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Peer Review File

N-terminal α-Amino SUMOylation Promotes Phosphorylation-Independent Cofilin-1 Translocation to the Mitochondrial Matrix and Induces Apoptosis

Corresponding Author: Professor Yong Li

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors challenge the conventional paradigm that CFL1 activity is driven solely by phosphorylation and actin binding. Instead, they reveal N-terminal sumoylation of CFL1 as actin-independent mechanism influencing mitochondrial integrity and apoptosis initiation. This is an interesting novel paper as follow up of the previously published paper on N-terminal sumoylation of CFL1.

1 - Overall, the figures are extremely small and therefore virtually impossible to read. It would be important to use full-size pages for each figure.

2 - It is important to show the relative amount of CFL1 sumoylation. Therefore please show for all immunoblot panels that deal with CFL1 sumoylation both non-modified CFL1 and SUMO-modified CFL1 on the same blot.

3 - Many immunoprecipitation experiments lack IgG negative controls. The IgG negative control is however used in Figure 7A. Please include IgG negative controls in all immunoprecipitation experiments.

Reviewer #2

(Remarks to the Author)
Review of Nature Communications NCOMMS-25-35241-T

Title: "N-terminal α-Amino SUMOylation Promotes Phosphorylation-Independent Cofilin-1 Translocation to the Mitochondrial Matrix and Induces Apoptosis"
Authors: Qi Deng, Xiaokun Gu, Jiaqian Feng, Jinhua Xiang, Xinyue Li, Weiji Weng, Ou Huang, Si-Jian Pan and Yong Li

Summary of Critique: This manuscript follows up work from the same laboratory which characterized an α-amino-SUMOylated cofilin, the first N-terminal SUMOylation of any protein. In this current paper, the authors focus on the role of this SUMOylation in cofilin function, particularly its targeting to mitochondria and initiating the mitochondrial-dependent apoptotic pathway first reported in 2003 by Chua et al. Many studies since then have focused on ser3 phosphorylation of cofilin regulating mitochondrial targeting of cofilin-actin along with the cofilin-dependent remodeling of actin surrounding mitochondria as being important for mitochondrial fission events that have been suggested to be responsible for the release of matrix proteins such as cytochrome c, triggering apoptosis via cytoplasmic caspase activation. In this manuscript, the authors utilize expression of a number of cofilin point mutations in cells silenced for expression of endogenous cofilin along with various SUMO-cofilin mutants to convincingly demonstrate that mitochondrial targeting of cofilin is independent of both cofilin's phosphorylation state and its actin binding ability, but is totally dependent upon N-terminal SUMOylation. Furthermore, they show convincing evidence that SUMO-cofilin is transported into the mitochondrial matrix, the first characterization of this location through both electron microscopy and co-immunoprecipitation with matrix proteins from lysates of purified mitochondria. Either through co-immunoprecipitation and mass spectrometry, direct binding studies, or expression knock downs, the authors identify roles for protein-folding chaperones (HSP70), specific outer (TOM20/TOM70)

and inner (Tim23) mitochondrial membrane proteins, and show a direct interaction of SUMO-cofilin with cytochrome c. Of major significance is the fact that all these functions of cofilin appear to be totally independent of actin binding since SUMOylated mutant forms of cofilin that lack the ability to bind actin undergo identical targeting. siRNA-induced decrease in cofilin-1 also decreases cytochrome c release from mitochondria and reduces caspase activity. Cells in brains of mice deficient in deSUMOylating activity (Senp1) undergo increased apoptosis, contain mitochondria with reduced membrane potential and demonstrate increased embryonic lethality that is increased to 100% by expression of cofilin-1 K34R, a mutation that undergoes enhanced N-terminal SUMOylation.

Critique: Both reviewers have read the manuscript thoroughly and checked the validity of the citations. Although the citations generally do not go back to the first reports of the items being referenced, the ones cited are adequate and most contain some relevant new information. However, ref 86 in line 875 of the manuscript is not in the reference list which ends at 82. This number was probably inserted accidentally from copying the information on plasmid construction from another manuscript and should either be included in the citations or removed.

Scientifically this is a very sound paper containing an enormous amount of high-quality research. Most of the work was performed in a variety of cultured mammalian cell lines, including CHO, HeLa, HEK293, and mouse embryonic fibroblasts. For some research questions that might involve cell-lineage dependent behavior, switching cell lines might be considered a detriment, but with cofilin sequence and function showing extremely high conservation across mammalian cells, the use of multiple cell lines does not present a problem in interpretation. Controls for each experiment are contained within each figure and several figures of Extended Data are provided for support. Most figures contain a huge amount of information including immunoblots, their quantified comparisons, immunofluorescence images and quantified co-localizations using Pearson's correlation coefficient of multiple fields, etc. Many are difficult to read in a printed version of the paper but can be enlarged on screen in the pdf to show clear results. Band quantification from blots (three replicates per sample) and immunofluorescence (six fields from three biological replicates) are used to provide statistical significance between treatments. The sample blots provided do support the quantifications shown in plots. We agree with the authors' interpretation of their data and with their overall conclusions.

Although the text is understandable, there are several grammatical errors throughout the manuscript. Some are issues with tense. It is better to use present than past tense for statements such as in line 69: "Alternatively, recent studies suggested that … " (should be suggest). Many other items deal with faulty sentence construction leading to sentence fragments- these are easily corrected. For example, line 168 starting with "Furthermore, expressed …" needs to have "we expressed". Lines 555-556- add comma after fertile and eliminate However while continuing the sentence. These may seem very minor (they are) but there are many throughout the text that can make reading it a bit awkward even though the ideas are well conveyed.

The biggest issue we had with the manuscript is the discussion, which is a restatement of the results focusing on how the new findings change the interpretation of previous work. This has already been stated in the results and could be summed up in 2-3 pages of the discussion. What is missing is addressing an extension of this knowledge. For example many recent papers have shown a relationship between the severity of cancers and and increased expression of Senp1. Of course this relationship could be explained by a decreased apoptotic activity in cancer cells due to decreased SUMO-cofilin. Would this not warrant a paragraph which would also highlight the importance of this current work to cancer research?

Additionally, previous work from the Li lab showed that SUMOylation of cofilin requires the N-terminal Met residue. Do the authors have any information on the relative abundance of the N-terminal Met form versus the N-acetylated Ala form? Most cytoplasmic proteins undergo this demethionylation but does cofilin-1? Characterization of the ser3 phosphorylation site on cofilin was done through isolation of a his-tagged N-terminal peptide (Moriyama et al., 1996) thus providing no information on the relative abundance of the Met vs N-Ac-Ala form. Have any other papers addressed this issue? It seems to be significant since chicken actin-depolymerizing factor (ADF), the first member of the ADF/cofilin family to be identified, is identical in sequence to cofilin in the first 5 N-terminal amino acids but sequencing of the ser-3 phosphopeptide from ADF showed it had an N-terminal N-Ac-Ala and is thus demethionylated (Agnew et al., 1995). If cofilin-1 does not undergo demethionylation, this feature alone may provide a reason for the evolution of these isoforms with different subcellular targeting abilities. If the majority of cofilin in cells does contain N-terminal N-Ac-Ala, it suggests the mitochondrial targeted pool arises from newly synthesized cofilin. Such a finding would be very significant and suggest a whole new area of focus relating translation to apoptosis. Also, chickens do not express cofilin-1, so would this imply a completely different mitochondrial dependent apoptotic pathway in avians?

Reviewer #3

(Remarks to the Author)
N-terminal α-Amino SUMOylation Promotes Phosphorylation-Independent Cofilin-1 Translocation to the Mitochondrial Matrix and Induces Apoptosis
Authors: Qi Deng, Xiaokun Gu, Jiaqian Feng, Jinhua Xiang, Xinyue Li, Weiji Weng, Ou Huang, Si-Jian Pan and Yong Li

Nature Communications NCOMMS-25-35241-T

General comments:
Overall, the science presented in this manuscript is very sound based on earlier findings from this laboratory, the first to demonstrate N-terminal SUMOylation of cofilin. All the critical controls for experiments were provided in full disclosure and the team seems very proficient in all techniques. The presentation of the data follows a clearly delineated narrative that is

easy to follow. Each data figure contains a massive amount of data, which could be difficult to see in the print format, however figures are easily enlarged in a PDF version and, importantly, maintain a very high resolution. The quintessential findings of this research demonstrates very convincingly that N-terminal SUMOylation of cofilin overrides the serine 3 phosphorylation hence targeting cofilin to mitochondria. Import requires a role for HSP70 and the TOM20/70 transport system in conjunction with TIM23. Cofilin interaction with cytochrome C initiates apoptosis and capsase activation. Using a combination of mutations of cofilin, these pathways could be disrupted and dissected. Notably, this action of cofilin is completely independent of actin. This reviewer agrees with the authors on their interpretations of each data set and the overall conclusion. This finding is actually quite interesting.

Specific comments:

Citations are by and large correct and adequate for the purpose of statement. The reference list stops at #82 but citations in the manuscript run up to #86.

Discussion: this section is simply a summary of the results. Authors should address the acquired knowledge in the context of the many functions of cofilin family members as well as the actin-independent/dependent roles of cofilin and mitochondria.

Results:
1. Phosphorylation and dephosphorylation of cofilin (shown to be dominated by SUMOylation) was shown with TAT peptides that block LIM kinase or SSL. Since they are making the point that phosphorylation was shown to determine mitochondrial translation, but SUMOylation overrides this signal, one wonders what LIMkinase and SSL activities are in their system. Simply, is the peptide assay sufficient?
2. Is the kinetics of the N-terminal methionine removal of cofilin known at all and if so, this could vastly impact the degree or level of cofilin SUMOylation. Could that kinetics be manipulated or what if, after knocking out endogenous cofilin, a cofilin protein is introduced without a methionine, or use an N-terminal peptide of cofilin with the Met to compete for the actual newly made cofilin thereby redirecting the SUMOylation process.
3. Lastly, cofilin message is translated in different compartments in different cell types. That could also affect SUMOylation.

Reviewer #4

(Remarks to the Author)

This manuscript seeks to identify mechanisms by which Cofilin (CFL1), a regulator of actin dynamics, influences mitochondrial biology. Previous work had linked the phosphorylation of Cofilin to mitochondrial dynamics, and the authors propose that rather than phosphorylation, that N-terminal SUMOylation of CFL1, facilitates mitochondrial translocation and altered susceptibility to cell death. The authors perform many experiments and use many mutants to map CFL1 to mitochondria and to put forth a model that CFL1 translocates to the mitochondrial matrix to influence cytochrome c release and cell death. Unfortunately, I found this manuscript to be dense, highly correlative, and presented in a way that made it challenging to rigorously evaluate the claims put forth by the authors. I detail these challenges below.

1. The authors use an impressive number of orthogonal methods to test their hypotheses. However, often times the rationale is not mentioned in the manuscript, nor are the hypotheses being tested. For instance, in Figure 1, the authors perform experiments in Senp1 knockout MEFs, but do not clarify what Senp1 does, what might be expected to occur in this experiment, or what hypothesis is being tested. This occurs throughout the manuscript, and it seems as though the authors assume that the readers will be familiar with very nuanced conclusions from their former papers, which likely will not be true for the general readership of Nature Communications. To me, this manuscript reads more as a list of things that were done rather than addressing how the experiments fit into the narrative of the field.

