EMBO reports

The AAA-ATPase Atad1 and its partners promote degradation of desmin intermediate filaments in muscle

Dina Aweida and Shenhav Cohen
DOI: 10.15252/embr.202050852

Corresponding author(s): Shenhav Cohen (shenhavc@technion.ac.il)

Review Timeline:

Transfer from Review Commons:	4th Apr 22
Editorial Decision:	10th May 22
Revision Received:	13th Sep 22
Accepted:	4th Oct 22

Editor: Martina Rembold

Transaction Report: This manuscript was transferred to EMBO Reports following peer review at Review Commons.

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The manuscript reports the identification of a novel protein complex involved in denervation-induced desmin degradation. The first protein to be identified was the ATPAse Atad1. A clever isolation strategy was based on the fact that the ATPAse p97/VCP is involved in the extraction of ubiquitinated myofibrillar proteins but is not required for the removal of ubiquitinated desmin filaments. The authors reasoned that a related ATPAse might be specifically required for desmin filaments. Atad1 was identified by treating desmin filaments with a nonhydolyzable ATP analog and looking for ATPases that are associated with desmin filaments by proteomics. Knockdown of Atad1 causes a loss of desmin degradation and led to a loss of denervation-induced muscle atrophy. It seems that Atad1 binds desmin in a phsphorlation-dependent manner, although the binding maybe mediated by a protein that hasn't yet been identified. The authors went on and identified two additional proteins which together with Atad1 form a protein complex involved in recruiting calpain for desmin degradation.

Overall, this study is very convincing providing novel important insight. I have only some minor comments

Minor comments

1. I wondered whether Aatd1 is expressed at higher-than-normal levels in muscle and heart. I looked that expression pattern up and it seems that they are especially abundant in muscle and heart and expressed at lesser levels in smooth muscle and overall have a restricted expression. Maybe you have some data on their expression in muscle tissue. Did you perform some staining of muscle tissue at baseline and after denervation with regard to the protein localization by immunostaining?
2. The string data presented in Figure 3C needs some further explanation with regard to the colors used for the different proteins. While the authors explained the meaning of the proteins labeled in red, there is no explanation for the other colors.
3. Molecular weights in Fig. 2E, 3D needs to be 'repaired' and additional MW information is required in case of the ubiquitin blot shown in 3D.
4. Fibre size distributions shown in Fig. 1D and 4F. Have the differences been statistically tested?
5. For my taste the referral to the individual data (Fig. numbers) in the discussion section is too detailed and becomes a second results section. This should be substituted by a summary paragraph before the implications are discussed.
6. The summary slide is very good. However, could you please add information, which protein of the three in the Atad1 complex is depicted by each symbol?

2. Significance:

Significance (Required)

Novel insight into the proteins involved in desmin filament degradation. Since this is an important subject both in muscle and heart and plays an important role in muscle and heart disease, it is of significant clinical importance. Currently it has only been implicated in denervation-induced skeletal muscle atrophy, but it is likely that desmin filament metabolisms is also similarly regulated in the heart.

I am a researcher mainly focusing on the cardiac biology with some expertise also on muscle, however no specific knowledge about desmin filament biology.

Referee Cross-commenting

Overall, I think all three reviewers agree that this is a significant and important paper. I think that the comments made by the reviewers are fair and probably add to the quality of the manuscript.

Thus, both myself and reviewer 2 agree that it would be useful to visualize Atad1 and partners localization in muscle fibers by immunofluorescence. These data would provide independent support to the model the authors are proposing, which currently is only based on biochemical analysis.

I also support the proposed use of proximity ligation to provide further evidence of the presence of the Atad1, Ubxn4 and PLAA in a complex. However, this experiment depends on the quality of the available antibodies and I would consider this not absolutely required.

I also agree that some further information on the proteomics data (as suggested by reviewer 3) is required with regard to the method of filtering for UPS components was performed.

The proposed request for further information on the electroporation approach is a valid comment and if the authors have this information, it would be good to provide. However, I do not recommend further experiments as overall the data are very consistent and the findings are very significant and represent a major advance in our understanding of desmin degradation.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In their manuscript the authors show the involvement of the AAA ATPase Atad1 in Desmin degradation. They identify PLAA and Ubxn4 as partners of Atad1 that participate to its function in desmin degradation.

A general comment is that some conclusions are overstated. The authors mention several times that Atad1 depolymerises desmin filaments. The data show that Atad1 participates to the degradation of Desmin and to its solubilization. "Depolymerisation" should be kept for the model presented in figure 8 but not used in the result section.
Major comments:

1) It would be useful to visualize Atad1 and partners localization in muscle fibers in immunofluorescence. Do they colocalize with desmin filaments, with calpain?

2) In the same line, interactors were obtained from large crosslinked complexes. It would make the model more convincing if direct interactions with Atad1 were shown, for example using Proximity Ligation Assays.

3) Evaluation of atrophy is made on cross-sections of muscles electroporated with shRNAs. Histology pictures should be shown.

4) What is the percentage of electroporated fibers? To evaluate the effect of shRNAs it is important to have this information. For example, if the efficiency is 50% it means that the reduction in expression of the target in electroporated fibers is twice the value reported for the whole muscle. Alternatively, immunofluorescence could be provided to see the decrease in targeted proteins in electroporated fibers.

5) The same is true for all the experiments quantifying the effect of shRNAs in western blot. Since quantifications are probably made on whole muscles (ie a mix between electroporated and non electroporated fibers) and since the percentage of electroporated fibers is not given it is not possible to estimate the efficiency of the shRNAs in electroporated fibers.

6) Figure 2C: by decreasing solubilization of desmin, one would expect a decrease in the levels of soluble desmin. Conversely the authors observe an increase in both insoluble and soluble desmin. Of course, this can be explained by reduced desmin degradation once solubilized but this should be demonstrated at least by showing that UPS inhibitors induces an increase in soluble ubiquitinated Desmin.

7) Figure 2E: the levels of Atad1 in the insoluble fraction seem to be the same in the shLacZ and GSK3DN conditions, whereas the phosphor Ser is different. In other words, there should be more Atad1 in the insoluble fraction with shLacZ than with GSAK3DN since the phosphorylation level with shLacZ is significantly higher.

8) Figure 4E: the authors state that phosphorylation decreases because of increased degradation (lanes 6-8). However, Calpain also increases degradation and phosphorylation is increased (lanes 2-4), so increasing degradation does not systematically cause a decrease in phosphorylation. Similarly, lane 5 Atad1 induces less degradation than Calpain, however, it causes a decrease in phosphorylation. Explain.

