation was maximal (9-11 days after denervation).

Minor Concerns:

1) The manuscript uses the word disuse atrophy (in several places) when actually they have only looked at denervation atrophy. For example page 9 "thus inhibition of p97 prevented completely disuse atrophy." No experiments were done using disuse models.

Finally, we have replaced the word disuse with denervation atrophy as referee 3 suggested. However, our prior studies (Sacheck et al, 2007) indicated very similar changes in denervated muscle and purely disused muscles during the first two weeks after onset.

2) The increase in ubiquitinated conjugates in figure 5c is not very convincing. A positive control with proteasome inhibitors would be helpful.

As requested, we have added in the blot shown in Fig. 5C an additional control experiment showing cells treated with the proteasome inhibitor, as referee 3 asked.

3) In the abstract: VCP mutations more correctly cause "an inclusion body myopathy phenotype" within the context of IBMPFD.

The text has been modified and we do not include such a statement in the revised version.

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2nd Editorial Decision 19 December 2011

Thank you for submitting a new version of your manuscript (previously EMBOJ-2011-77537) on p97/VCP in muscle atrophy. It has now been seen once more by the three original reviewers, whose comments are copied below. Although the referees acknowledge that the study has on the whole improved, they also express their indignation with the responses given to their originally raised criticisms and constructive reviewer input. This is especially clear from the report of referee 3, who retains major technical issues as well as concerns regarding data interpretation; and from the points of referee 1, who remains critical about discussion and interpretation of the presented results, and who further indicated his/her full agreement with the criticisms of referee 3 during our routine process of pre-decision cross-referee commenting.

In light of these criticisms, I am afraid the study is at the current stage still not ready for publication in The EMBO Journal. Given the improvements made and the fact that the remaining problems should be in principle addressable, I would be inclined to allow you one additional opportunity to tackle these issues through one more, ultimate round of regular revision. I should make it clear, however, that this will be the last revision we can consider for this study, and that we will have to reject the manuscript should this final version and response fail to satisfy referees 1 and 3. In order to not further jeopardize the overall support/interest of the referees for this work, may I therefore suggest to carefully consider their requests (interpretational and experimental) and balance your responses accordingly. When resubmitting the final version, please also make sure to include a 'Conflict of Interest' statement in the manuscript as required in our author guidelines.

I hope you will find these final sets of comments valuable for ultimately make the study suitable for publication in The EMBO Journal. Should you have any further questions that require clarification in this regard, please do not hesitate to contact me.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors have considerably improved the previously submitted manuscript. However there are still two major concerns that must be addressed by the authors.

Major points:

1. The authors wrote (page 4 lines 5-9) that 'During disuse atrophy, thick filament components (e.g. myosin) are selectively ubiquitinated by MuRF1 while in the myofibrils and degraded (Cohen et al, 2009), but other ubiquitin ligases appear to catalyze the degradation of thin filament components (e.g. actin) (Kudryashova et al, 2005)' and page 23 lines 6-10) that 'Thin filament components (e.g. actin) are then degraded via a distinct ubiquitin ligase, probably Trim32 (Cohen et al, 2009; Kudryashova et al, 2005)' (see also page 17 second paragraph). All these statements must be corrected.
a) First a recent paper (Cosper & Leinwand Int J Cancer 2011 Jul 27. doi: 10.1002/ijc.26298. [Epub ahead of print]) does show that the selective breakdown of myosin heavy chain (which is supported in the Cohen's paper and to a much lesser extend here) is purely artefactual.
b) Second Trim 32 monoubiquitinates actin (Kudryashova et al, 2005) and cannot target actin for proteasome breakdown.
c) Third a recent paper does show that actin is indeed also targeted for breakdown by the proteasome

by MuRF1 (Polge et al. FASEB J. 2011 Nov;25(11):3790-802), as is troponin I (another component of the thin filament).
d) Last but not least, the own data of the authors (Fig. 8E) do show that actin is degraded within hours (and not within days as claimed in the Cohen's paper).