2. The figures are often too small to be able to be interpreted in a rigorous fashion. The microscopy images showing co-localization of CFL1 constructs are almost impossible to interpret, other than to demonstrate yellow overlap in the channel. As there is significant signals for overexpressed CFL1 in other compartments (e.g., the nucleus), it seems important for the reader to have better access to these images to evaluate. Similar problems occur with many of the blots. For instance, in Figure 2C, there are trace bands in some of the lanes, but I have a hard time distinguishing these from noise because of how small the figures are. This is true for many other figures as well. The APEX data presented in Figure 4D shows dense mitochondria with red arrows, but I cannot see what the arrows refer to. The structural modeling in Figure 7E is challenging to interpret because of the small size of the font and labels, as well as the similar colors. Many bar graphs are so small that it is hard to distinguish the distribution of the data. Because of this, I do not feel as though I can evaluate many of the claims of this paper due to the presentation of the data.

3. Despite the authors using many orthogonal methods and mutants in the paper, many of the experiments in the paper are correlative, and thus it is hard to distinguish the model put forth by the authors from other potential models that could explain the data. This happens persistently, with some problematic examples outlined below:
A. While the authors show that Senp1 KO decreases mitochondrial association of CFL1, this could be due to many reasons, only one of which is direct, increased SUMOylation. As a general regulator of SUMOylation, the influence of Senp1 knockout on CFL1 may also be due to non-specific or indirect effects on its function or on mitochondrial function. Similar comments can be made for experiment suing ML-792; while this compound may affect CFL1 N-terminal SUMOylation, it is not specific and thus the effects cannot be attributed specifically to the alteration of this specific modification.

B. The authors use mutants of CFL1 that they previously demonstrate have lower SUMOyation, K112/114R, and they confirm that SUMOylation is at the N-terminus in this study. However, the use of these mutants is problematic, as lysines often carry other post-translational modifications, and the use of a K/R mutant cannot distinguish effects from the differential N-terminal SUMOylation from these potential effects. Notably, the Phosphosite plus website notes 73 different studies that have identified ubiquitnation on CFL1 at K112, and another 61 studies that have identified ubiquitnation on K114. Thus, the effects of these mutants cannot be attributated solely to SUMOylation.
C. The authors show that CFL1 interacts with the mitochondrial receptors TOM20 and TOM70, and that siRNA mediated knockdown of these proteins decreases mitochondrial translocation. TOM20 and TOM70 are highly abundant proteins at the outer mitochondrial membrane, and immunoprecipitations do not necessarily demonstrate that these proteins facilitate mitochondrial recruitment. (Notably the authors state “CFL1 translocates to mitochondria via direct interactions with Tom20 and Tom70” on lines 366-67, but immunoprecipitations do not demonstrate direct interactions.) Furthermore, the knockdown of Tom20 and Tom70 would be expected to have highly pleiotrophic effects, disrupting much of protein import and compromising mitochondrial function. Thus, these studies do not effectively demonstrate the recruitment mechanism of CFL1. I have similar comments for the Tim23 experiments.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have properly addressed my comments.

Reviewer #2

(Remarks to the Author)
The authors have done an outstanding job in responding to each comment of every reviewer, considerably strengthening what was already an excellent paper. In my 55 years of reviewing manuscripts, I have never seen such a complete revision of figures to include everything asked for by the reviewers. Figures were vastly improved, new IP controls were added, and writing was improved to eliminate tense errors. Furthermore. The authors responded to reviewers’ questions that they found both intellectually and functionally significant by performing new sets of experiments that added significantly to the impact of this manuscript.
One significant set of new experiments provided in the authors’ response to reviewers demonstrated that in chicken DF-1 cells, which express ADF and cofilin-2 but NOT cofilin-1, only cofilin-2 undergoes N-terminal SUMOylation and mitochondrial targeting. This is significant in at least two respects: 1) ADF and Cofilin 1 and Cofilin 2 from both mammals and birds contain the identical N-terminal 5 amino acids (MASGV) and the new results show that the specificity for N-terminal SUMOylation requires additional recognition sites; and 2) that in avians where ADF substitutes for cofilin-1 for most general cell functions involving actin dynamics in motility and cytokinesis and cofilin-2 is mainly involved in muscle development as it is in mammals, it is cofilin-2 that carries out the mitochondrial targeted responses involved in triggering apoptosis. It appears that authors had planned to include these new data into this manuscript because the use of the DF-1 cells was added to the Materials and Methods sections (lines 606-607, 610, 676-679 and 744-746) but they eventually settled on just adding: “other functional comparisons between ADF/cofilin family members will be essential to determine whether similar SUMO-mediated pathways operate in other contexts” as stated in lines 528-531. Perhaps the lack of adding the additional results is due to the authors’ desire to include experiments using a chicken CFL2 antibody which “was held up by customs” and thus their results rely only on the HA tag for SUMO detection? Because these findings are significant in showing a very specific difference between ADF and cofilin in terms of a cell function that does not require actin-interaction, I hope that they will include these results provided the authors are comfortable with the veracity of their findings, and that a paragraph in the discussion be devoted to this finding. Otherwise, I suggest the additions to the methods that reference the experiments using DF-1 cells be removed.
Regardless of the inclusion or removal of the ADF/cofilin-2 comparison in chick cells, I strongly support the publication of this manuscript.

Reviewer #3

(Remarks to the Author)
After a thorough review of this revised manuscript, it is apparent that the authors went through great length in their revision. Authors addressed all the suggestions raised in depth and with great rigor. Large portions of the manuscript were completely rewritten such as the discussion and their results put into the larger context of the cofilin reserach. Many figures were revised with additional data targeting very accurately the 'concerns' raised initially. Analysis of the data and interpretation provides a substantial and solid foundation of their conclusions. This manuscript does add a substantial degree of new and important knowledge of the role of cofilin independent of its crucial action on actin dynamics.

Reviewer #4

(Remarks to the Author)
The authors have adequately addressed my concerns from the initial manuscript. This, in combination with substantial work

to address the other reviewer's critiques, render this manuscript acceptable for publication in Nature Communications.

Authors' Responses to the Reviewers' Comments

NCOMMS-25-35241-T Decision Letter
Deng et al., **N-terminal α-Amino SUMOylation Promotes Phosphorylation-Independent Cofilin-1 Translocation to the Mitochondrial Matrix and Induces Apoptosis**

We thank the reviewers for their careful evaluation of our manuscript and for providing thoughtful and constructive feedback. Below, we present a detailed, point-by-point response to each comment. Reviewers' comments are shown in *italics*, and our responses are provided in plain text. All textual changes in the revised manuscript are highlighted in blue.

Reviewer #1 (Remarks to the Author):

The authors challenge the conventional paradigm that CFL1 activity is driven solely by phosphorylation and actin binding. Instead, they reveal N-terminal sumoylation of CFL1 as actin-independent mechanism influencing mitochondrial integrity and apoptosis initiation. This is an interesting novel paper as follow up of the previously published paper on N-terminal sumoylation of CFL1.

We sincerely thank Reviewer #1 for recognizing the novelty and significance of our work, as well as for providing valuable suggestions that have greatly improved the quality of our manuscript. In response to these comments, we have conducted additional experiments, reanalyzed the relevant data, and revised the text accordingly to address all concerns raised.

1 - Overall, the figures are extremely small and therefore virtually impossible to read. It would be important to use full-size pages for each figure.

Response: We gratefully acknowledge this valuable suggestion and apologize for the inadequate presentation quality in the original figures. In the revised manuscript, we have resized all images in accordance with the *Nature Communications* formatting guidelines, ensuring that each figure now occupies a full page for optimal readability and visual clarity. Figs. 1–7 have been replaced with Revised Figs. 1–7, and Extended Data Figs. 1–7 have been updated accordingly (now Revised Supplementary Figs. 1–7).

2 - It is important to show the relative amount of CFL1 sumoylation. Therefore please show for all immunoblot panels that deal with CFL1 sumoylation both non-modified CFL1 and SUMO-modified CFL1 on the same blot.

Response: We apologize for not initially presenting both unmodified and SUMOylated forms of cofilin-1 (CFL1) on the same Western blot membrane in our SUMOylation assays. To address this concern, we have reperformed all relevant experiments and included quantitative analyses of the results. Accordingly, Fig. 1C, E, 2A–E, 6I, 7C have been replaced with Revised Fig. 1C,

E, 2A–E, 6I, 7C. In addition, Extended Data Fig. 1G, 5G has been updated as Revised Supplementary Fig. 1G, 5G.

3 - Many immunoprecipitation experiments lack IgG negative controls. The IgG negative control is however used in Figure 7A. Please include IgG negative controls in all immunoprecipitation experiments.

Response: We thank the reviewer for pointing out this important issue and sincerely apologize for the omission in the original submission. To address this concern, we have reperformed all immunoprecipitation experiments that previously lacked IgG negative controls and have now included the corresponding quantitative and statistical analyses. Accordingly, Fig. 1C, E, 2A–D, 5A, B, 6I, and 7B–D have been replaced with Revised Fig. 1C, E, 2A–D, 5A, B, 6I, and 7B–D in the updated manuscript. In addition, Extended Data Fig. 1G, 5G has been updated as Revised Supplementary Fig. 1G, 5G.

Reviewer #2 (Remarks to the Author):

Summary of Critique: This manuscript follows up work from the same laboratory which characterized an α-amino-SUMOylated cofilin, the first N-terminal SUMOylation of any protein. In this current paper, the authors focus on the role of this SUMOylation in cofilin function, particularly its targeting to mitochondria and initiating the mitochondrial-dependent apoptotic pathway first reported in 2003 by Chua et al. Many studies since then have focused on ser3 phosphorylation of cofilin regulating mitochondrial targeting of cofilin-actin along with the cofilin-dependent remodeling of actin surrounding mitochondria as being important for mitochondrial fission events that have been suggested to be responsible for the release of matrix proteins such as cytochrome c, triggering apoptosis via cytoplasmic caspase activation. In this manuscript, the authors utilize expression of a number of cofilin point mutations in cells silenced for expression of endogenous cofilin along with various SUMO-cofilin mutants to convincingly demonstrate that mitochondrial targeting of cofilin is independent of both cofilin's phosphorylation state and its actin binding ability, but is totally dependent upon N-terminal SUMOylation. Furthermore, they show convincing evidence that SUMO-cofilin is transported into the mitochondrial matrix, the first characterization of this location through both electron microscopy and co-immunoprecipitation with matrix proteins from lysates of purified mitochondria. Either through co-immunoprecipitation and mass spectrometry, direct binding studies, or expression knock downs, the authors identify roles for protein-folding chaperones (HSP70), specific outer (TOM20/TOM70) and inner (Tim23) mitochondrial membrane proteins, and show a direct interaction of SUMO-cofilin with cytochrome c. Of major significance is the fact that all these functions of cofilin appear to be totally independent of actin binding since SUMOylated mutant forms

of cofilin that lack the ability to bind actin undergo identical targeting. siRNA-induced decrease in cofilin-1 also decreases cytochrome c release from mitochondria and reduces caspase activity. Cells in brains of mice deficient in deSUMOylating activity (Senp1) undergo increased apoptosis, contain mitochondria with reduced membrane potential and demonstrate increased embryonic lethality that is increased to 100% by expression of cofilin-1 K34R, a mutation that undergoes enhanced N-terminal SUMOylation.

We sincerely thank Reviewer #2 for the positive and encouraging evaluation of our work. We also greatly appreciate the constructive suggestions, which have been invaluable for improving the clarity and overall quality of our manuscript.

Critique: Both reviewers have read the manuscript thoroughly and checked the validity of the citations. Although the citations generally do not go back to the first reports of the items being referenced, the ones cited are adequate and most contain some relevant new information. However, ref 86 in line 875 of the manuscript is not in the reference list which ends at 82. This number was probably inserted accidentally from copying the information on plasmid construction from another manuscript and should either be included in the citations or removed.