9) The AAA ATPase VCP shares partners with Atad1 and is involved in muscle atrophy. It would greatly add to the manuscript if the authors inhibited VCP to compare its effect to Atad1

Minor comments:

1) The soluble fraction contains a large number of ubiquitinated proteins. Please explain how it can be stated that an increase in total soluble polyubiquitinated proteins corresponds to an increase in ubiquitinated desmin.

2) Page 11: the authors conclude that denervation enhance the interactions with Atad1. Figure 3D indeed show an increase for Ubxn4, but it is not clear for the other proteins.

3) Figure 4 F: show muscle sections

4) Page 21 in vivo transfection: it is stated "see details under immunofluorescence" but there is no immunofluorescence section in materials and methods.

5) The authors show that Atad1 inhibition in innervated muscle is sufficient to induce muscle hypertrophy (Figure 4E). They conclude that the hypertrophic effect of Atad1 is due to the inhibition of Desmin degradation. However, this hypertrophic effect could be independent of the action of Atad1 on Desmin.

2. Significance:

Significance (Required)

This is new information in the field since calpain cannot hydrolyze desmin insoluble filaments and that the mechanisms that give calpain access to desmin are not known.

The authors already made important contribution in the study of muscle atrophy and especially in desmin degradation. This work constitutes a new advance in their attempts to understand the molecular mechanisms leading to desmin degradation and muscle atrophy.

Audience: desmin is the main intermediate filament in skeletal muscle. This work will therefore interest scientists working on skeletal muscle.

Expertise of the reviewer: molecular and cellular biology of skeletal muscles, muscle atrophy.

Referee Cross-commenting

I fully agree with reviewer 1.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

The manuscript by Aweida & Cohen introduces a novel complex formed by the AAA-ATPase ATAD1 and its interacting partners PLAA and UBXN4 as initiator of calpain-1-mediated disassembly of ubiquitylated desmin intermediate filaments (IF) during muscle atrophy. The authors use a denervation model of murine tibialis anterior muscles as their main resource for experimentation. They apply a kinase trap-assay and co-immunoprecipitation method followed by mass spectrometry as starting point for identifying novel interactors of desmin IF (Aweida et al. 2018 in JCB). They continue to analyze their candidates using immunoblotting, co-immunoprecipitation, shRNA-mediated intramuscular knock-down, gel filtration, mass spectrometry, and enzyme assays. In their experiments, thee authors show an accumulation of ATAD1 in the insoluble desmin filament fraction of denervated muscle fibers together with an increase in ubiquitylation of desmin filaments. Both proteomics experiments of size-exclusion chromatography of denervated muscles and ATAD1 immunoprecipitation identify several components of the ubiquitin-proteasome system as novel interactors of ATAD1, that are also bound to insoluble desmin filaments after muscle denervation. Following additional co-immunoprecipitation and knock-down experiments, the authors confirm PLAA and UBXN4 as novel cofactors of Atad1 that help in extracting previously GSK3-β-phosphorylated and TRIM32-ubiquitylated (Aweida et al. 2018 in JCB, Volodin et al. 2017 in PNAS) desmin from desmin IF. The authors further show that ATAD1 encourages calpain-1-dependent proteolysis of soluble desmin after extraction from the desmin IF in an in vitro enzymatic proteolysis assay.

Major comments:

The authors present clear and convincing arguments from in vivo and in vitro experiments for their proposed model of ATAD1/PLAA/UBXN4-aided calpain-1-mediated proteolysis of desmin IF.
In my opinion, no additional experimental evidence is essential to underlining their statement.
Data and methods are presented clearly and understandably to allow for the reproduction and the reapplication of the utilized methods for verifying the presented data and analyzing complementary aspects in a similar fashion.

A concern is with the presentation of mass spectrometry results, particularly regarding Table

I: I am wondering whether the presented UPS components were the only proteins found in the proteomics screens or whether any filtering has taken place to only show UPS components in this manuscript. If so, please note the total number of proteins identified in the respective proteomics analyses and explain how filtering for UPS components was performed. This comment goes in line with the first minor comment on Figure 1A, see below. The relatively small number of individuals analyzed per experiment is owing to the limiting nature of mouse research and therefore acceptable. The observed alignment of the individual results is commendable, underlines the experimentator's ability, and strengthens the reached conclusion of the study.

Minor comments:

Figure 1A seems redundant, since the experimental approaches are described in the text and the Venn diagram does not integrate the identification of ATAD1 into the setting of the conducted screens, e.g. by showing how many additional proteins were identified in these two screens before the authors tended to their candidate ATAD1.

Word order mistake on page 6 in the sentence: "To test whether Atad1 is important for atrophy, we suppressed...".

Figure 1D: statistical analysis of the significance of the fiber area difference missing

Figure 2A: desmin ubiquitylation is not shown in these samples by immunoblotting against (poly-)ubiquitin, but only by the identification of high molecular weight bands of the desmin blot. I wonder about the specificity of the desmin antibody in this case and about the manner of sample extraction/isolation for this particular blot, as a detailed description is missing. There seems not to have been any muscle tissue fractionation beforehand, if I am correct?

Orthography mistake "demin" instead of "desmin" on page 7 in sentence "It is noteworthy that the amount of ubiquitinated demin..."

Figure 3C: image quality is insufficient; some protein names are rather difficult to decipher

Word missing on page 13 in sentence "In addition, by 10 minutes of incubation, phosphorylated ... due to their processive cleaveage *by* calpain-1 ..."

Figure 4F: statistical analysis of the significance of the fiber area difference missing

"ug" on page 21 in "Briefly, 20ug of plasmid DNA..." is probably supposed to be "µg". In general, please be aware of correct unit declaration and space character usage before units.