2. It is totally premature to suggest that p97 is an atrogene. Thus, the second paragraph page 22 must be suppressed.

Referee #2 (Remarks to the Author):

Piccirillo and Goldberg investigated the function of p97 and its cofactors during muscle atrophy when highly organized myofibrils are rapidly degraded. The manuscript reveals a novel function for p97 in the disassembly of the myofibrillar apparatus. Their findings evidently demonstrate that p97 is responsible for the loss of muscle mass upon denervation and food deprivation. Consequently, in normal adult muscles and myotubes p97 promotes muscle protein degradation to limit muscle growth. The data may help to understand the mechanism of muscle atrophy and VCP related muscle diseases.
Authors revealed that the production of p97 and its cofactors increase in atrophying muscles. Interestingly, expression of a dominant negative p97 (DNp97) in adult mouse muscles reduced fiber atrophy. It was observed that DNp97 caused accumulation of polyubiquitinated components of both thick and thin filaments and blocked their proteasomal and lysosomal degradation induced by FoxO3 (Has accumulation of polyubiquitinated components of both thick and thin filaments in muscles expressing DNp97 an effect on sarcomeric function/integrity?). In addition, it is shown that p97 forms distinct complexes with different contractile proteins and cofactors. Authors concluded that p97 influences the progression of the atrophy process and the size of muscles.

The manuscript shows novel and interesting data supported by well executed experiments. This version clarified all criticism raised before. The revised version has been properly reorganized to emphasize the main findings and conclusions. In my opinion this data should be published.

Minor concern:
Authors observed no increase in the amount or phosphorylation of AKT (Fig. S5A and B). It seems that the ratio of phosphorylated and nonphosphorylated AKT differs between WTp97 and DNp97. This should be clarified/discussed.

Referee #3 (Remarks to the Author):

The manuscript by Piccirillo and Goldberg address an important question and propose an innovative answer. However not all of their claims are supported by the data which is in some cases of poor quality. Importantly, the revision has been restructured and new data has been added which significantly strengthen the manuscript. However the tone of the rebuttal letter dampens this reviewers enthusiasm.

1) The shRNA knockdown western blots need to be quantified just stating 40% is not quantified. I need error bars etc. In addition, I need to see confirmation in vivo in the tissue measured. This should be feasible with an antibody to VCP especially with such a robust finding. It needs to be explicitly stated that the GIPZ vector has a GFP marker allowing one to identify the shRNA KD fibers. In addition, correlating VCP fluorsecenc in transfected vs. non transduced fibers should be feasible in vivo.

The next few statements encompass the largest problem with the manuscript. In particular the evidence that VCP is truly degrading myofibrillar proteins and is directed to them in a regulated manner is not convincing. VCP has been shown to be a specific binding partner in some scenarios (Hif1a or Unc45) but a promiscous binder in other scenarios (i.e all ERAD proteins) therefore to convincingly identify myofibrillar proteins as being specific VCP client proteins is a difficult task.

2) Please show the ATP dependency data for binding.

3) I do not agree that colocalization studies cannot be performed or are difficult. One can tag VCP constructs with RFP or mCherry as others have done. I would like to see this data. This is an important issue since VCP has been shown to be exclusively subsarcolemmal and in another paper exclusively nuclear in skeletal muscle. If one were to find more KA mutant on striations this would strengthen the findings. Showing me changes in associations with extracts is one thing but actually demonstrating this in tissue sections is essential.

4) Figure 8E needs to be performed with a WT control as previously requested. In addition, the blots have been cut up making it diffult to assess them. I would like to see the full blot at a short and long exposure. I am not convinced that these proteins are being ubiquitinated and then migrating at a higher weight. The paper would have me believe that all of the MyLC2 non ubiquitinated becomes ubiquitinated and accumulates at a higher weight. I find this implausible. Actin levels go up then down it is all puzzling. Perhaps quantitation would help. This a critical figure as it proves one of the main hypotheses of the paper (VCP specifically is involved in the degradation of ubiquitinated myofibrillar proteins). If this cannot be shown convincingly than their hypothesis is wrong. Even if VCP can bind to some of these proteins, if they do not convincingly accumulate than it is not proven.