Response: We thank the reviewer for carefully identifying this error and sincerely apologize for the oversight. The incorrect citation “ref. 86” has been removed, and the reference has been corrected in the revised manuscript (ref. 76). In addition, we have conducted a comprehensive audit of all references throughout the main text, figure legends, methods, and supplementary information. Every in-text citation was cross-checked against the reference list, and all numbering and formatting inconsistencies have been corrected to ensure full accuracy and consistency.

Scientifically this is a very sound paper containing an enormous amount of high-quality research. Most of the work was performed in a variety of cultured mammalian cell lines, including CHO, HeLa, HEK293, and mouse embryonic fibroblasts. For some research questions that might involve cell-lineage dependent behavior, switching cell lines might be considered a detriment, but with cofilin sequence and function showing extremely high conservation across mammalian cells, the use of multiple cell lines does not present a problem in interpretation. Controls for each experiment are contained within each figure and several figures of Supplementary are provided for support. Most figures contain a huge amount of information including immunoblots, their quantified comparisons, immunofluorescence images and quantified co-localizations using Pearson’s correlation coefficient of multiple fields, etc. Many are difficult to read in a printed version of the paper but can be enlarged on screen in the pdf to show clear results. Band quantification from blots (three replicates per sample) and immunofluorescence (six

fields from three biological replicates) are used to provide statistical significance between treatments. The sample blots provided do support the quantifications shown in plots. We agree with the authors' interpretation of their data and with their overall conclusions.

Response: We appreciate the reviewer's insightful observation and positive evaluation. As noted, human and mouse cofilin-1 (CFL1) are highly conserved and both undergo N-terminal α-amino SUMOylation (Weng et al., 2023). Accordingly, we selected specific cell lines for distinct experimental purposes:

- **CHO cells** were used to map SUMOylation sites of mouse CFL1 and to validate SUMOylation levels of point mutants.
- **HEK 293T cells** were chosen for most WB experiments because of their high transfection and knockdown efficiency.
- **HeLa cells** were used for immunofluorescence imaging due to their strong adherence and flat morphology.
- ***Senp1*$^{+/+}$ and *Senp1*$^{-/-}$ MEFs**, derived from embryos, were employed to verify results in primary cells.

This experimental design ensured that each assay was conducted in the most suitable model system for its specific objective.

Reference:

Weng, W. et al. *Nat. Commun*. 14, 5688 (2023).

Although the text is understandable, there are several grammatical errors throughout the manuscript. Some are issues with tense. It is better to use present than past tense for statements such as in line 69: "Alternatively, recent studies suggested that …"(should be suggest). Many other items deal with faulty sentence construction leading to sentence fragments- these are easily corrected. For example, line 168 starting with "Furthermore, expressed …" needs to have "we expressed". Lines 555-556- add comma after fertile and eliminate However while continuing the sentence. These may seem very minor (they are) but there are many throughout the text that can make reading it a bit awkward even though the ideas are well conveyed.

Response: We thank the reviewer for this valuable comment. We agree that these issues affected readability. We have carefully revised the manuscript throughout：

1. Unified verb tense—factual statements have been standardized to the present tense (e.g., Page 4, line 85: "suggested" → "suggest").

2. Completed sentence fragments by adding missing subjects (e.g., Page 8, line 178: "we expressed").

3. Refined punctuation and transitions to enhance fluency (e.g., corrected as suggested on Pages 17–18, lines 425–426).

These revisions substantially improve the clarity and readability of the text. All corresponding changes are highlighted in blue in the revised manuscript.

The biggest issue we had with the manuscript is the discussion, which is a restatement of the results focusing on how the new findings change the interpretation of previous work. This has already been stated in the results and could be summed up in 2-3 pages of the discussion. What is missing is addressing an extension of this knowledge. For example many recent papers have shown a relationship between the severity of cancers and increased expression of Senp1. Of course this relationship could be explained by a decreased apoptotic activity in cancer cells due to decreased SUMO-cofilin. Would this not warrant a paragraph which would also highlight the importance of this current work to cancer research?

Response: We thank the reviewer for this insightful comment. We agree that the original Discussion was overly repetitive and did not sufficiently emphasize the broader biological implications of our findings. Following the reviewer's suggestion, we have substantially revised this section to enhance clarity and conceptual depth. Specifically, we added a new paragraph linking SENP1 expression to tumor severity and discussed how reduced SUMO-modified CFL1 activity may contribute to impaired apoptosis in cancer cells, thereby establishing a mechanistic connection between our findings and tumor biology.

The revised text (Pages 22–23, lines 541–553) now reads:

"Recent transcriptomic and clinical studies consistently show that elevated SENP1 expression correlates with higher tumor grade and poor prognosis in breast, renal, and prostate cancers (Shangguan et al., 2021; Gao et al., 2022; Lee et al., 2022). SENP1 promotes tumorigenesis by de-SUMOylating substrates that normally restrain proliferation or promote apoptosis (Sheng et al., 2025; Gu et al., 2025). Our findings provide a mechanistic explanation for this correlation: excessive SENP1 activity would de-SUMOylate CFL1, thereby preventing its mitochondrial translocation and reducing apoptosis sensitivity in tumor cells. Conversely, restoring CFL1 SUMOylation or pharmacologically inhibiting SENP1 could resensitize cancer cells to intrinsic apoptotic cues. This interpretation aligns with the growing view that impaired SUMO homeostasis contributes to oncogenic survival pathways. Thus, the SENP1-CFL1 axis represents a potential therapeutic target linking post-translational regulation of cytoskeletal proteins to mitochondrial apoptosis and cancer progression."

References:

Shangguan, X. et al. *Nat. Commun*. 12, 1812 (2021).

Gao, Y. et al. *Int. J. Biol. Sci*. 18, 2186–2201 (2022).

Lee, M. H. et al. *Oncogenesis* 11, 65 (2022).

Sheng, Z. et al. *Oncogene* 44, 1361–1374 (2025).

Gu, J. et al. *Cell. Oncol*. 48, 67–81 (2025).

Additionally, previous work from the Li lab showed that SUMOylation of cofilin requires the N-terminal Met residue. Do the authors have any information on the relative abundance of the N-terminal Met form versus the N-acetylated Ala form? Most cytoplasmic proteins undergo this demethionylation but does cofilin-1? Characterization of the ser3 phosphorylation site on cofilin was done through isolation of a his-tagged N-terminal peptide (Moriyama et al., 1996) thus providing no information on the relative abundance of the Met vs N-Ac-Ala form. Have any other papers addressed this issue? It seems to be significant since chicken actin-depolymerizing factor (ADF), the first member of the ADF/cofilin family to be identified, is identical in sequence to cofilin in the first 5 N-terminal amino acids but sequencing of the ser-3 phosphopeptide from ADF showed it had an N-terminal N-Ac-Ala and is thus demethionylated (Agnew et al., 1995). If cofilin-1 does not undergo demethionylation, this feature alone may provide a reason for the evolution of these isoforms with different subcellular targeting abilities. If the majority of cofilin in cells does contain N-terminal N-Ac-Ala, it suggests the mitochondrial targeted pool arises from newly synthesized cofilin. Such a finding would be very significant and suggest a whole new area of focus relating translation to apoptosis. Also, chickens do not express cofilin-1, so would this imply a completely different mitochondrial dependent apoptotic pathway in avians?

We thank the reviewer for raising this fundamental point and for the insightful, stimulating comments. To address it comprehensively, we provide a six-part response aligned with the specific questions raised:

1. General rules of N-terminal processing

Reviewer's question:

Most cytoplasmic proteins undergo N-terminal methionine excision (NME). Does cofilin-1 also follow this rule?

Response: Yes. More than half of all newly synthesized proteins undergo N-terminal methionine excision (NME) (Nguyen et al., 2019; Yang et al., 2019). When the penultimate residue is a small amino acid such as Ala, Ser, Thr, Val, or Cys, the exposed N-terminus is frequently acetylated by N-terminal acetyltransferases (Hwang et al., 2010; McTiernan et al., 2025). CFL1 contains an alanine at position 2, which conforms to this canonical substrate rule, strongly suggesting that CFL1 undergoes NME followed by N-terminal acetylation.

References:

Nguyen, K. T. et al. *J. Biol. Chem.* 294, 4464–4476 (2019).

Yang, C. I. et al. *Proc. Natl. Acad. Sci. U.S.A.* 116, 23050–23060 (2019).

Hwang, C. S. et al. *Science* 327, 973–977 (2010).

McTiernan, N. et al. *Nat. Commun.* 16, 703 (2025).

2. Evidence for CFL1 NME and N-acetylation

Reviewer's question:

Have any other papers directly addressed whether cofilin-1 undergoes NME or N-acetylation?

Response: Yes. Proteomic studies have directly identified N-acetylated Ala peptides derived from CFL1 (Jovceva et al., 2007; Weng et al., 2023), consistent with our interpretation that CFL1 undergoes removal of the initiator methionine followed by N-terminal acetylation at Ala2. This post-translational sequence—methionine excision and subsequent acetylation—is typical for proteins beginning with Met-Ala, as in CFL1. However, to date, no study has quantitatively compared the relative abundance of Met-initiated versus Ala-initiated forms of CFL1 in cells. Such quantitative analysis would help determine the proportion of CFL1 molecules that remain unprocessed and therefore potentially available for alternative N-terminal modifications such as α-amino SUMOylation.

References:

Jovceva, E. et al. *J. Cell Sci.* 120, 1888–1897 (2007).

Weng, W. et al. *Nat. Commun.* 14, 5688 (2023).

3. Relative abundance of N-terminal forms

Reviewer's question:

What is the relative abundance of the N-terminal Met form compared to the N-acetylated Ala form in cofilin-1?

Response: N-terminal acetylation states are most reliably determined by high-resolution mass spectrometry (Van Damme et al., 2012; Woo et al., 2024). To directly assess the modification status of CFL1, we reanalyzed three in-house LC–MS/MS datasets. The extracted ion chromatograms revealed a consistent ratio of approximately 2:5 for Met-initiated versus Ala-initiated CFL1 peptides. Notably, no acetylation was detected on Met-initiated peptides, whereas 3 of 7 Ala-initiated peptides were N-acetylated (Reviewer Table 1). These results strongly support that the majority of cellular CFL1 undergoes NME, followed by N-acetylation of Ala2, establishing the biochemical context for subsequent α-amino SUMOylation.

Protein Accession	Peptides
P23528 (CFL1)	A(+42.01)SGVAVSDGVIK
	A(+42.01)SGVAVSDGVIK
	ASGVAVSDGVIK
	M(+15.99)ASGVAVSDGVIK
	A(+42.01)SGVAVSDGVIK
	ASGVAVSDGVIK
	MASGVAVSDGVIK

Reviewer Table 1. Identification of CFL1 N-terminal processing forms by immunoprecipitation–mass spectrometry (IP–MS).

HEK 293T cell lysates were immunoprecipitated with an anti-CFL1 antibody. The immunoprecipitated proteins were digested with trypsin and analyzed by LC–MS/MS. Two distinct N-terminal peptide species of CFL1 were consistently

detected in three independent experiments: the unprocessed methionine-retaining peptide (M-A-S-G-V-A-V-S-D-G-V-I-K) and the N-acetylated alanine peptide (Ac-A-S-G-V-A-V-S-D-G-V-I-K, the acetylation modification is characterized by a mass shift of +42.01 Da). These findings demonstrate that CFL1 exists in both Met-retaining and demethionylated N-acetylated forms, consistent with co-translational N-terminal processing in mammalian cells.

References:

Van Damme, P. et al. *Proc. Natl. Acad. Sci. U.S.A*. 109, 12449–12454 (2012).