Please be aware of the usage of correct nucleic acid and protein nomenclature and style: When referring to gene or transcript levels mark the candidate characters in italic, e.g. Atad1 mRNA levels, shUbxn4, versus ATAD1 protein etc. In addition, please be aware to use the correct gene and protein name styles: e.g. shCapn1 instead of shCAPN1 for shRNA targeting the murine Capn1 transcript in Figure 4 in comparison to CAPN1 the protein. Helpful link: https://www.biosciencewriters.com/Guidelines-for-Formatting-Gene-and-Protein-Names.aspx

2. Significance:

Significance (Required)

Aweida & Cohen present evidence for the involvement of the AAA-ATPase ATAD1 not only in regulation of synaptic plasticity and the extraction of mislocalized proteins from the mitochondrial membrane, but also in a collaboration with the ubiquitin-binding proteins PLAA and UBXN4 in the disassembly of desmin intermediate filaments in muscle atrophy. The authors compare this newly discovered function of the AAA-ATPase ATAD1 to the numerous functions of the AAA+ ATPase p97/VCP and raise compelling arguments for their statement. Previously, E3 ligases that ubiquitylate sarcomere components in muscle atrophy have been identified, such as MuRF1 (Bodine et al. 2001 in Science) and TRIM32 (reviewed in Bawa et al. 2021 in Biomolecules), but the complete extraction mechanism of monomers from the diverse macromolecular fibrillary structures in muscle has been lacking.

Both, researchers of general proteostasis mechanisms, in particular their impact on muscle function and metabolism, as well as medical researcher investigating therapeutic roads may appreciate the authors' work. This study opens up various roads to follow with complementing investigations on the many functions of the UPS in the regulation of muscle fiber architecture and functionality.

I am working on proteostasis and particularly the UPS. I have a long-standing track record on muscle assmebly mechanisms, the regulation of E3 ligases and p97/VCP functions.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

Full Revision

1. General Statements [optional]

This section is optional. Insert here any general statements you wish to make about the goal of the study or about the reviews.

2. Point-by-point description of the revisions

This section is mandatory. Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

The manuscript reports the identification of a novel protein complex involved in denervation-induced desmin degradation. The first protein to be identified was the ATPAse Atad1. A clever isolation strategy was based on the fact that the ATPAse p97/VCP is involved in the extraction of ubiquitinated myofibrillar proteins but is not required for the removal of ubiquitinated desmin filaments. The authors reasoned that a related ATPAse might be specifically required for desmin filaments. Atad1 was identified by treating desmin filaments with a nonhydolyzable ATP analog and looking for ATPases that are associated with desmin filaments by proteomics. Knockdown of Atad1 causes a loss of desmin degradation and led to a loss of denervation-induced muscle atrophy. It seems that Atad1 binds desmin in a phsphorlation-dependent manner, although the binding maybe mediated by a protein that hasn't yet been identified. The authors went on and identified two additional proteins which together with Atad1 form a protein complex involved in recruiting calpain for desmin degradation.

Overall, this study is very convincing providing novel important insight. I have only some minor comments

Minor comments

1. I wondered whether Aatd1 is expressed at higher-than-normal levels in muscle and heart. I looked

that expression pattern up and it seems that they are especially abundant in muscle and heart and expressed at lesser levels in smooth muscle and overall have a restricted expression.

We now analyzed ATAD1 levels in various tissues by Western Blotting and the new data is presented as Fig. S2. ATAD1 is present in many tissues and thus may have many cellular roles.

Maybe you have some data on their expression in muscle tissue. Did you perform some staining of muscle tissue at baseline and after denervation with regard to the protein localization by immunostaining?

The new associations between ATAD1 and its protein partners reported herein were further validated by an immunofluorescence staining of longitudinal sections from 7 d denervated muscles and super-resolution Structured illumination microscopy (SIM). The new data presented as Fig. 3E demonstrate colocalization of ATAD1 with calpain-1, PLAA and UBXN4. To confirm that these proteins in fact colocalize, we measured the average colocalization of ATAD1 with calpain-1, PLAA and UBXN4 using the spots detection and colocalization analysis of the Imaris software (Fig. 3E). Only spots that were within a distance threshold of less than 100 nm were considered colocalized (Fig. 3E, graph).

2. The string data presented in Figure 3C needs some further explanation with regard to the colors used for the different proteins. While the authors explained the meaning of the proteins labeled in red, there is no explanation for the other colors.

These were arbitrary colors assigned to protein nodes by the STRING database. The current color code we use is only meant to group the UPS enzymes based on function (e.g. E2s, E3s, DUBs etc). This information has now been added to figure legend.

3. Molecular weights in Fig. 2E, 3D needs to be 'repaired' and additional MW information is required in case of the ubiquitin blot shown in 3D.

All molecular weight values and protein ladders have been added.

4. Fiber size distributions shown in Fig. 1D and 4F. Have the differences been statistically tested?

We thank the reviewer for raising this important point because we just established an approach to quantitate these effects statistically using Vargha-Delaney A-statistics test and Brunner-Manzel test. Our new paper on this topic entitled "A semi-automated measurement of muscle fiber size using the Imaris software" by Gilda et al. was recently published in the AJP Cell Physiol. As requested by the reviewer, we now also apply A-statistics test and Brunner-Manzel test on the fiber size measurements presented in our current manuscript (Figs. 1C, 4F and Table I), which show a significant difference in size distributions of fibers expressing shAtad1 vs. adjacent non-transfected fibers. As indicated in our paper (Gilda et al, 2021), the A-statistics is a direct measure of the fiber size effect, and it shows significant beneficial

effects on cell size by shAtad1 (Table I). Such effects can be simply missed by traditional measurements of median, average, and Student's t-test.

5. For my taste the referral to the individual data (Fig. numbers) in the discussion section is too detailed and becomes a second results section. This should be substituted by a summary paragraph before the implications are discussed.

We agree and revised the discussion section accordingly.

6. The summary slide is very good. However, could you please add information, which protein of the three in the Atad1 complex is depicted by each symbol?

The model slide has been revised to include all enzymes studied in this paper, and a legend to improve clarity.

Reviewer #1 (Significance (Required)):

Novel insight into the proteins involved in desmin filament degradation. Since this is an important subject both in muscle and heart and plays an important role in muscle and heart disease, it is of significant clinical importance. Currently it has only been implicated in denervation-induced skeletal muscle atrophy, but it is likely that desmin filament metabolisms is also similarly regulated in the heart.

I am a researcher mainly focusing on the cardiac biology with some expertise also on muscle, however no specific knowledge about desmin filament biology.

Referee Cross-commenting

Overall, I think all three reviewers agree that this is a significant and important paper. I think that the comments made by the reviewers are fair and probably add to the quality of the manuscript.

We are pleased that the reviewers found our paper novel and important.

Thus, both myself and reviewer 2 agree that it would be useful to visualize Atad1 and partners localization in muscle fibers by immunofluorescence. These data would provide independent support to the model the authors are proposing, which currently is only based on biochemical analysis.

These data have been added as new Fig. 3E.