5) Figure 9A, the RGSH4 blot is way overexposed. Blots 9A and B are poor quality. Can the reverse experiment be performed. pulling down on VCP instead of actin and mycLC2.

Finally if the paper is going to suggest that the increase in myofiber size, the changes in protein degradation in muscle are related specifically to VCP selectively degrading myofibrillar proteins than I am not convinced. I am convinced that VCP is essential for muscle atrophy and protein degradation in skeletal muscle. Perhaps this is statement enough. It still seems conceivable to me that VCP could be essential for degrading an essential intermediate that then starts the atrophy cascade.

1st Revision - authors' response 28 February 2012

Response to the comments of the reviewers:
The referees' critiques clearly indicated ways for us to strengthen the manuscript and improve our data presentation. The revised version includes new data and a number of changes in the text. These additions should resolve the various concerns of the referees and also provide further insights into how the p97 complex influences muscle fiber size. We are convinced that this version is much more improved by the changes made.

Referee #1:
The authors have considerably improved the previously submitted manuscript. However there are still two major concerns that must be addressed by the authors.

We are pleased that the referee found our manuscript considerably improved, and that the only remaining concerns were with our citations of prior work. However, we believe that our statements are in fact accurate, and that the referee's comments reflect some misimpressions about the prior literature or these experimental systems.

Major points:

1. The authors wrote (page 4 lines 5-9) that 'During disuse atrophy, thick filament components (e.g. myosin) are selectively ubiquitinated by MuRF1 while in the myofibrils and degraded (Cohen et al, 2009), but other ubiquitin ligases appear to catalyze the degradation of thin filament components (e.g. actin) (Kudryashova et al, 2005)' and page 23 lines 6-10) that 'Thin filament components (e.g. actin) are then degraded via a distinct ubiquitin ligase, probably Trim32 (Cohen et al, 2009; Kudryashova et al, 2005)' (see also page 17 second paragraph). All these statements must be corrected.

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c) Third a recent paper does show that actin is indeed also targeted for breakdown by the proteasome by MuRF1 (Polge et al. FASEB J. 2011 Nov;25(11):3790-802), as is troponin I (another component of the thin filament).

d) Last but not least, the own data of the authors (Fig. 8E) do show that actin is degraded within hours (and not within days as claimed in the Cohen's paper).

A) The referee questioned our statement that "thick filament components are selectively ubiquitinated by MuRF1 while in the myofibrils..." based upon the recent paper of Cosper and Leinwand (Cosper & Leinwand, 2011). There must be some misunderstanding of our results because our paper (Cohen et al, 2009) does not report selective loss of myosin, as had been claimed to occur in cancer cachexia by one group (Acharyya et al, 2004), and that specific claim was challenged by Cosper and Leinwand. The latter workers actually report findings in agreement with ours, which they explicitly state. Our prior paper (Cohen et al, 2009) actually demonstrated an ordered pattern of protein loss after denervation, in which MuRF1 selectively degrades first Myosin light chain 1 and 2 and Myosin binding protein C and then Myosin heavy chain and ultimately actin by another ubiquitin ligase. Also, the artifact reported by Cosper and Leinwand could not apply to our results, which always compared denervated and control muscles in the same animal.

B) The reviewer states that Trim32 cannot target actin for proteasome breakdown because it only monoubiquitinates actin, as was reported by (Kudryashova et al, 2005). However, that report is inconsistent with our recent results. In a manuscript presently under review, Cohen and Goldberg demonstrate that Trim32 can polyubiquitinate actin (in contrast to the prior report), both as a soluble protein and in the myofibril. (It should also be noted that whether an ubiquitin ligase attaches one or a chain of ubiquitins often depends simply on the E2 that is used and on the exact *in vitro* conditions).