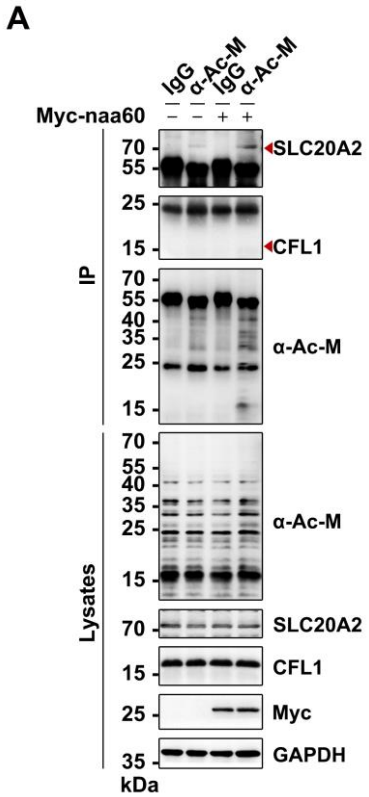
Woo, H. et al. *Sci. Adv*. 10, eadl6280 (2024).

4. Validation using Western blotting

Reviewer's question:

Can this be biochemically validated?

Response: We attempted to validate CFL1 N-terminal acetylation status by Western blotting (WB) using an antibody specific for N-acetyl-methionine, as no antibody recognizing N-acetyl-alanine is currently available. As a positive control, the known N-acetyl-Met substrate SLC20A2 was robustly detected, and its acetylation signal increased following Naa60 overexpression, as indicated by the red arrows in Reviewer Fig. 1A, consistent with previous reports (Chelban et al., 2024; Siggervåg et al., 2025). In contrast, no N-acetyl-Met signal was observed for CFL1 (Reviewer Fig. 1A), supporting the conclusion that CFL1 rarely retains and acetylates its initiator methionine. These results reinforce that CFL1 predominantly undergoes methionine excision followed by N-acetylation of Ala2.



Reviewer Figure 1. N-terminal acetylation of methionine on CFL1 compared with SLC20A2.

(A) HEK 293T cells were transfected with or without Myc-Naa60 plasmid, which encodes the N-terminal acetyltransferase Naa60. Cell lysates were subjected to immunoprecipitation (IP) using either control IgG or anti-α-Ac-M (N-acetyl-methionine) antibody, followed by immunoblotting with anti-α-Ac-M, anti-SLC20A2, and anti-CFL1 antibodies. Input lysates were analyzed in parallel to confirm protein expression. The membrane regions containing CFL1 and SLC20A2 were excised from the same PVDF membrane and separately probed with anti-CFL1 and anti-SLC20A2 antibodies. The red arrows indicate the positions of the acetylated SLC20A2 and the putative acetylated CFL1 bands; however, CFL1 showed no detectable acetylation signal. Overexpression of Myc-Naa60 enhanced the α-Ac-M signal of SLC20A2 but not CFL1, indicating that CFL1's N-terminal methionine is not acetylated. GAPDH served as a loading control (n = 2 biological replicates).

References:

Chelban, V. et al. *Nat. Commun*. 15, 2269 (2024).

Siggervåg, A. et al. *Brain* 148, 3085–3094 (2025).

5. Functional implications

Reviewer's question:

If most cofilin-1 exists in the N-acetyl-Ala form, what does this mean for SUMOylation and apoptosis?

Response: Our data indicate that CFL1 predominantly exists in the N-acetyl-Ala form, with only a minor fraction retaining the initiator methionine (Reviewer Table 1). This finding suggests that the SUMO-competent, mitochondria-targeted pool of CFL1 likely originates from newly synthesized molecules, thereby establishing a mechanistic link between translation, N-terminal processing, SUMOylation, mitochondrial import, and apoptosis.

6. Evolutionary considerations

Reviewer's question:

Also, chickens lack cofilin-1 but express ADF with an N-acetyl-Ala N-terminus, does this imply a distinct apoptotic pathway in avians?

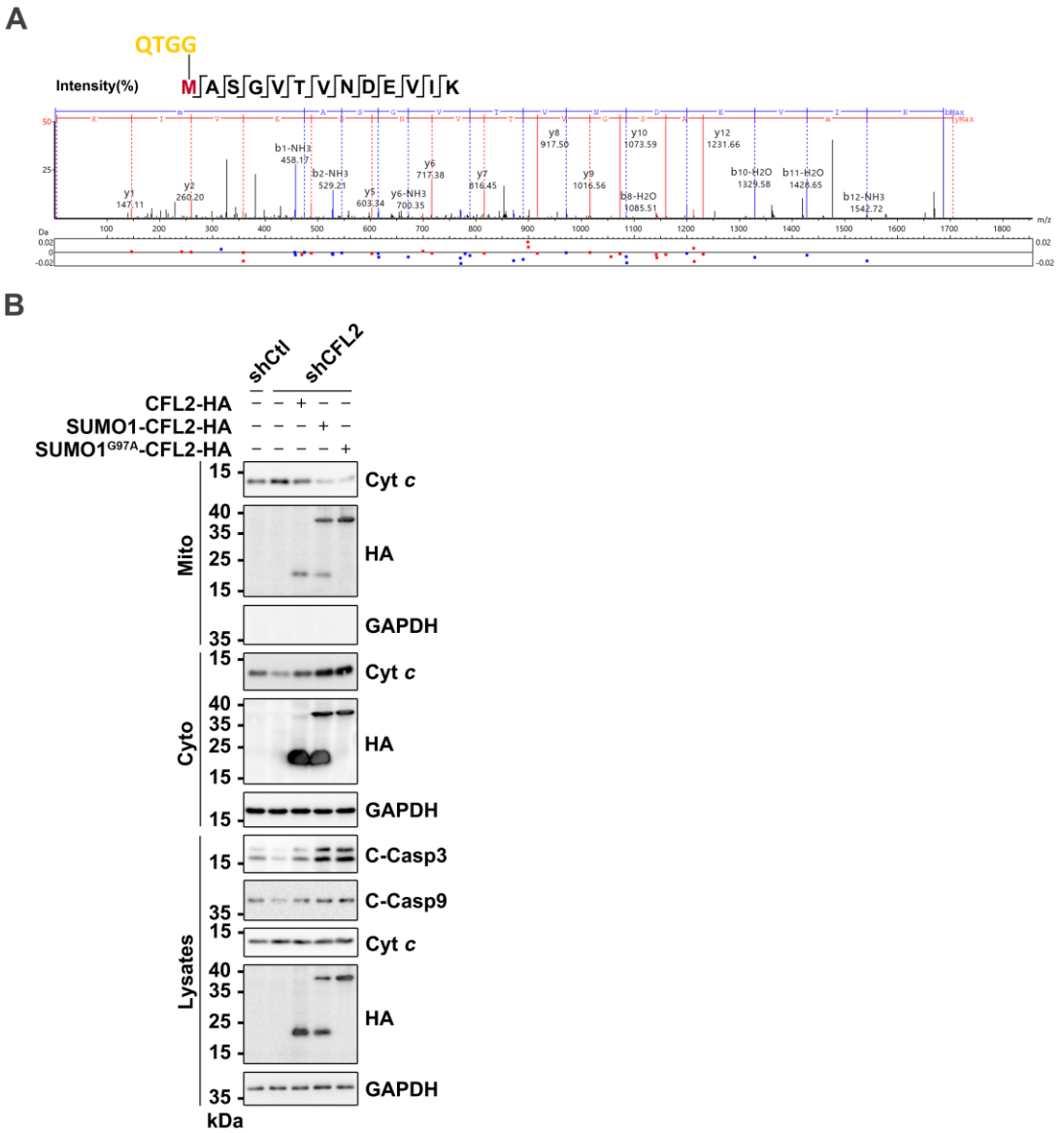
Response: We thank the reviewer for raising this important point. Following the suggestion, we compared the sequence homology of ADF/CFL family proteins across species. In mice and humans, CFL1 and cofilin-2 (CFL2) share more than 80% amino acid sequence identity, and both show >70% homology with actin-depolymerizing factor (ADF) (Vartiainen MK et al., 2002). Although chickens do not express CFL1, they do express both CFL2 and ADF (Abe H et al., 1990; Adams et al., 1990). Sequence alignment revealed that chicken CFL2 shares >80% identity with mouse CFL1, while chicken ADF shows >70% identity, with highly conserved N-terminal regions. These results raise the

possibility that chicken CFL2 or ADF may functionally substitute for CFL1, including undergoing N-terminal α-amino SUMOylation and participating in CFL-mediated mitochondrial apoptosis.

To test this hypothesis, we cloned chicken CFL2-HA, ADF-HA, and His-SUMO1^{E93R} constructs and expressed them in chicken embryonic fibroblast cell line (DF-1). Co-expression of His-SUMO1^{E93R} enabled MS analysis to detect SUMOylation sites. The results demonstrated that chicken CFL2 is modified at its N-terminal methionine by the addition of a glutamine-threonine-glycine-glycine (QTGG) fragment, confirming N-terminal α-amino SUMOylation (Reviewer Fig. 2A). In contrast, ADF exhibited no detectable N-terminal SUMOylation under the same conditions.

Furthermore, previous studies have shown that avian species are capable of undergoing mitochondrial-dependent apoptosis (Duan et al., 2020; Wang et al., 2024). However, whether this involves CFL-mediated mechanisms has not been reported. To directly test this, we transfected DF-1 cells with CFL2-HA, SUMO1-CFL2-HA, or SUMO1^{G97A}-CFL2-HA plasmids, isolated mitochondrial fractions, and examined CFL2 translocation, cytochrome *c* release, and caspase activation. We observed that N-terminal SUMO-fused CFL2 showed increased mitochondrial localization, enhanced cytochrome *c* release, and stronger activation of caspase-dependent apoptotic signaling (Reviewer Fig. 2B).

Taken together, these findings suggest that avian CFL2, can undergo N-terminal α-amino SUMOylation and promote mitochondrial-dependent apoptosis. Thus, although chickens lack CFL1, they retain a CFL-mediated apoptotic pathway through CFL2, suggesting that this SUMO-dependent mitochondrial regulatory mechanism is evolutionarily conserved across vertebrates.



Reviewer Figure 2. N-terminal SUMOylation of CFL2 promotes mitochondrial-dependent apoptosis in avian cells.

(A) DF-1 cells were co-transfected with CFL2-HA and His-SUMO1^{E93R} plasmids. Cell lysates were subjected to immunoprecipitation using anti-HA beads, followed by LC–MS/MS analysis. The MS/MS spectrum shows a tryptic peptide containing the glutamine-threonine-glycine-glycine (QTGG) remnant attached to the N-terminal methionine of CFL2, confirming N-terminal SUMOylation.

(B) CFL2 SUMOylation enhances mitochondrial translocation and activation of apoptotic signaling. DF-1 cells were transfected with CFL2-HA, SUMO1-CFL2-HA, or SUMO1^{G97A}-CFL2-HA plasmids. Mitochondrial (Mito), cytoplasmic (Cyto), and total lysate fractions were analyzed by Western blotting (WB) using antibodies against HA (since the import and customs clearance process for a commercial chicken CFL2 antibody is still pending, HA-tag detection was used to monitor exogenous CFL2-HA expression),

cytochrome *c* (Cyt *c*), cleaved caspase-3 (C-Casp3), and cleaved caspase-9 (C-Casp9). SUMO-fused CFL2 promoted Cyt *c* release from mitochondria to cytosol and increased caspase activation, indicating that N-terminal SUMOylation facilitates CFL2-mediated mitochondrial apoptosis. GAPDH served as the loading control (n = 3 biological replicates).

References:

Vartiainen, M. K. et al. *Mol. Biol. Cell* 13, 183–194 (2002).

Abe, H. et al. *Biochemistry* 29, 7420–7425 (1990).

Adams, M. E. et al. *Biochemistry* 29, 7414–7420 (1990).