I also support the proposed use of proximity ligation to provide further evidence of the presence of the Atad1, Ubxn4 and PLAA in a complex. However, this experiment depends on the quality of the available antibodies and I would consider this not absolutely required.

Because our antibodies are not suitable for proximity ligation assay (PLA), we used a super-resolution SIM microscope, immunofluorescence, and the spots detection and colocalization analysis of the Imaris software to confirm colocalization of ATAD1 and its partners (new Fig. 3E). Similar to PLA (where signal is generated only if two antibodies used for staining are 100nm apart), only spots that were within a distance threshold of less than 100 nm were considered colocalized (Fig. 3E, graph). In addition, we present immunoprecipitation (Fig. 3D) and use three independent mass spectrometry-based proteomic approaches to validate these new associations.

I also agree that some further information on the proteomics data (as suggested by reviewer 3) is required with regard to the method of filtering for UPS components was performed.

We agree and thank the reviewer for this comment. More information on the proteomics data have been added to the text and legend to Table II.

The proposed request for further information on the electroporation approach is a valid comment and if the authors have this information, it would be good to provide. However, I do not recommend further experiments as overall the data are very consistent and the findings are very significant and represent a major advance in our understanding of desmin degradation.

With regard to the electroporation approach, i) representative images have been added to Figs. 1C and 4F, ii) a statement was added to Methods under "in vivo electroporation" about the percent of transfection routinely used in our experiments (60-70%), iii) we determine transfection efficiency by dividing the number of transfected fibers (also express GFP) by the total number of fibers in the same muscle cross section (using the Imaris software). This approach was fully validated in our recent papers by Goldbraikh et al EMBO Rep, 2020 (see supplementary material) and Gilda et al AJP-Cell Physiol, 2021.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In their manuscript the authors show the involvement of the AAA ATPase Atad1 in Desmin degradation. They identify PLAA and Ubxn4 as partners of Atad1 that participate to its function in desmin degradation.

A general comment is that some conclusions are overstated. The authors mention several times that Atad1 depolymerises desmin filaments. The data show that Atad1 participates to the degradation of Desmin and to its solubilization. "Depolymerisation" should be kept for the model presented in figure 8 but not used in the result section.

We respectfully disagree with the reviewer that our conclusions are overstated. Early studies from Fred Goldberg's group showed that filaments are not accessible to the catalytic core of the proteasome (Solomon and Goldberg, JBC, 1996), and therefore must depolymerize before degradation. Accordingly,

more recent studies by us and others identified distinct enzymes and cellular steps promoting disassembly and subsequent degradation of ubiquitinated desmin filaments (Cohen, JCB, 2012; Aweida, JCB, 2018) and myofibrils (Cohen, JCB, 2009; Volodin, PNAS, 2017). In the current manuscript, we employed a similar approach as we used before to analyze disassembly of filamentous myofibrils by p97/VCP (Volodin, PNAS, 2017), and demonstrate a critical role for ATAD1-PLAA-UBXN4 complex in promoting desmin IF disassembly and loss (figures 2C, 3D, 3G, 4C, 4G, 4H). We show that ATAD1 binds intact insoluble desmin filaments in an early phase during atrophy (3 d after denervation)(figures 2B, 2F) and later accumulates in the cytosol bound to soluble ubiquitinated desmin (figure 3D). Moreover, downregulation of ATAD1, PLAA or UBXN4 in mouse muscles prevents the solubilization of desmin IF (figures 2C, 3G, 4C) because in these muscles desmin accumulates as ubiquitinated insoluble filaments. Based on these data we conclude that Atad1 complex promotes desmin IF disassembly and subsequent loss.

Major comments:

1) It would be useful to visualize Atad1 and partners localization in muscle fibers in immunofluorescence. Do they colocalize with desmin filaments, with calpain?

As requested, the new associations between ATAD1 and its protein partners reported herein were further validated by an immunofluorescence staining of longitudinal sections from 7 d denervated muscles and super-resolution Structured illumination microscopy (SIM). The new data presented as Fig. 3E demonstrate colocalization of ATAD1 with calpain-1, PLAA and UBXN4. To confirm that these proteins in fact colocalize, we measured the average colocalization of ATAD1 with calpain-1, PLAA and UBXN4 using the spots detection and colocalization analysis of the Imaris software (Fig. 3E). Only spots that were within a distance threshold of less than 100 nm were considered colocalized (Fig. 3E, graph). Given the antibodies in hand and new ones that we purchased, as well as the species of the antibodies, we were able to perform and optimize the staining only for the presented combinations of antibodies.

2) In the same line, interactors were obtained from large crosslinked complexes. It would make the model more convincing if direct interactions with Atad1 were shown, for example using Proximity Ligation Assays.

Because our antibodies are not suitable for proximity ligation assay (PLA), we used a super-resolution SIM microscope, immunofluorescence, and the spots detection and colocalization analysis of the Imaris software to confirm colocalization of ATAD1 and its partners (new Fig. 3E). Similar to PLA (where signal is generated only if two antibodies used for staining are 100nm apart), only spots that were within a distance threshold of less than 100 nm were considered colocalized (Fig. 3E, graph). In addition, we present immunoprecipitation (Fig. 3D) and use three independent mass spectrometry-based proteomic approaches to validate these new associations.

3) Evaluation of atrophy is made on cross-sections of muscles electroporated with shRNAs. Histology pictures should be shown.

As requested, representative images of transfected muscles were added to figures 1C and 4F.

4) What is the percentage of electroporated fibers? To evaluate the effect of shRNAs it is important to have this information. For example, if the efficiency is 50% it means that the reduction in expression of the target in electroporated fibers is twice the value reported for the whole muscle. Alternatively, immunofluorescence could be provided to see the decrease in targeted proteins in electroporated fibers.

We determine transfection efficiency by dividing the number of transfected fibers (also express GFP) by the total number of fibers in the same muscle cross section (using the Imaris software). This approach is fully validated in our recent papers by Goldbraikh et al EMBO Rep, 2020 (see supplementary material) and Gilda et al AJP-Cell Physiol, 2021. For our biochemical studies we always analyze muscles that are at least 60-70% transfected (added to methods).

As shown in figures 1B, 3F, and 4A-B, our shRNAs reduced gene expression by at least 40-50%, which in a whole muscle was sufficient to promote the beneficial effects on muscle (as mentioned in the text, shCAPN1 was validated in Aweida, JCB, 2018). Similar reduction in gene expression is commonly seen by the in vivo electroporation of a fully developed mouse muscles because transfection efficiency is never 100%. This means that the beneficial effects on muscle by the electroporated shRNA must underestimate the actual protective effects by gene downregulation. To prove that these beneficial effects on muscle result from specific gene downregulation, we compare and analyze in parallel in each experiment muscles transfected with shLacz scrambled control.