C) The referee argues that actin can be targeted to the proteasome by MuRF1, based upon the recent report of (Polge et al, 2011). While that group and our own (see Supplement material of (Cohen et al, 2009)) showed that pure MuRF1 can ubiquitylate pure soluble actin and also troponin I (see also other papers from our group, including (Uchiki et al, 2009), (Kim et al, 2007), (Kim et al, 2009)), *in vivo*, we saw no effect of MuRF1 knockout on the loss of actin or troponin I during denervation atrophy. On the contrary, Cohen and Goldberg (in revision for J Cell Biol) show that loss of actin and troponin I during fasting and denervation atrophy is dependent upon Trim32. In the ubiquitin-proteasome field, it is now well appreciated that ubiquitylation of a substrate by a pure E3 or by an overexpressed E3 does not mean that this enzyme serves that function *in vivo* at physiological concentrations.

D) The referee senses a possible contradiction between the present findings in Fig 8E about actin being degraded within hours and not within days, as the referee states was the conclusion of the Cohen paper. There is in fact no disagreement, and we would like to clarify how the experiments were done. Neither of these studies actually measured actin degradation. In fact, the statement about actin degradation taking days was never made in our paper, which only looked at the net loss of actin and other myofibrillar components at different times after nerve section. Thus, that paper indicates the difference between rates of protein synthesis and degradation (not rates of proteolysis), and the time lag before actin degradation is activated. In addition, the half-life of these proteins depends on the physiological conditions and differs in adult muscle and myotubes. (In fasting for example, actin loss occurs much more rapidly than upon denervation atrophy). The present findings involve myotube cultures, where the proteins are less present in myofibrils and turn over very much more rapidly than in mature muscles (even after denervation).

2. It is totally premature to suggest that p97 is an atrogene. Thus, the second paragraph page 22 must be suppressed.

2) The reviewer states that it is premature to suggest that p97 is an atrogene. We are a bit surprised by this criticism, since we are only suggesting that this possibility merits further study (based upon the increase in p97 mRNA in both fasting and denervation). However, because of the reviewer's concern, in the revised version, we have tried to make this proposal more tentatively. We specifically state that it will be necessary to assay this protein's levels in the four other disease states, which we originally studied to formulate the concept of "Atrogenes" as well as disused and denervated muscles (Lecker et al, 2004) (Sacheck et al, 2007) (See pages 19 and 22 of the last version). We also point out that p97 expression occurs later than typical atrogenes, and thus would require a distinct signalling mechanism.

Referee #2:

Piccirillo and Goldberg investigated the function of p97 and its cofactors during muscle atrophy when highly organized myofibrils are rapidly degraded. The manuscript reveals a novel function for p97 in the disassembly of the myofibrillar apparatus. Their findings evidently demonstrate that p97 is responsible for the loss of muscle mass upon denervation and food deprivation. Consequently, in normal adult muscles and myotubes p97 promotes muscle protein degradation to limit muscle growth. The data may help to understand the mechanism of muscle atrophy and VCP related muscle diseases.
Authors revealed that the production of p97 and its cofactors increase in atrophying muscles. Interestingly, expression of a dominant negative p97 (DNp97) in adult mouse muscles reduced fiber atrophy. It was observed that DNp97 caused accumulation of polyubiquitinated components of both thick and thin filaments and blocked their proteasomal and lysosomal degradation induced by FoxO3 (Has accumulation of polyubiquitinated components of both thick and thin filaments in muscles expressing DNp97 an effect on sarcomeric function/integrity?). In addition, it is shown that p97 forms distinct complexes with different contractile proteins and cofactors. Authors concluded that p97 influences the progression of the atrophy process and the size of muscles.

The manuscript shows novel and interesting data supported by well executed experiments. This version clarified all criticism raised before. The revised version has been properly reorganized to emphasize the main findings and conclusions. In my opinion this data should be published.