Duan, X. et al. *J. Virol*. 94, e01724-19 (2020).

Wang, X. et al. *Environ. Toxicol*. 39, 2052–2063 (2024).

Reviewer #3 (Remarks to the Author):

General comments:

Overall, the science presented in this manuscript is very sound based on earlier findings from this laboratory, the first to demonstrate N-terminal SUMOylation of cofilin. All the critical controls for experiments were provided in full disclosure and the team seems very proficient in all techniques. The presentation of the data follows a clearly delineated narrative that is easy to follow. Each data figure contains a massive amount of data, which could be difficult to see in the print format, however figures are easily enlarged in a PDF version and, importantly, maintain a very high resolution. The quintessential findings of this research demonstrates very convincingly that N-terminal SUMOylation of cofilin overrides the serine 3 phosphorylation hence targeting cofilin to mitochondria. Import requires a role for HSP70 and the TOM20/70 transport system in conjunction with TIM23. Cofilin interaction with cytochrome C initiates apoptosis and caspase activation. Using a combination of mutations of cofilin, these pathways could be disrupted and dissected. Notably, this action of cofilin is completely independent of actin. This reviewer agrees with the authors on their interpretations of each data set and the overall conclusion. This finding is actually quite interesting.

We sincerely thank Reviewer #3 for the encouraging and positive evaluation of our work and for the constructive suggestions. In response, we have resized all figures according to the *Nature Communications* guidelines to ensure optimal presentation in both print and digital formats. We also deeply appreciate the reviewer's recognition of the novelty and soundness of our findings, which has motivated us to further strengthen the clarity and impact of our study.

Specific comments:

Citations are by and large correct and adequate for the purpose of statement. The reference list stops at #82 but citations in the manuscript run up to #86.

Response: We thank the reviewer for carefully pointing out this error and sincerely apologize for the oversight. The incorrect “ref 86” has been removed, and the citation has been corrected in the Revised manuscript (ref. 76). In addition, we conducted a comprehensive audit of all references across the main text, figure legends, methods, and supplementary Information. Every in-text citation was cross-checked against the reference list, and numbering/formatting inconsistencies were corrected.

Discussion: this section is simply a summary of the results. Authors should address the acquired knowledge in the context of the many functions of cofilin family members as well as the actin-independent/dependent roles of cofilin and mitochondria.

Response: We thank the reviewer for this insightful suggestion. Following the recommendation, we have revised the Discussion by (i) removing redundant restatements of results and (ii) adding a new section that situates our findings within the broader context of ADF/cofilin (CFL) family biology, emphasizing both actin-dependent and actin-independent functions of CFL in mitochondrial regulation.

The revised text (Pages 21–22, lines 507–540) now reads:

“CFL has long been viewed as an actin-binding protein that regulates cytoskeletal turnover by severing F-actin and recycling G-actin (Ono et al., 1994; Niwa et al., 2012; Hamill et al., 2016). Yet accumulating evidence indicates that CFL also exerts actin-independent functions, particularly in mitochondrial dynamics and apoptosis (Xu et al., 2021). Early studies suggested that dephosphorylated CFL1 associates with Drp1 and F-actin at mitochondria-associated membranes (MAMs), modulating mitochondrial fission and apoptotic sensitivity (Li et al., 2018; Hu et al., 2020). Our findings refine this concept: even when actin binding is disrupted, SUMO-modified CFL1 is still efficiently imported into mitochondria, revealing an actin-independent route (Revised Fig. 1 and Revised Supplementary Fig. 2). This expands the functional landscape of CFL1 from a cytoskeletal modulator to a signal-responsive mitochondrial effector that integrates post-translational inputs to control cell fate.

Among the three ADF/CFL family members (CFL1, CFL2, and ADF), each exhibits distinct expression and physiological specialization. CFL1 is ubiquitous and essential for embryogenesis and neuronal morphogenesis (Grego-Bessa et al., 2015), CFL2 is enriched in muscle tissue and linked to congenital myopathies (Ockeloen et al., 2012), and ADF is implicated in epithelial cell migration and cancer metastasis (Zhang et al., 2020). The discovery that CFL1 undergoes N-terminal α-amino SUMOylation raises the possibility that this modification could confer family-specific regulation. Given that ADF/CFL,

contains residues compatible with SUMO conjugation and Tom20/Tom70 binding motifs, N-terminal SUMOylation may represent an evolutionary innovation that equips ADF/CFL with a unique mitochondrial signaling function. Comparative studies across the ADF/CFL family will be essential to determine whether similar SUMO-mediated pathways operate in other contexts, such as muscle homeostasis or epithelial remodeling.

At the cellular level, CFL1 N-terminal SUMOylation functions as a molecular switch linking cytoskeletal dynamics to mitochondrial fate (Weng et al., 2023). By enhancing CFL1 translocation into mitochondria, this modification amplifies Cyt *c* release, caspase-3/9 activation, and mitochondrial membrane depolarization (Revised Figs. 1, 6). These results position SUMOylated CFL1 as a pro-apoptotic mediator acting independently of actin disassembly. Conceptually, N-terminal SUMOylation can be viewed as a "mitochondrial targeting license" that allows newly synthesized CFL1 molecules—before N-terminal methionine excision and acetylation are complete—to enter mitochondria and engage the apoptotic machinery."

References:

Ono, S. et al. *J. Biol. Chem*. 269, 15280-15286 (1994).

Niwa, R. et al. *Cell* 108, 233-246 (2002).

Hamill, S. et al. *Mol. Cell* 62, 397-408 (2016).

Xu, J. et al. *Front. Cell Dev. Biol*. 9, 599065 (2021).

Li, G. B. et al. *Oncogene* 37, 1485-1502 (2018)

Hu, J. et al. *J. Exp. Clin. Cancer Res*. 39, 37 (2020)

Grego-Bessa, J. et al. *Development* 142, 1305–1314 (2015).

Ockeloen, C. W. et al. *Neuromuscul. Disord*. 22, 632–639 (2012).

Zhang, H. J. et al. *Mol. Cancer Res.* 18, 1789–1802 (2020).

Weng, W. et al. *Nat. Commun*. 14, 5688 (2023).

Results:

1. Phosphorylation and dephosphorylation of cofilin (shown to be dominated by SUMOylation) was shown with TAT peptides that block LIM kinase or SSL. Since they are making the point that phosphorylation was shown to determine mitochondrial translation, but SUMOylation overrides this signal, one wonders what LIM kinase and SSL activities are in their system. Simply, is the peptide assay sufficient?

Response: We thank the reviewer for this critical point, which helps us rigorously establish our central conclusion that SUMOylation predominates over phosphorylation in regulating CFL1 mitochondrial translocation.

LIMK and SSH activity in our system:

LIMK and SSH activities are reliably reflected by their phosphorylation status (Edwards et al., 1999; Singla et al., 2019) and by the phosphorylation state of their primary substrate, CFL1 (Arber et al., 1998; Aizawa et al., 2001; Lee et al., 2021). To directly test whether these kinases or phosphatases were altered

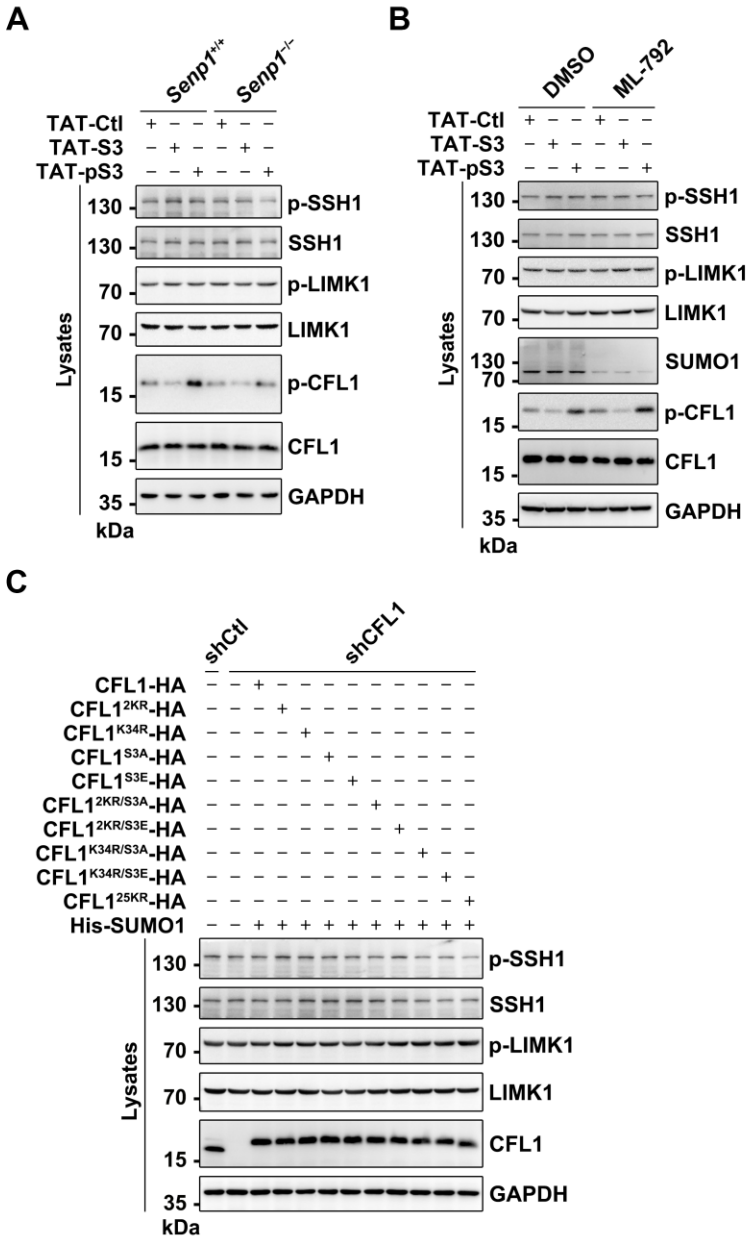
under our experimental conditions, we examined LIMK1 (Thr508) and SSH1 (Ser978) phosphorylation following TAT peptide treatments, SENP1 deficiency, and ML-792-mediated SUMOylation inhibition. Across all conditions, neither LIMK/SSH phosphorylation nor total protein levels changed (Reviewer Fig. 3A–C), indicating that LIMK/SSH activity remained stable and was not confounded by our manipulations.

Specificity and sufficiency of the peptide assay:

The TAT-S3 and TAT-pS3 peptides provide precise biochemical control over CFL1 phosphorylation dynamics. The TAT-S3 peptide (corresponding to the N-terminal 16 amino acids of CFL1 fused to the TAT sequence) competitively inhibits LIMK1-mediated phosphorylation of CFL1, while the TAT-pS3 peptide mimics phosphorylated CFL1 and competes for SSH1 binding, thereby preventing dephosphorylation (Arber et al., 1998; Aizawa et al., 2001; Lee et al., 2021). These assays are highly specific since CFL1 is the predominant physiological substrate of LIMK/SSH.

To complement the peptide-based approach, we also generated phosphorylation-deficient (S3A) and phosphomimetic (S3E) CFL1 mutants and combined them with SUMOylation-reduced (2KR) or SUMOylation-enhanced (K34R) variants. Across all combinations, mitochondrial translocation consistently correlated with SUMOylation status rather than phosphorylation state (Revised Fig. 2B, D–F).

Together, these independent approaches—biochemical (TAT-S3/pS3 peptides), genetic (S3A/S3E and SUMO mutants), and molecular readouts (LIMK/SSH phosphorylation)—converge to demonstrate that LIMK/SSH activity does not influence our observed effects. Therefore, the peptide assays are both specific and sufficient within the experimental framework, and the results robustly support that N-terminal SUMOylation, rather than Ser3 phosphorylation, governs CFL1 mitochondrial translocation.