5) The same is true for all the experiments quantifying the effect of shRNAs in western blot. Since quantifications are probably made on whole muscles (ie a mix between electroporated and non electroporated fibers) and since the percentage of electroporated fibers is not given it is not possible to estimate the efficiency of the shRNAs in electroporated fibers.

As mentioned above and now also in the text, for our biochemical studies we always analyze muscles that are ~60-70% transfected. This methodology is very well established in our lab, and a reduction of 40-50% in gene expression by our shRNAs is sufficient to promote the beneficial effects on mouse muscle (see our papers in JCB, PNAS, Nat Comm, EMBO rep).

6) Figure 2C: by decreasing solubilization of desmin, one would expect a decrease in the levels of soluble desmin. Conversely the authors observe an increase in both insoluble and soluble desmin. Of course, this can be explained by reduced desmin degradation once solubilized but this should be demonstrated at least by showing that UPS inhibitors induces an increase in soluble ubiquitinated Desmin.

The reviewer raises an important point that we now discuss in the text. Soluble pool of desmin, its homolog vimentin as well as other Type III IF proteins is small as these proteins mostly exist in the cell assembled within filaments (see papers by RA Quinlan and WW Franke). This soluble pool of desmin may

function either as precursors to the mature filament or as components released during filament turnover. Because we block desmin IF disassembly by downregulating Atad1, the soluble desmin that accumulates in the cytosol likely represents new precursors whose degradation also requires ATAD1. Therefore, we conclude that ATAD1 promotes degradation of desmin filaments and of soluble proteins (see also figures 2E and 4D).

As requested by the reviewer, we inhibited proteasome activity by injecting mice with Bortezommib and measured the effects on desmin content in denervated muscle (new figure 2D). Our new data clearly demonstrate accumulation of ubiquitinated desmin in atrophying muscles where proteasome activity was inhibited, indicating that in denervated muscles desmin is degraded by the proteasome.

7) Figure 2E: the levels of Atad1 in the insoluble fraction seem to be the same in the shLacZ and GSK3DN conditions, whereas the phosphor Ser is different. In other words, there should be more Atad1 in the insoluble fraction with shLacZ than with GSAK3DN since the phosphorylation level with shLacZ is significantly higher.

To quantitate the changes in ATAD1 association with desmin and avoid confusion by the reader, we performed densitometric measurements of ATAD1 and desmin, and depict in a graph the ratio of ATAD1 to desmin in the insoluble fraction. The new data was added to figure 2F and clearly demonstrate that ATAD1 association with desmin is significantly reduced in muscles expressing GSK3β-DN. These findings further support our conclusions that Atad1 association with desmin IF requires desmin phosphorylation.

8) Figure 4E: the authors state that phosphorylation decreases because of increased degradation (lanes 6-8). However, Calpain also increases degradation and phosphorylation is increased (lanes 2-4), so increasing degradation does not systematically cause a decrease in phosphorylation. Similarly, lane 5 Atad1 induces less degradation than Calpain, however, it causes a decrease in phosphorylation. Explain.

Here we use a cleavage assay, which was established and validated in our recent JCB paper (Aweida 2018). Desmin filaments were isolated from mouse muscle and the obtained preparation was divided between 9 tubes (hence there is no situation for "increase in phosphorylation" as indicated by the reviewer). Recombinant calpain-1 was then added to the tubes and cleavage of phosphorylated desmin was analyzed over time. Because the substrate for calpain-1 is phosphorylated desmin, we measured the content of both desmin and its phosphorylated form in the tube throughout the duration of the experiment. Only when cleavage of phosphorylated desmin by calpain-1 was accelerated (i.e., in the presence of Atad1), a rapid reduction in the amount of phosphorylated desmin could be detected (compare lanes 6-8 with 5) concomitantly with accumulation of small desmin fragments in short incubation times (compare lanes 6-7 with 2-3).

With respect to the reviewer's comment that "Atad1 induces less degradation than Calpain" in lane 5, please note that Atad1 is not a protease and cleavage of desmin occurs in this experiment only in the presence of calpain-1. However, if there is a slight reduction in phosphorylated desmin, it should

account for the ability of ATAD1 appears to slowly disassemble desmin IF (as our in vivo data by shATAD1 show).

9) The AAA ATPase VCP shares partners with Atad1 and is involved in muscle atrophy. It would greatly add to the manuscript if the authors inhibited VCP to compare its effect to Atad1

As stated in the text, we previously demonstrated that p97/VCP is not required for desmin filament loss: "the AAA-ATPase, p97/VCP disassembles ubiquitinated filamentous myofibrils and promotes their loss in muscles atrophying due to denervation or fasting (Piccirillo and Goldberg, 2012; Volodin et al., 2017). However, desmin IF are lost by a mechanism not requiring p97/VCP (Volodin et al., 2017). We show here that their degradation requires a distinct AAA-ATPase, ATAD1". Therefore, our current studies were undertaken to specifically identify the AAA-ATPase that is involved in desmin filament disassembly and loss. Accordingly, p97/VCP was not detected by our mass spectrometry-based proteomic analyses presented here (stated in the discussion).

We did identify PLAA and UBXN4 as ATAD1 partners and show they are required for desmin loss, and therefore state in the text that "PLAA and UBXN4 are also known cofactors for p97/VCP (Liang et al., 2006; Papadopoulos et al., 2017), a AAA-ATPase that was not in our datasets, indicating that p97/VCP adaptors can bind and function with other AAA-ATPases".

Minor comments:

1) The soluble fraction contains a large number of ubiquitinated proteins. Please explain how it can be stated that an increase in total soluble polyubiquitinated proteins corresponds to an increase in ubiquitinated desmin.

We do not state in the text that "an increase in total soluble polyubiquitinated proteins corresponds to an increase in ubiquitinated desmin". We state that "stabilization of desmin filaments attenuates overall proteolysis. The reduced structural integrity of desmin filaments on denervation is likely the key step in the destabilization of insoluble proteins (e.g. myofibrils) during atrophy, leading to the enhanced solubilization and degradation in the cytosol". We invite the reviewer to read our papers about this topic by Cohen 2012, Volodin 2017, and Aweida 2018. Using a dominant negative of desmin polymerization we show that disassembly of desmin filaments is sufficient to trigger myofibril destruction and consequently overall proteolysis (because myofibrils comprise ~70% of muscle proteins).