We are pleased that the referee found our manuscript considerably improved and is ready to be published. Regarding his/her minor concerns, we never measured whether the possible accumulation of polyubiquitinated components of both thick and thin filaments in muscles expressing DNp97 may impact sarcomeric function because such questions are beyond the scope of our biochemical study. However, it shall be noted that only a very small fraction of myofibrillar proteins are ubiquitinated at any instant, and to detect them *in vivo* would be harder because only a fraction (60-80%) of fibers are transfected. Nonetheless, in this new version we provide evidence that muscles expressing DNp97 still retain somehow sarcomeric organization, as shown by the striated pattern of the DNp97 in longitudinal muscle sections (See Figure 8D of the latest version).

Minor concern:
Authors observed no increase in the amount or phosphorylation of AKT (Fig. S5A and B). It seems that the ratio of phosphorylated and nonphosphorylated AKT differs between WTp97 and DNp97. This should be clarified/discussed.

We thank the referee for raising this issue since it allows us to further improve our data presentation. In Figure S5A, although the phosphorylated AKT may suggest a possible trend over time only when DNp97 is overexpressed, these data refer to single samples for each time point, and multiple samples for each time point are necessary for any rigorous conclusions. In fact, when many samples were analyzed, there was no difference in phospho-AKT levels in the WT and DN groups and it did not correlate with change of phosphorylation of 4E-BP1 or of S6 kinase. Specifically, because each time point in S5A is based on a single measurement (i.e. cells lysed from a well of a 6-well plate), we further tested the levels of phosphorylated AKT in triplicates for the 48h timepoint, when most

of our measurements were carried out and found no difference (Fig. S5B). Because the phosphorylated AKT in Figure S5A was misleading, we have removed it from the revised version.

Referee #3:
The manuscript by Piccirillo and Goldberg address an important question and propose an innovative answer. However not all of their claims are supported by the data which is in some cases of poor quality. Importantly, the revision has been restructured and new data has been added which significantly strengthen the manuscript. However the tone of the rebuttal letter dampens this reviewer's enthusiasm.

We are pleased that the reviewer thought that our new data significantly strengthened the manuscript. We were quite surprised, however, that he/she found the tone of our response inappropriate or in some way impolite. We apologize for this impression and thank the reviewer for having given us another opportunity to improve our manuscript.

1) The shRNA knockdown western blots need to be quantified just stating 40% is not quantified. I need error bars etc. In addition, I need to see confirmation in vivo in the tissue measured. This should be feasible with an antibody to VCP especially with such a robust finding. It needs to be explicitly stated that the GIPZ vector has a GFP marker allowing one to identify the shRNA KD fibers. In addition, correlating VCP fluorsecenc in transfected vs. non transduced fibers should be feasible in vivo.

We agree with the referee that these important controls and quantitation should have been presented in the prior version. As requested by the referee, we have added proper quantitation of the p97 downregulation in the new Figure S2 (error bars and T test). In this revised version, we have also changed the text to state clearly that the fibers expressing shRNA for p97 were recognized by means of the GFP marker present in the GIPZ vector (See page 9). Importantly, we provide also a western blot showing the downregulation of p97 in adult muscle electroporated for 7 days with the shRNA p97 (Fig. S2E). While this downregulation was presumably quite dramatic in these transfected fibers, it should be noted that the efficiency of electroporation and thus the downregulation varied in the different experiments. Consequently, it was not always possible to determine by western blot on the total muscle to demonstrate silencing of the endogenous p97. We here show in Figure S2E of the revised version a lysate from a muscle that expressed high levels of the shRNA for p97 for 7 days, as indicated by GFP immunoblotting and by disappearance of the p97 band. Unfortunately, in sectioned fibers, we could not observe downregulation of p97 because the fixation method to efficiently decorate endogenous p97 by IHC (Methanol/Aceton fixation) resulted in a great loss of GFP signal.