Reviewer Figure 3. LIMK and SSH activities are unaffected by SUMOylation manipulations in our experimental system.

(A) SENP1 deficiency does not alter LIMK/SSH activity. *Senp1*$^{+/+}$ and *Senp1*$^{-/-}$ MEF cells were treated with TAT-Ctl, TAT-S3, or TAT-pS3 peptide (10 μM, 16 h). Whole-cell lysates were analyzed by Western blotting (WB) using antibodies against phospho-SSH1 (Ser978), SSH1, phospho-LIMK1 (Thr508), LIMK1, phospho-CFL1 (Ser3) and CFL1. No significant differences were observed in the phosphorylation of SSH1 or LIMK1 (n = 2 biological replicates).

(B) Pharmacological inhibition of SUMOylation does not affect LIMK/SSH activity. HEK 293T cells were treated with TAT-Ctl, TAT-S3, or TAT-pS3 peptide (10 μM, 16 h), followed by DMSO or ML-792 (0.5 μM, 4 h). Cell lysates were subjected to WB with the indicated antibodies. ML-792

efficiently reduced global SUMOylation but did not alter SSH1 or LIMK1 phosphorylation, indicating that SUMOylation inhibition does not influence LIMK/SSH activity (n = 3 biological replicates).

(C) LIMK/SSH activities remain stable across CFL1 SUMOylation and phosphorylation mutants. HEK 293T cells with endogenous CFL1 knockdown were transfected with the indicated CFL1-HA constructs (WT, 2KR, K34R, S3A, S3E, 2KR/S3A, 2KR/S3E, K34R/S3A, K34R/S3E, or 25KR) together with His-SUMO1 plasmids. WB analysis revealed that neither LIMK1 nor SSH1 phosphorylation was affected by CFL1 mutations or SUMO manipulations, confirming that LIMK/SSH signaling remains unaltered across all conditions. GAPDH served as a loading control (n = 2 biological replicates).

References:

Edwards, D. C. et al. *Nat. Cell Biol*. 1, 253–259 (1999).

Singla, B. et al. *Cell. Signal*. 53, 111–121 (2019).

Arber, S. et al. *Nature* 393, 805–809 (1998).

Aizawa, H. et al. *Nat. Neurosci*. 4, 367–373 (2001).

Lee, K. Y. et al. *EMBO Rep*. 22, e52645 (2021).

2. Is the kinetics of the N-terminal methionine removal of cofilin known at all and if so, this could vastly impact the degree or level of cofilin SUMOylation. Could that kinetics be manipulated or what if, after knocking out endogenous cofilin, a cofilin protein is introduced without a methionine, or use an N-terminal peptide of cofilin with the Met to compete for the actual newly made cofilin thereby redirecting the SUMOylation process.

Response: We thank the reviewer for this stimulating question, which addresses a key mechanistic aspect of our study. While global N-terminal methionine excision (NME) kinetics have been extensively investigated (Nguyen et al., 2019; Klein et al., 2024), the processing kinetics of CFL1 specifically have not been systematically characterized. To begin addressing this, we performed three complementary experiments:

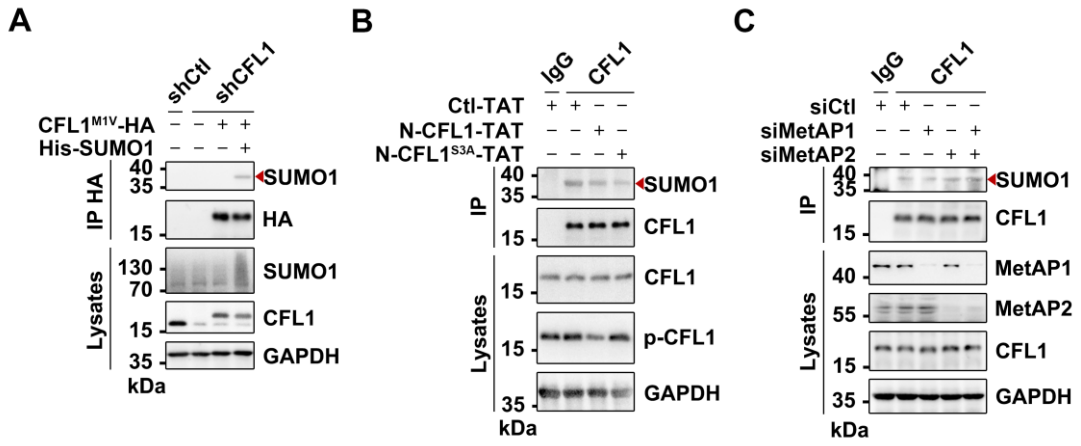
1. CFL1 M1V mutant analysis: We generated a CFL1 M1V mutant in which the initiator methionine (Met1) was replaced by valine (Val), which can serve as an alternative start codon (Drabkin et al., 1998). After lentiviral shRNA-mediated knockdown of endogenous CFL1, $CFL1^{M1V}$-HA was expressed together with His-SUMO1. Consistent with our previous report (Weng et al., 2023), $CFL1^{M1V}$ retained an accessible N-terminal α-amino group and underwent SUMOylation (Reviewer Fig. 4A), supporting that SUMO1 conjugation occurs at the N-α-NH_2 group of CFL.

2. N-terminal TAT peptide competition assays: To competitively probe SUMOylation, we designed N-terminal peptides fused with a TAT sequence—N-CFL1-TAT and N-$CFL1^{S3A}$-TAT—based on strategies reported by Rrustemi et al. (2024) for engineering N-terminal TAT peptides to modulate post-

translational modifications. After 16 h of treatment, both peptides reduced endogenous CFL1 SUMOylation. Notably, N-CFL1^{S3A}-TAT did not alter SSH/LIMK-dependent phosphorylation, confirming that the observed effects specifically reflected SUMOylation interference (Reviewer Fig. 4B).

3. Manipulating NME kinetics via MetAP knockdown: To directly modulate NME efficiency, we knocked down MetAP1 or MetAP2, which catalyze removal of the initiator methionine from nascent proteins (Gamerdinger et al., 2023; Rrustemi et al., 2024). Knockdown of MetAP1 and MetAP2 led to increased retention of the N-terminal Met and elevated CFL1 SUMOylation levels (Reviewer Fig. 4C). These findings indicate that NME efficiency controls the proportion of SUMO-competent CFL1.

Collectively, these results demonstrate that CFL1 SUMOylation preferentially occurs on nascent molecules with intact N-terminal methionine. Manipulation of NME kinetics—via mutagenesis, competitive N-terminal peptides, or MetAP inhibition—significantly altered SUMOylation levels. Thus, the SUMO-competent, mitochondria-targeted pool of CFL1 likely arises from newly synthesized proteins prior to complete NME and acetylation, establishing a mechanistic link between translation, N-terminal processing, SUMOylation, and mitochondrial import.



Reviewer Figure 4. Regulation of CFL1 N-terminal α-amino SUMOylation.

(A) CFL1 retains SUMOylation in the absence of its N-terminal methionine. HEK 293T cells with endogenous CFL1 knockdown were transfected with CFL1^{M1V}-HA (Met→Val substitution at the initiator codon) and His-SUMO1 plasmids. Immunoprecipitation (IP) with anti-HA beads followed by WB using anti-SUMO1, ant-HA, and anti-CFL1 antibodies demonstrated that CFL1^{M1V} still undergoes SUMO conjugation at its N-terminal α-amino group (n = 3 biological replicates).

(B) N-terminal CFL1 peptides competitively inhibit endogenous SUMOylation. HEK 293T cells were treated with Ctl-TAT, N-CFL1-TAT, or phosphorylation-deficient N-CFL1^{S3A}-TAT peptide (20 μM, 16 h). IP with control IgG or anti-CFL1 antibody followed by WB using anti-SUMO1, anti-phospho-CFL1

(Ser3), and anti-CFL1 antibodies revealed that both N-CFL1-TAT and N-CFL1^{S3A}-TAT peptides reduced endogenous CFL1 SUMOylation without altering phosphorylation (n = 3 biological replicates).

(C) N-terminal methionine excision modulates CFL1 SUMOylation. Knockdown of MetAP1 or MetAP2 in HEK 293T cells by siRNA increased the SUMOylation of CFL1, indicating that reduced N-terminal methionine removal enhances CFL1 N-terminal SUMO conjugation (n = 2 biological replicates).

References:

Nguyen, K. T. et al. *J. Biol. Chem*. 294, 4464–4476 (2019).

Klein, M. A. et al. *Nat. Commun*. 15, 716 (2024).

Drabkin, H. J. et al. *Mol. Cell. Biol*. 18, 5140–5147 (1998).

Weng, W. et al. *Nat. Commun*. 14, 5688 (2023).

Gamerdinger, M. et al. *Science* 380, 1238–1243 (2023).

Rrustemi, T. et al. *Nat. Commun.* 15, 3146 (2024).

3. Lastly, cofilin message is translated in different compartments in different cell types. That could also affect SUMOylation.

Response: We thank the reviewer for this constructive comment. Indeed, CFL1 has been reported to localize and function in multiple subcellular compartments, and such compartment-specific translation and distribution could influence its SUMOylation status.

1. **Cytoplasmic translation and actin dynamics**

   CFL1 is primarily synthesized in the cytoplasm, where it regulates actin dynamics to promote axonal and dendritic growth in neurons (Willis et al., 2007; Choi et al., 2018).

2. **Nuclear localization**

   Beyond cytoplasmic roles, CFL1 can translocate into the nucleus to regulate nuclear actin transport, transcription, and DNA damage repair (Obrdlik et al., 2011; Munsie et al., 2012; Hurst et al., 2019).

3. **Mitochondrial targeting**

   Under apoptotic stimuli, CFL1 translocates to mitochondria, where it modulates mitochondrial dynamics, cytochrome *c* release, and apoptosis (Luo et al., 2025).

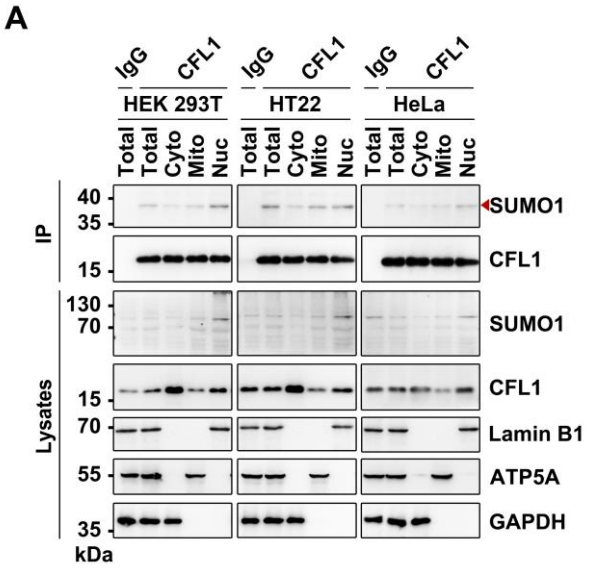
4. **Experimental evidence for compartment-specific SUMOylation**

   To directly address the reviewer's concern, we isolated nuclear, cytoplasmic, and mitochondrial fractions from human embryonic kidney cell line (HEK 293T), cervical carcinoma cell line (HeLa), and mouse hippocampal neuronal cell line (HT22). Western blotting revealed that both CFL1 abundance and SUMOylation varied across compartments and cell types.

   In particular:

HeLa cells showed overall lower levels of SUMOylated CFL1 compared with HEK 293T and HT22 cells. Within the same cell type, cytoplasmic CFL1 exhibited lower SUMOylation relative to nuclear and mitochondrial pools (Reviewer Fig. 5A).