2) Page 11: the authors conclude that denervation enhance the interactions with Atad1. Figure 3D indeed show an increase for Ubxn4, but it is not clear for the other proteins.

Figure 3D shows that in 7 d denervated muscles there is an increase in associations between ATAD1 and ubiquitinated desmin, UBXN4, PLAA and calpain-1.

3) Figure 4 F: show muscle sections

Review COMMONS

A representative image was added as requested.

4) Page 21 in vivo transfection: it is stated "see details under immunofluorescence" but there is no immunofluorescence section in materials and methods.

Thank you. An immunofluorescence section has been added to Methods.

5) The authors show that Atad1 inhibition in innervated muscle is sufficient to induce muscle hypertrophy (Figure 4E). They conclude that the hypertrophic effect of Atad1 is due to the inhibition of Desmin degradation. However, this hypertrophic effect could be independent of the action of Atad1 on Desmin.

We believe the reviewer refers to figure 4F-H, where we show that downregulation of ATAD1 prevents the basal turnover of desmin and of soluble proteins and causes muscle fiber growth. Based on this data we speculate in the text that "ATAD1 attenuated normal muscle growth most likely by promoting the loss of desmin filaments and of soluble proteins … Thus, ATAD1 seems to function in normal postnatal muscle to limit fiber growth, and suppression of its activity alone can induce muscle hypertrophy". We agree with the reviewer that in addition to these beneficial effects on desmin and soluble proteins, ATAD1 downregulation may contribute to muscle growth by additional mechanisms.

Reviewer #2 (Significance (Required)):

This is new information in the field since calpain cannot hydrolyze desmin insoluble filaments and that the mechanisms that give calpain access to desmin are not known.

The authors already made important contribution in the study of muscle atrophy and especially in desmin degradation. This work constitutes a new advance in their attempts to understand the molecular mechanisms leading to desmin degradation and muscle atrophy.

Audience: desmin is the main intermediate filament in skeletal muscle. This work will therefore interest scientists working on skeletal muscle.

Expertise of the reviewer: molecular and cellular biology of skeletal muscles, muscle atrophy.

Referee Cross-commenting

I fully agree with reviewer 1.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

The manuscript by Aweida & Cohen introduces a novel complex formed by the AAA-ATPase ATAD1 and its interacting partners PLAA and UBXN4 as initiator of calpain-1-mediated disassembly of ubiquitylated desmin intermediate filaments (IF) during muscle atrophy. The authors use a denervation model of murine tibialis anterior muscles as their main resource for experimentation. They apply a kinase trap-assay and co-immunoprecipitation method followed by mass spectrometry as starting point for identifying novel interactors of desmin IF (Aweida et al. 2018 in JCB). They continue to analyze their candidates using immunoblotting, co-immunoprecipitation, shRNA-mediated intramuscular knock-down, gel filtration, mass spectrometry, and enzyme assays. In their experiments, thee authors show an accumulation of ATAD1 in the insoluble desmin filament fraction of denervated muscle fibers together with an increase in ubiquitylation of desmin filaments. Both proteomics experiments of size-exclusion chromatography of denervated muscles and ATAD1 immunoprecipitation identify several components of the ubiquitin-proteasome system as novel interactors of ATAD1, that are also bound to insoluble desmin filaments after muscle denervation. Following additional co-immunoprecipitation and knock-down experiments, the authors confirm PLAA and UBXN4 as novel cofactors of Atad1 that help in extracting previously GSK3-β-phosphorylated and TRIM32-ubiquitylated (Aweida et al. 2018 in JCB, Volodin et al. 2017 in PNAS) desmin from desmin IF. The authors further show that ATAD1 encourages calpain-1-dependent proteolysis of soluble desmin after extraction from the desmin IF in an in vitro enzymatic proteolysis assay.

Major comments:

The authors present clear and convincing arguments from in vivo and in vitro experiments for their proposed model of ATAD1/PLAA/UBXN4-aided calpain-1-mediated proteolysis of desmin IF.
In my opinion, no additional experimental evidence is essential to underlining their statement.
Data and methods are presented clearly and understandably to allow for the reproduction and the reapplication of the utilized methods for verifying the presented data and analyzing complementary aspects in a similar fashion.

A concern is with the presentation of mass spectrometry results, particularly regarding Table I: I am wondering whether the presented UPS components were the only proteins found in the proteomics screens or whether any filtering has taken place to only show UPS components in this manuscript. If so, please note the total number of proteins identified in the respective proteomics analyses and explain how filtering for UPS components was performed. This comment goes in line with the first minor comment on Figure 1A, see below.

We thank the reviewer for this valuable comment, as it helps clarify a point that was not completely lucid in the previous version of this manuscript. Because our paper focuses on protein degradation, we extracted from our datasets only UPS components that were identified with ≥2 unique peptides using

DAVID annotation tool-derived categories (Table II). Column 1 includes UPS components that were co-purified with ATAD1 by size exclusion chromatography (SEC)(20 out of 427 total proteins), and column 2 includes UPS components that were co-purified with ATAD1 by immunoprecipitation from muscle homogenates (17 out of 592 total proteins). These two proteomics experiments were oriented specifically towards identifying ATAD1-binding partners. To further validate our observations, we compared these lists of ATAD1-interacting components to our previous kinase-trap assay dataset (Aweida 2018, 1552 total proteins were identified) and included in column 3 only the proteins that overlapped with the other two proteomics approaches. The kinase trap assay was used to identify proteins that utilize ATP for their function and act on desmin, and as mentioned in the text, ATAD1 was one of the most abundant proteins in the sample. Of note is UBXN4, which was identified only by our kinase trap assay, and accumulated on desmin after denervation. These interactions between active enzymes in vivo must be transient and very dynamic, hence using three approaches did not identify the exact same subset of putative adaptors (see "discussion"). These points are now further elaborated in the text and the legend for Table II.

The relatively small number of individuals analyzed per experiment is owing to the limiting nature of mouse research and therefore acceptable. The observed alignment of the individual results is commendable, underlines the experimentator's ability, and strengthens the reached conclusion of the study.

We thank the reviewer for this comment.