The next few statements encompass the largest problem with the manuscript. In particular the evidence that VCP is truly degrading myofibrillar proteins and is directed to them in a regulated manner is not convincing. VCP has been shown to be a specific binding partner in some scenarios (Hif1a or Unc45) but a promiscous binder in other scenarios (i.e all ERAD proteins) therefore to convincingly identify myofibrillar proteins as being specific VCP client proteins is a difficult task.

Perhaps, there was a misunderstanding. We did not mean to imply a highly specific role in p97 taking apart the myofibrillar apparatus. The data is consistent with a general role in myofibrillar degradation since we have demonstrated a sparing of the bulk of muscle proteins. Our *in vitro* data on p97 in myotubes focuses on two abundant proteins but these cells lack many components present in mature myofibrils.

2) Please show the ATP dependency data for binding.

As asked by the referee, in this version, we provide evidence that actin does not pull down DNp97 in myotubes, when ATP is omitted from the lysis buffer, and the washing steps in immunoprecipitation experiments (See Figure S4C of the revised version).

3) I do not agree that colocalization studies cannot be performed or are difficult. One can tag VCP constructs with RFP or mCherry as others have done. I would like to see this data. This is an important issue since VCP has been shown to be exclusively subsarcolemmal and in another paper exclusively nuclear in skeletal muscle. If one were to find more KA mutant on striations this would strengthen the findings. Showing me changes in associations with extracts is one thing but actually demonstrating this in tissue sections is essential.

We agree with the reviewer about the importance to show by colocalization studies the change in distribution of the DNp97. Consequently, we have put a lot of effort into these experiments, because as previously stated, we could not detect DNp97 tagged with GFP since GFP itself shows a striation-like pattern. We have therefore employed another DNp97 mutant (p97E578Q) that is myc-His tagged and electroporated it or its WT version in muscle for 7 days. Immunohistochemistry on longitudinal muscle sections with anti-myc antibodies was performed (See Figure 8D of the latest version). This use of a different DN in place of the p97K524A was also done because this reviewer had requested previously that we repeat our experiments with p97E578Q, as we now have done in this colocalization studies.

4) Figure 8E needs to be performed with a WT control as previously requested. In addition, the blots have been cut up making it diffult to assess them. I would like to see the full blot at a short and long exposure. I am not convinced that these proteins are being ubiquitinated and then migrating at a higher weight. The paper would have me believe that all of the MyLC2 non ubiquitinated becomes ubiquitinated and accumulates at a higher weight. I find this implausible. Actin levels go up then down it is all puzzling. Perhaps quantitation would help. This a critical figure as it proves one of the main hypotheses of the paper (VCP specifically is involved in the degradation of ubiquitinated myofibrillar proteins). If this cannot be shown convincingly than their hypothesis is wrong. Even if VCP can bind to some of these proteins, if they do not convincingly accumulate than it is not proven.

We apologize to the referee for not having provided this information earlier. We do agree on the importance of comparing the effects of the DN mutant with the WT, and in fact showed that there isn't a difference in the rate of proteolysis between GFP and p97WT (See Fig. 4A and B). In the revised version, we provide evidence that both actin and MyLC2 accumulate as ubiquitinated species but not Myosin heavy chain in the control conditions requested by the referee. Because of the limited space related by the journal, we prefer to show the entire blots at different exposure times in the supplement (See Fig. S6C). On repeating these experiments, the disappearance of the unmodified MyLC2 has been found again at 72h of expression of the DN mutant, but is not reproducible for reasons that we do not understand (for this reason, we have changed the text accordingly on page 16). The accumulation as ubiquitinated species is instead reproducible as shown in the Figure 8F, S6A and B of this version.

5) Figure 9A, the RGSH4 blot is way overexposed. Blots 9A and B are poor quality. Can the reverse experiment be performed. pulling down on VCP instead of actin and mycLC2.