These findings support the reviewer's point that the site of translation and subcellular compartmentalization significantly affect the SUMOylation status of CFL1. Such differences likely contribute to context-specific functions of CFL1, including actin regulation in the cytoplasm, nuclear activities, and mitochondrial apoptosis.



Reviewer Figure 5. Subcellular distribution of CFL1 SUMOylation.

(A) CFL1 SUMOylation levels were examined in total, cytoplasmic (Cyto), mitochondrial (Mito), and nuclear (Nuc) fractions of HEK 293T, HT22, and HeLa cells by IP-WB using anti-CFL1 and anti-SUMO1 antibodies. Fractionation purity was verified with ATP5A (mitochondrial marker), Lamin B1 (nuclear marker), and GAPDH (cytoplasmic marker) (n = 2 biological replicates).

References:

Willis, D. E. et al. *J. Cell Biol*. 178, 965–980 (2007).

Choi, J. H. et al. *EMBO J*. 37, e95266 (2018).

Obrdlik, A. et al. *Nucleus* 2, 72–79 (2011).

Munsie, L. N. et al. *J. Cell Sci*. 125, 3977–3988 (2012).

Hurst, V. et al. *Trends Cell Biol*. 29, 462–476 (2019).

Luo, Y. et al. *Biochem. Pharmacol*. 241, 117173 (2025).

Reviewer #4 (Remarks to the Author):

This manuscript seeks to identify mechanisms by which cofilin-1 (CFL1), a regulator of actin dynamics, influences mitochondrial biology. Previous work had linked the phosphorylation of CFL1 to mitochondrial dynamics, and the authors propose that rather than phosphorylation, that N-terminal

SUMOylation of CFL1, facilitates mitochondrial translocation and altered susceptibility to cell death. The authors perform many experiments and use many mutants to map CFL1 to mitochondria and to put forth a model that CFL1 translocates to the mitochondrial matrix to influence cytochrome c release and cell death. Unfortunately, I found this manuscript to be dense, highly correlative, and presented in a way that made it challenging to rigorously evaluate the claims put forth by the authors. I detail these challenges below.

We sincerely thank Reviewer #4 for the detailed and constructive evaluation of our manuscript. The reviewer correctly identified several important issues regarding logical flow, causality, and data presentation, which have been highly valuable in helping us revise and strengthen the study. In response, we have (i) reorganized the Results section to follow a clear, hypothesis-driven narrative; (ii) performed additional targeted experiments to establish stronger causal relationships; and (iii) substantially improved figure organization, labeling, and resolution for clarity and readability.

Below, we provide a detailed point-by-point response addressing each of the reviewer's comments.

1. The authors use an impressive number of orthogonal methods to test their hypotheses. However, often times the rationale is not mentioned in the manuscript, nor are the hypotheses being tested. For instance, in Figure 1, the authors perform experiments in Senp1 knockout MEFs, but do not clarify what Senp1 does, what might be expected to occur in this experiment, or what hypothesis is being tested. This occurs throughout the manuscript, and it seems as though the authors assume that the readers will be familiar with very nuanced conclusions from their former papers, which likely will not be true for the general readership of Nature Communications. To me, this manuscript reads more as a list of things that were done rather than addressing how the experiments fit into the narrative of the field.

Response: We sincerely thank the reviewer for this constructive and insightful comment. We fully agree that the rationale and hypotheses underlying key experiments were not always made explicit, which could make the manuscript appear descriptive rather than hypothesis-driven. To address this important issue, we have carefully revised the Results section to clearly state the experimental rationale, expected outcomes, and how each experiment tests specific mechanistic hypotheses.

In particular:

1. In Fig. 1, we now explicitly explain that SENP1 is a SUMO-specific protease that counteracts global SUMOylation, we next examined whether SENP1 deficiency affects CFL1 mitochondrial translocation. *Senp1*$^{-/-}$ MEFs were used to test the hypothesis that loss of SENP1 increases CFL1

SUMOylation, leading to enhanced mitochondrial translocation if the modification is functionally relevant (Pages 7–8, lines 151–158).

2. Each major experiment is now introduced with a concise "rationale–hypothesis–test" structure. For example, Fig. 2 clarifies the interplay between SUMOylation and phosphorylation; Figs. 3–5 define the mechanistic role of Tom/Tim receptors and HSP70 in CFL1 import; and Figs. 6, 7 address how SUMOylation affects Cyt *c* release and apoptosis.
3. We also revised the Discussion to better connect our findings with the broader context of ADF/CFL family functions and actin-independent roles in mitochondrial regulation.
4. Redundant descriptions were removed, and transitions between sections were strengthened to ensure a concise and logically coherent presentation.

We believe these revisions have substantially improved the narrative flow and readability of the manuscript.

The figures are often too small to be able to be interpreted in a rigorous fashion. The microscopy images showing co-localization of CFL1 constructs are almost impossible to interpret, other than to demonstrate yellow overlap in the channel. As there are significant signals for overexpressed CFL1 in other compartments (e.g., the nucleus), it seems important for the reader to have better access to these images to evaluate. Similar problems occur with many of the blots. For instance, in Figure 2C, there are trace bands in some of the lanes, but I have a hard time distinguishing these from noise because of how small the figures are. This is true for many other figures as well. The APEX data presented in Figure 4D shows dense mitochondria with red arrows, but I cannot see what the arrows refer to. The structural modeling in Figure 7E is challenging to interpret because of the small size of the font and labels, as well as the similar colors. Many bar graphs are so small that it is hard to distinguish the distribution of the data. Because of this, I do not feel as though I can evaluate many of the claims of this paper due to the presentation of the data.

Response: We appreciate this feedback and have revised all figures according to *Nature Communications* guidelines. Specifically:

All figure panels were enlarged and reformatted for clarity (Revised Figs. 1–7, and Revised Supplementary Figs. 1–7).

Immunofluorescence (IF) images were replaced with higher-resolution, enlarged single-channel and merged panels.

Several immunoblots were repeated and replaced with clearer replicates (Revised Fig. 1C, E, 2A–E, 5A, B, 6I, 7B–D and Revised Supplementary Fig. 1G).

We thank the reviewer for highlighting the limitations in the quality and interpretability of our original electron microscope (EM) images. We acknowledge that the contrast in our initial APEX-labeled sections was

insufficient, making it difficult to rigorously evaluate mitochondrial structures and labeling points.

To address this issue, we optimized our EM staining protocol. In addition to the standard uranyl acetate staining, we incorporated an additional lead citrate staining step, following the protocol described by Wong et al. (2014) and Martell et al. (2017). Uranyl acetate primarily enhances contrast of nucleic acids and certain protein structures, whereas lead citrate preferentially binds to negatively charged groups such as phosphates and carboxyl groups, thereby significantly increasing the contrast of membranes and proteinaceous structures. This dual-staining approach provides superior visualization of mitochondrial cristae and matrix architecture, while also improving the clarity of APEX labeling.

As shown in the Revised Fig. 4D, these optimizations yield EM images with markedly enhanced contrast and structural definition. Mitochondrial boundaries and cristae are now more clearly resolved, and the APEX-labeled signals can be reliably interpreted. These improvements ensure that readers can rigorously assess the mitochondrial localization of CFL1, directly addressing the reviewer's concern.

The revised text (Page 33, lines 829–832) now reads:

"To improve the interpretability of APEX-EM labeling, we optimized the staining protocol by adding a lead citrate staining step after uranyl acetate, this modification enhanced the contrast of mitochondrial membranes and matrix structures." (Revised Fig. 4D).

Structural modeling panels were reformatted with larger fonts and distinct color codes (Revised Fig. 7E).

All statistical bar graphs were enlarged to clearly display data distributions (Revised Fig. 1B, F, 2B, F, 3C, 4F, 5F, 6A, B, D, F, H, I, 7B and Revised Supplementary Fig. 2C, 5A, B, G, 6A, B).

These revisions ensure the data can now be rigorously evaluated.

References:

Wong, M. et al. *Science* 346, 1256898 (2014).

Martell, J. D. et al. *Nat. Protoc*. 12, 1792–1816 (2017).

3. Despite the authors using many orthogonal methods and mutants in the paper, many of the experiments in the paper are correlative, and thus it is hard to distinguish the model put forth by the authors from other potential models that could explain the data. This happens persistently, with some problematic examples outlined below:

A. While the authors show that Senp1 KO decreases mitochondrial association of CFL1, this could be due to many reasons, only one of which is direct, increased SUMOylation. As a general regulator of SUMOylation, the influence of Senp1 knockout on CFL1 may also be due to non-specific or indirect effects on its function or on mitochondrial function. Similar comments can be made for experiment suing ML-792; while this

compound may affect CFL1 N-terminal SUMOylation, it is not specific and thus the effects cannot be attributed specifically to the alteration of this specific modification.

Response: We fully agree with the reviewer that SENP1 deficiency or ML-792 treatment alters global SUMOylation and thus may cause indirect effects. To address this concern and directly test whether CFL1 SUMOylation per se regulates mitochondrial localization, we performed multiple complementary approaches:

1. Mutational analysis of CFL1 SUMOylation

- We generated SUMOylation-reduced (2KR) and SUMOylation-enhanced (K34R) mutants, as well as SUMO-fusion constructs (SUMO1-CFL1 and SUMO1^{G97A}-CFL1).
- Across these mutants, mitochondrial localization consistently tracked with CFL1 SUMOylation status, not global SUMOylation changes (Revised Fig. 1).

This provides direct genetic evidence that CFL1 SUMOylation specifically promotes its mitochondrial import.

2. Rescue experiments in *Senp1*$^{-/-}$ cells

- We expressed CFL1-GFP alongside a doxycycline-inducible construct encoding GNb-SENP1, which consists of an anti-GFP nanobody fused to the catalytic domain of SENP1 (Ramesh et al., 2025). We reasoned that doxycycline–induced GNb-SENP1 expression will target SENP1 to deSUMOylate CFL1-GFP.
- In *Senp1*$^{-/-}$ MEFs, this targeted rescue reduced CFL1 SUMOylation and mitochondrial localization, even under global SENP1 deficiency (Reviewer Fig. 6A).

This confirms that the phenotype in *Senp1*$^{-/-}$ cells stems directly from CFL1 SUMOylation rather than indirect effects of global SUMOylation.

3. Rescue experiments under ML-792 treatment

- ML-792 is an SAE inhibitor that reduces global SUMOylation, raising concerns about specificity.
- To test causality, we expressed SUMO-fused CFL1 in CFL1-depleted HEK 293T cells and treated with ML-792.
- SUMO-fused CFL1 retained robust mitochondrial localization, counteracting the inhibitory effect of ML-792 (Reviewer Fig. 6B).

This demonstrates that ML-792's effect on CFL1 localization is mediated through CFL1 SUMOylation rather than indirect global effects.