Minor comments:

Figure 1A seems redundant, since the experimental approaches are described in the text and the Venn diagram does not integrate the identification of ATAD1 into the setting of the conducted screens, e.g. by showing how many additional proteins were identified in these two screens before the authors tended to their candidate ATAD1.

We agree and therefore removed Fig. 1A.

Word order mistake on page 6 in the sentence: "To test whether Atad1 is important for atrophy, we suppressed...".

Corrected.

Figure 1D: statistical analysis of the significance of the fiber area difference missing

Statistics for these effects is now included in new Table I. We quantitated the effects statistically using Vargha-Delaney A-statistics test and Brunner-Manzel test, based on our recent methodology paper in AJP Cell Physiol: "A semi-automated measurement of muscle fiber size using the Imaris software" (Gilda et al. 2021). The new statistical analyses show a significant difference in size distributions of fibers

expressing shAtad1 vs. adjacent non-transfected fibers (Table I). As indicated in our paper (Gilda et al, 2021), the A-statistics is a direct measure of the fiber size effect.

Figure 2A: desmin ubiquitylation is not shown in these samples by immunoblotting against (poly-)ubiquitin, but only by the identification of high molecular weight bands of the desmin blot. I wonder about the specificity of the desmin antibody in this case and about the manner of sample extraction/isolation for this particular blot, as a detailed description is missing. There seems not to have been any muscle tissue fractionation beforehand, if I am correct?

This blot presents an analysis of desmin filaments isolated from mouse muscle, which are purified with associated proteins. In order to specifically detect ubiquitinated desmin filaments we must use a specific desmin antibody (antibody and methodology are validated in Cohen 2012 JCB, Volodin 2017 PNAS, and Aweida 2018 JCB). An antibody against ubiquitin conjugates will detect all proteins that are ubiquitinated in this insoluble preparation (e.g. proteins that bind desmin).

Orthography mistake "demin" instead of "desmin" on page 7 in sentence "It is noteworthy that the amount of ubiquitinated demin..."

Corrected.

Figure 3C: image quality is insufficient; some protein names are rather difficult to decipher

The figure has been revised to improve clarity.

Word missing on page 13 in sentence "In addition, by 10 minutes of incubation, phosphorylated ... due to their processive cleaveage *by* calpain-1 ..."

We thank the reviewer for reading the paper thoroughly and carefully. The missing word was added to the text.

Figure 4F: statistical analysis of the significance of the fiber area difference missing

Statistics is now included in new Table I. Asmentioned above, we quantitated the effects statistically using Vargha-Delaney A-statistics test and Brunner-Manzel test, based on our recent methodology paper in AJP Cell Physiol: "A semi-automated measurement of muscle fiber size using the Imaris software" (Gilda et al. 2021).

"ug" on page 21 in "Briefly, 20ug of plasmid DNA..." is probably supposed to be "µg". In general, please be aware of correct unit declaration and space character usage before units.

Review COMMONS

Corrected.

Please be aware of the usage of correct nucleic acid and protein nomenclature and style: When referring to gene or transcript levels mark the candidate characters in italic, e.g. Atad1 mRNA levels, shUbxn4, versus ATAD1 protein etc. In addition, please be aware to use the correct gene and protein name styles: e.g. shCapn1 instead of shCAPN1 for shRNA targeting the murine Capn1 transcript in Figure 4 in comparison to CAPN1 the protein. Helpful link: https://www.biosciencewriters.com/Guidelines-for-Formatting-Gene-and-Protein-Names.aspx

We thank the reviewer for this comment. The nomenclature for all genes and proteins have been revised accordingly.

Reviewer #3 (Significance (Required)):

Aweida & Cohen present evidence for the involvement of the AAA-ATPase ATAD1 not only in regulation of synaptic plasticity and the extraction of mislocalized proteins from the mitochondrial membrane, but also in a collaboration with the ubiquitin-binding proteins PLAA and UBXN4 in the disassembly of desmin intermediate filaments in muscle atrophy. The authors compare this newly discovered function of the AAA-ATPase ATAD1 to the numerous functions of the AAA+ ATPase p97/VCP and raise compelling arguments for their statement. Previously, E3 ligases that ubiquitylate sarcomere components in muscle atrophy have been identified, such as MuRF1 (Bodine et al. 2001 in Science) and TRIM32 (reviewed in Bawa et al. 2021 in Biomolecules), but the complete extraction mechanism of monomers from the diverse macromolecular fibrillary structures in muscle has been lacking.

Both, researchers of general proteostasis mechanisms, in particular their impact on muscle function and metabolism, as well as medical researcher investigating therapeutic roads may appreciate the authors' work. This study opens up various roads to follow with complementing investigations on the many functions of the UPS in the regulation of muscle fiber architecture and functionality.

I am working on proteostasis and particularly the UPS. I have a long-standing track record on muscle assmebly mechanisms, the regulation of E3 ligases and p97/VCP functions.

1st Editorial Decision 10th May 2022

Dear Prof. Cohen,

Thank you for the submission of your revised manuscript to EMBO reports. Two of the original referees at Review Commons have looked at the paper and now consider that you have properly dealt with all of their major concerns. Referee #2 expresses, however, some minor concerns regarding figures 1C and 4F that will require your attention (see below). Additionally, before we can proceed with the acceptance of your study, there are a few minor editorial issues that need to be corrected:

- The manuscript needs a Data Availability Section where you describe the databases in which datasets have been made available. For transparency, this section is required even if just to mention that there are no public datasets associated to the results in the paper.
- While you provide author contributions, they must be specified in the manuscript submission system using the the CRediT contributor roles taxonomy (https://casrai.org/credit/).
- Reference "JE, G., K. JH, E. AY, T. N, P. A, A. B, J. W, and C. S. 2021. A semiautomated measurement of muscle fiber size using the Imaris software. American journal of physiology. Cell physiology. 321:C615-C631. doi:10.1152/AJPCELL.00206.2021" must be corrected to match the style of the rest of the references, including surnames instead of just initials.
- Main manuscript figures must be uploaded as individual files and each figure must fit on a single page. The number of figures can be increased if subdivision is required. Make sure manuscript callouts are modified accordingly in that case.
- Please include a callout for Figure 1A in the manuscript text. The red box in figure 3B needs to be defined in the figure legend. Scale bars must be thicker for better visibility and their size must be removed from the figure and mentioned in the corresponding figure legend.
- Tables I and II must be renamed to Table 1 and 2.
- Supplementary information (2 figures and 1 table) should be renamed as Appendix and should be presented as a single file starting with a Table of Contents. Correct nomenclature for Appendix figures and tables is "Appendix Figure S1" or "Appendix Table S1" respectively. Please rename figures and tables and manuscript callouts accordingly.
- The journal requires a completed author checklist (which covers animal welfare, human subjects, data deposition and ethics), to be filled and returned to the editorial office at revision. All information covered in the checklist should be included in the manuscript. Please note that the author checklist will be included in the transparent process information. Go to https://www.embopress.org/page/journal/14693178/authorguide#submissionofrevisions to download the checklist.