We agree with the referee and have changed Figure 9A accordingly. Since the reverse immunoprecipitation would further strengthen our findings, we had tried multiple times to pull down exogenous VCP by means of RGS-His tag, but we always failed (despite trying several conditions), most probably because the antibody against this tag is not optimal for immunoprecipitation. To circumvent this issue, we have performed immunoprecipitation of the cofactors that most probably are involved in myofibrillar disassembly, such as p47 and Ufd1 (Figure 9) (i.e. because both are able to bind ubquitinated proteins, and because we have found them to be increased in conditions of accelerated proteolysis in adult muscle (Figure 10)).

Finally if the paper is going to suggest that the increase in myofiber size, the changes in protein degradation in muscle are related specifically to VCP selectively degrading myofibrillar proteins than I am not convinced. I am convinced that VCP is essential for muscle atrophy and protein degradation in skeletal muscle. Perhaps this is statement enough. It still seems conceivable to me that VCP could be essential for degrading an essential intermediate that then starts the atrophy cascade.

We hope that we have successfully answered the referee's concerns in a way to have convinced him about the role of p97 in myofibrllar disassembly by direct action on some contractile proteins. To further convince the referee, we have provided additional data (see Fig. S6).

We are pleased that the referee is "now convinced that VCP is essential for muscle atrophy and protein degradation in skeletal muscle". This was the primary finding in our paper. However, we are not sure if we understand the reviewer's concern and what he/she means by "selectively". We never meant to imply that these effects on muscle size are due to a "selective degradation of myofibrillar proteins" by the proteasome. On the contrary, we do show data for an effect on lysosomal proteolysis and FoxO-induced proteolysis, which in myotubes are largely through activation of autophagy. The disassembly and degradation of myofibrillar apparatus is in our view a new function for p97 complexes, but is only one of several p97-dependent processes. We agree that p97 may promote degradation of an "essential intermediate that then starts the atrophy cascade", but we believe it also directly interacts with certain key myofibrillar proteins and show data supporting such an interaction.

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Additional correspondence (editor) 10 April 2012

Please accept my apologies for the delay associated with the evaluation of your re- revised manuscript. Referee 3, who had raised the most substantive yet constructive comments throughout the earlier rounds of review, has assessed the study once more (see comments copied below), and still retains major reservations regarding some of the data and the conclusions drawn from them. This necessitated me to myself once more closely look into the manuscript, with particular focus on the data in question, and to further discuss the relevant issues with the referee. In conclusion, while the key findings of p97/VCP effects on muscle atrophy and proteolysis remain interesting, I am afraid we still cannot consider the current interpretation that p97/VCP specifically extracts or degrades certain myofibrillar proteins justified based on the ubiquitination and co-immunoprecipitation data you show in Figures 8F, 9 and S6; and given that these results could not be substantiated during two rounds of revision, we are not convinced that further experiments using these approaches will be able to better specify the exact molecular role of p97/VCP in the process. Therefore, we will unfortunately not be able to publish the manuscript in its current form and with the current dataset.

In this situation, I do feel the manuscript would be a stronger and more valuable contribution without these less conclusive ubiquitination and co- immunoprecipitation data in the figures mentioned above, and subsequently with toned-down conclusions on the exact role of p97/VCP. In my opinion, the key findings on p97/VCP roles in muscle atrophy would not be affected by this removal, and the proposal that it may have specific functions in binding and extracting ubiquitinated myofibrillar proteins could instead be presented in the discussion as a (quite likely) hypothesis that nevertheless awaits further conclusive experimental validation. Therefore, we should be able to offer publication if you would be willing to re-organize and streamline the manuscript in this way. Please let me know it you agree with this proposal - in which case I would formally return the study to you for a final round of modification, to allow you to remove the data in question, reorganize the figures, and tone down the conclusions (taking into account also the specific referee comments below).

I am looking forward to hearing from you.

Sincerely,
Editor

The EMBO Journal

Referee #3 (Remarks to the Author):

The manuscript is improved but one of the major conclusions is still not justified. The concept as stated in the abstract "Thus p97 appears to extract ubiquitinated proteins from myofibrils during atrophy" is not proven. I appreciate the distinction of "selectivity" but fundamentally the question is whether p97 is directing, extracting, selecting myofibrillar proteins for degradation during atrophy. Perhaps the authors can suggest a model for p97 that remains to be proven.
Many statements need to softened or removed. Page 16 "It seems very likely that this non-dissociating mutant blocked myofibrillar degradation by binding to ubiquitinated components and preventing their release from the sarcomere and delivery to the proteasome." Page 17 "most likely, these different p97 cofactor complexes associate with and extract these ubiquitinated proteins from the thin and thick filaments, and facilitate their degradation by the proteosome." Page 24 "Therefore it is very likely that these different p97 cofactor complexes catalyze the extraction of these contractile proteins from the sarcomere prior to delivery to the 26S Proteosome"/

It is clear from the manuscript that VCP is involved in muscle atrophy. VCP may also co-sediment with myofibrillar proteins. It is clear that loss of VCP may regulate the degradation of some myofibrillar proteins in C2C12 cells. However this appears to be a minority of proteins in C2C12 cells since the amount of "ubiquitinated myLC2 and actin" is far less than the total increase in ubiquitinated proteins. Whether VCP is truly the workhorse that "extracts" proteins from the myofibrillar apparatus remains to be determined and is not proven in this manuscript. In fact as

stated by the authors, C2C12 do not contain a completely organized myofibrillar network and thus may not be the best cells to perform these studies.

Additional correspondence (author) 17 April 2012

Thank you for your recent letter and your kind efforts to enable publication of our paper. While Dr. Piccirillo and I very much appreciate your special consideration, we remain totally mystified by the critique of the third reviewer. Although we are willing to modify our manuscript so as not to overstate our conclusions and recognize that our findings do not definitively resolve certain points, we are convinced that this referee's concerns are not valid and are not based upon clear scientific arguments. In our previous rebuttal, we did not directly criticize this Referee's arguments, since he/she had to our surprise taken personal offense at our initial attempts to point out his/her misimpressions. Yet, this reviewer still seems to misiconstrue our conclusions and to overlook aspects of the data and the changes we made in the text.

In my long career as an author or referee, I cannot remember a situation quite like this one. Consequently, I was wondering if I may phone you to discuss this reviewer's comments and your proposal. I could phone you at any time convenient for you since I am now in Europe on a mini-sabbatical as a Visiting Professor/Overseas Fellow at University of Cambridge,

I look forward to hearing your thoughts on these points and to speaking to you soon.

3rd Editorial Decision 26 April 2012

Following up our phone conversation, I am herewith returning your manuscript on p97 and muscle atrophy to you for a final round of minor revision, in order to allow to introduce the remaining modifications to text and figures. As discussed, please augment the protein interaction data in Figure 9 with electronic images of the original, uncropped scans to be included as supplementary source data; these should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type), and I would encourage you to include here also source files for other key panels, especially those currently displayed in Fig S6A/B. As further discussed, please remove figures S6C and 8F (maybe moving Fig S6A back into the latter's place in the main manuscript), and alter/qualify the mechanistic inferences accordingly.

Once we will have received the resubmitted final version, we should then be in a position to proceed with its acceptance and publication.

With kind regards,

Editor
The EMBO Journal

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2nd Revision - authors' response 29 May 2012

Enclosed at last is a further revision of our manuscript, in which we believe we have incorporated all your recommendations, as discussed on the telephone. The text has been altered in a number of places and the title changed to insure that our statements concerning the likely role of p97 in

degradation of myofibrillar proteins are only suggestions based upon our various observations. Also the gels that you specifically questioned have been removed and the full blots that you required have been added following your instructions. For your convenience, we have reported your last e-mail below.

We hope that it is now acceptable for publication, and are grateful to you for your personal attention and advice.

Acceptance letter 01 June 2012

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,
Editor
The EMBO Journal