Please provide the synopsis files. The paper's synopsis is composed of:
- a short 'blurb' text summarizing in two sentences the study (max. 250 characters) and three to four 'bullet points' highlighting the main findings. Bullet points and standfirst text should be submitted as a separate manuscript file in LaTeX, RTF or MS Word format. This text has not been provided.
- A "synopsis image", which can be used as a "visual title" for the synopsis section of your paper. While you provide this image, it needs to be resized. This figure must be in PNG or JPG format with pixel dimensions of 550 x 300-600 (width x height).

During a standard image analysis we detected potential aberrations in the figure set. In particular, Figure 2A, 4A and 4C are over-contrasted, while there is a likely compression artefeact in the top blot of figure 2B. Please send us the original data for these figures (source data, see below). If you make changes to the figures, please include a point-by-point describing what you changed.

Image source data should be provided as one file per figure that contains the original, uncropped and unprocessed scans of all or key gels/microscopy images used in the figure. The file(s) should be labeled with the appropriate figure/panel number, and should display molecular weight markers and indicate which areas are shown in the figure; further annotation may be useful but is not essential. Source data files will be published online with the article as supplementary "Source Data."

Let me know if you have any further questions regarding any of these points. Thank you again for giving us the chance to consider your manuscript for EMBO reports and congratulations!

I look forward to receiving the final version of your manuscript with these minor changes included.

Yours sincerely,

David del Alamo
Editor
EMBO reports

Referee #2:

The authors have adressed my comments.

I have a minor comment:
Figures 1C and 4F include photos of electroporated muscle fibers. These photos should show a whole muscle section, not only a few fibers.
The description of photos 1C and 4F are missing in the figure legends.

September 14, 2021

Dr. David Del Alamo

Editor

EMBO reports

Dear Dr. Del Alamo,

Enclosed is the final version of our manuscript "The AAA-ATPase ATAD1 and its partners promote degradation of desmin intermediate filaments in muscle".

We have addressed the remaining minor comment by reviewer #2, and added the requested data as new Appendix figures S1 and S2.

Paper corrections according to editorial requests:

- The manuscript needs a Data Availability Section where you describe the databases in which datasets have been made available. For transparency, this section is required even if just to mention that there are no public datasets associated to the results in the paper.

  A sentence has been added to text after Materials and methods section.

- While you provide author contributions, they must be specified in the manuscript submission system using the the CRediT contributor roles taxonomy.

  Author contributions have been modified according to CRediT.

- Reference "JE, G., K. JH, E. AY, T. N, P. A, A. B, J. W, and C. S. 2021. A semiautomated measurement of muscle fiber size using the Imaris software. American journal of physiology. Cell physiology. 321:C615-C631. doi:10.1152/AJPCELL.00206.2021" must be corrected to match the style of the rest of the references, including surnames instead of just initials.

  The reference has been corrected accordingly.

- Main manuscript figures must be uploaded as individual files and each figure must fit on a single page. The number of figures can be increased if subdivision is required. Make sure manuscript callouts are modified accordingly in that case.

  Done.

- Please include a callout for Figure 1A in the manuscript text. The red box in figure 3B needs to be defined in the figure legend. Scale bars must be thicker for better visibility and their size must be removed from the figure and mentioned in the corresponding figure legend.

  Done.

- Tables I and II must be renamed to Table 1 and 2.

  Done.

- Supplementary information (2 figures and 1 table) should be renamed as Appendix and should be presented as a single file starting with a Table of Contents. Correct nomenclature for Appendix figures and tables is "Appendix Figure S1" or "Appendix Table S1" respectively. Please rename figures and tables and manuscript callouts accordingly.

  Done.

- The journal requires a completed author checklist (which covers animal welfare, human subjects, data deposition and ethics), to be filled and returned to the editorial office at revision. All information covered in the checklist should be included in the manuscript. Please note that the author checklist will be included in the transparent process information. Go to https://www.embopress.org/page/journal/14693178/authorguide#submissionofrevisions to download the checklist.

  Done.

- Please provide the synopsis files. The paper's synopsis is composed of:
  a short 'blurb' text summarizing in two sentences the study (max. 250 characters) and three to four 'bullet points' highlighting the main findings. Bullet points and standfirst text

should be submitted as a separate manuscript file in LaTeX, RTF or MS Word format. This text has not been provided.

Done.

- A "synopsis image", which can be used as a "visual title" for the synopsis section of your paper. While you provide this image, it needs to be resized. This figure must be in PNG or JPG format with pixel dimensions of 550 x 300-600 (width x height).

  Done.

- During a standard image analysis we detected potential aberrations in the figure set. In particular, Figure 2A, 4A and 4C are over-contrasted, while there is a likely compression artefeact in the top blot of figure 2B. Please send us the original data for these figures (source data, see below). If you make changes to the figures, please include a point-by-point describing what you changed.

  The original data are included in the Source data file.

- Image source data should be provided as one file per figure that contains the original, uncropped and unprocessed scans of all or key gels/microscopy images used in the figure. The file(s) should be labeled with the appropriate figure/panel number, and should display molecular weight markers and indicate which areas are shown in the figure; further annotation may be useful but is not essential. Source data files will be published online with the article as supplementary "Source Data."

  Done.

We believe the manuscript is significantly clearer as a result of these final changes, and we are grateful to the reviewers for their helpful suggestions. We trust that this version is now appropriate for publication in *EMBO reports*.

Prof. Shenhav Cohen
Technion Institute of Technology
Biology
Faculty of Biology, Room 214, Technion city
Haifa, - 32000
Israel

Dear Prof. Cohen,

Thank you for sending the further revised figure files, which I uploaded. I am now very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

****

THINGS TO DO NOW:

Please note that you will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/er_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2022-55175V2 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO Press Author Checklist

Corresponding Author Name: Shenhav Cohen	
Journal Submitted to: EMBO Reports	
Manuscript Number: 55175V1	

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January 2022)
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures
1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Appendix table S1

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods, figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	