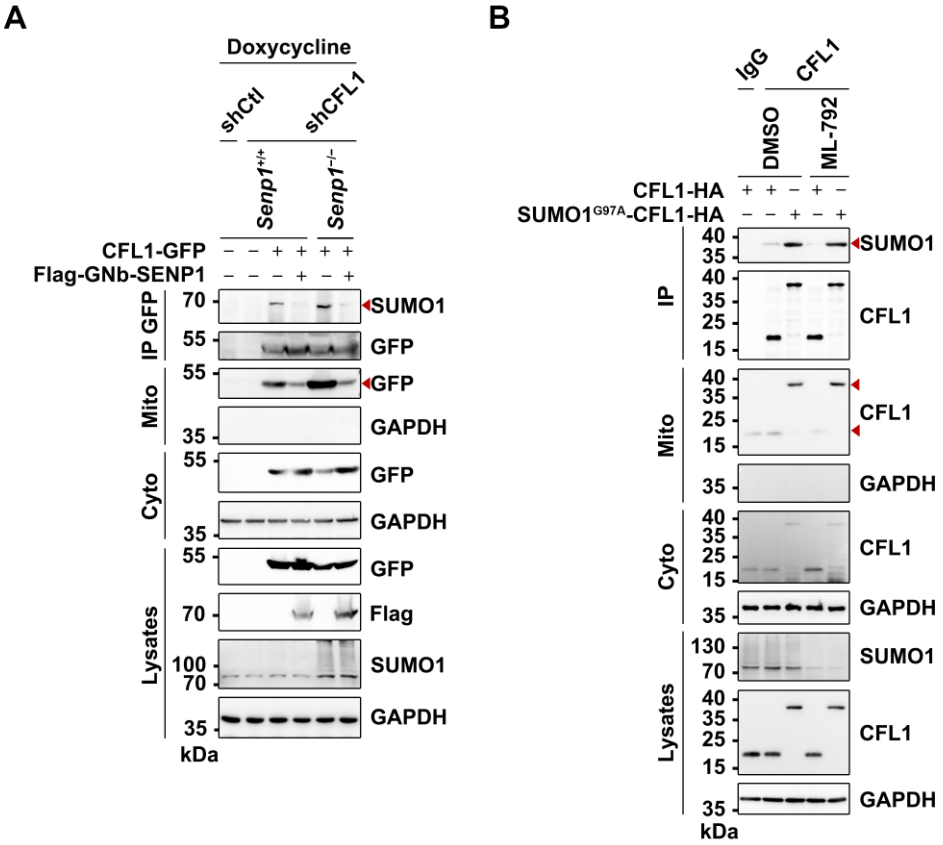
4. Conclusion: cross-method consistency

- The convergence of evidence from mutational analyses, SUMO-fusion constructs, *Senp1*$^{-/-}$ targeted rescue, and ML-792 rescue experiments

demonstrates that CFL1 N-terminal SUMOylation itself—not global SUMOylation perturbation—drives mitochondrial localization.

This cross-validation across orthogonal strategies strengthens causality and excludes alternative explanations based on indirect or off-target effects.

The revised text (Pages 9–10, lines 199–223; Page 15, lines 359–361; Page 17, lines 403–416) now clarifies this causal evidence.



Reviewer Figure 6. Rescue of CFL1 SUMOylation and mitochondrial localization under SENP1 deficiency or ML-792 inhibition.

(A) Selective removal of CFL1 SUMOylation in SENP1-deficient (*Senp1*$^{-/-}$) cells. *Senp1*$^{+/+}$ and *Senp1*$^{-/-}$ MEF cells were treated with doxycycline (2 μg/ml, 24 h) and then transfected with CFL1-GFP and Flag-Gnb-SENP1 plasmids. After 24 h, immunoprecipitation (IP) was performed using anti-GFP beads, followed by Western blotting (WB) with anti-SUMO1, anti-GFP, and anti-Flag antibodies. Mitochondrial (Mito), cytoplasmic (Cyto), and total lysate fractions from the same cells were analyzed by WB with anti-GFP antibody. Expression of Gnb-SENP1 selectively removed SUMO from CFL1 and reduced its mitochondrial localization, confirming that CFL1 SUMOylation mediates its translocation in *Senp1*$^{-/-}$ cells (n = 2 biological replicates).

(B) SUMO-fused CFL1 rescues ML-792–induced inhibition of mitochondrial localization. HEK 293T cells with endogenous CFL1 knockdown were transfected with CFL1-HA or SUMO1^{G97A}-CFL1-HA plasmids and treated with DMSO or ML-792 (0.5 μM, 4 h). Cell lysates were subjected to IP using

control IgG or anti-CFL1 antibody, followed by WB with anti-SUMO1 and anti-CFL1 antibodies. Mitochondrial, cytoplasmic, and total lysate fractions were analyzed by WB with anti-CFL1 antibody. Expression of SUMO-fused CFL1 restored mitochondrial localization suppressed by ML-792, demonstrating that the inhibitory effect of ML-792 on CFL1 translocation arises from blockade of CFL1 N-terminal SUMOylation. GAPDH served as the loading control (n = 3 biological replicates).

Reference:

Ramesh, N. S. et al. *bioRxiv* 2025.05.27.656300 (2025).

B. The authors use mutants of CFL1 that they previously demonstrate have lower SUMOyation, K112/114R, and they confirm that SUMOylation is at the N-terminus in this study. However, the use of these mutants is problematic, as lysines often carry other post-translational modifications, and the use of a K/R mutant cannot distinguish effects from the differential N-terminal SUMOylation from these potential effects. Notably, the Phosphosite plus website notes 73 different studies that have identified ubiquitnation on CFL1 at K112, and another 61 studies that have identified ubiquitnation on K114. Thus, the effects of these mutants cannot be attributated solely to SUMOylation.

Response: We thank the reviewer for this important point. To directly test whether ubiquitination or neddylation at K112/K114 could confound our interpretations, we performed the following analyses:

1. Ubiquitination at K112/K114 does not account for the phenotypes

- In CFL1-depleted cells reconstituted with CFL1 WT, K112R, K114R, or 2KR, we monitored CFL1 ubiquitination by IP-WB. K112R and K114R showed no reduction or redistribution of ubiquitination relative to WT (Reviewer Fig. 7A).
- Proteasome inhibition (MG132) increased global ubiquitination as expected, yet did not alter CFL1 SUMOylation (Reviewer Fig. 7B) and did not change SUMOylation across WT/K112R/K114R/2KR backgrounds (Reviewer Fig. 7C).

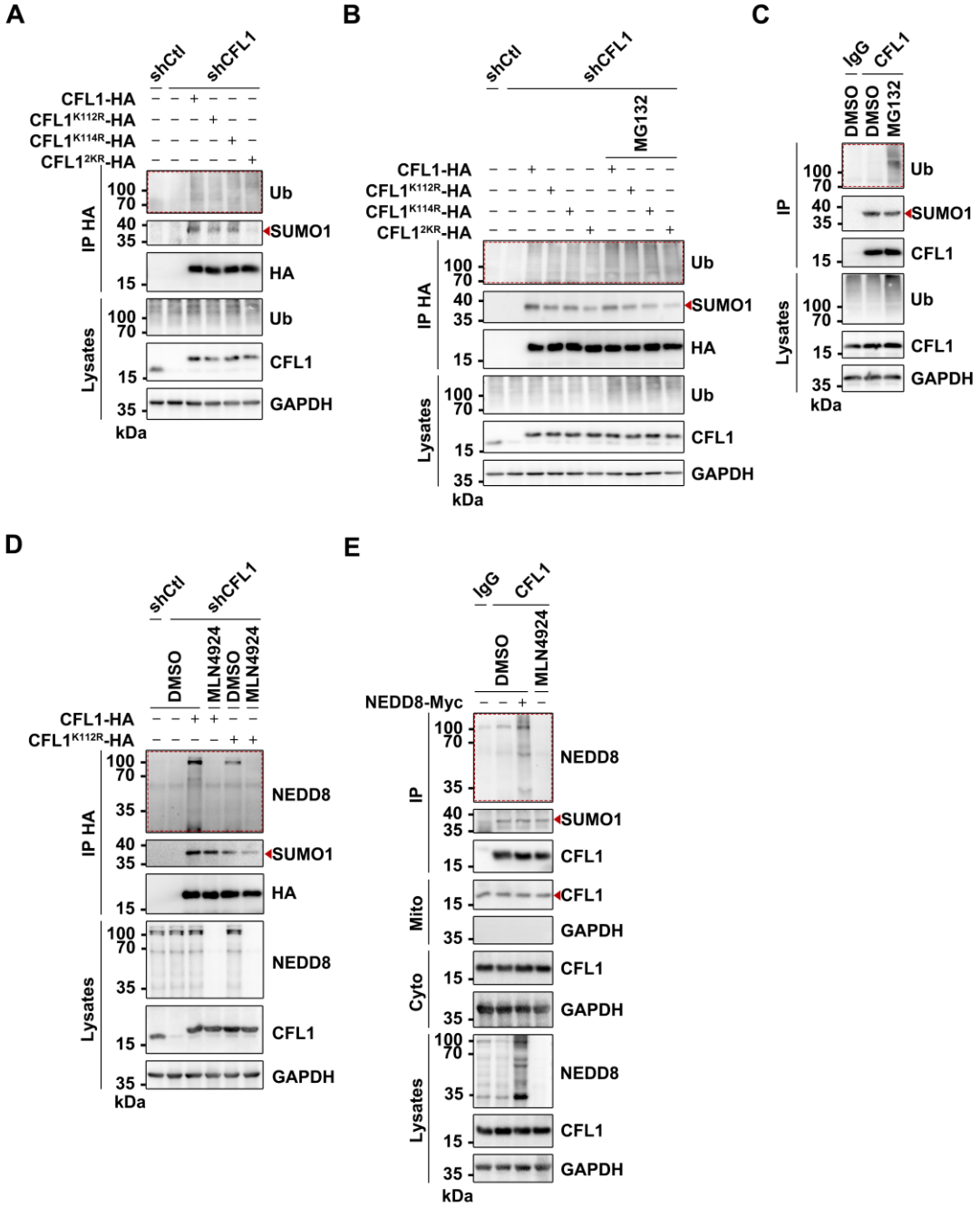
These results indicate that the K→R substitutions at 112/114 do not measurably change CFL1 ubiquitination in our system, and ubiquitination does not gate CFL1 SUMOylation.

2. Neddylation at K112 does not confound SUMOylation

- Previous work suggested K112 may undergo neddylation (Vogl et al., 2020). We therefore tested CFL1 WT and K112R under MLN4924 (neddylation inhibitor) and NEDD8 overexpression conditions.
- Both inhibition and enhancement of neddylation reduced neddylation as expected, but CFL1 SUMOylation and mitochondrial localization remained unchanged (Reviewer Fig. 7D, E).

Thus, neddylation does not interfere with SUMOylation or mitochondrial targeting of CFL1.

Together, these data demonstrate that the phenotypes observed with SUMOylation-reduced mutant (2KR) is not attributable to changes in ubiquitination or neddylation at K112/K114. Instead, they specifically reflect reduced SUMOylation. Importantly, these findings are consistent with results from independent approaches—including SUMOylation-enhanced mutants (K34R) and SUMO-fusion constructs—that also show SUMOylation dictates CFL1 mitochondrial localization. This convergence across multiple strategies strengthens the conclusion that N-terminal SUMOylation, rather than alternative lysine modifications, drives the observed phenotypes.



Reviewer Figure 7. Relationship between ubiquitination/neddylation and SUMOylation of CFL1.

(A) CFL1 K112R and K114R mutants do not alter ubiquitination. HEK 293T cells with endogenous CFL1 knockdown were transfected with CFL1-HA, $CFL1^{K112R}$-HA, $CFL1^{K114R}$-HA, or $CFL1^{2KR}$-HA plasmids. After 24 h, cell lysates were IP with anti-HA beads and analyzed by WB using anti-Ub, anti-SUMO1, anti-HA, and anti-CFL1 antibodies (n = 2 biological replicates).

(B) Proteasome inhibition does not affect CFL1 SUMOylation. HEK 293T cells transfected with the same CFL1 constructs were treated with MG132 (20 μM, 16 h) or vehicle. IP-WB analysis showed that although MG132 increased global ubiquitination, CFL1 SUMOylation remained unchanged across all mutants, suggesting no regulatory interplay between ubiquitination and SUMOylation (n = 2 biological replicates).

(C) Endogenous validation of the independence between ubiquitination and SUMOylation. Endogenous CFL1 was IP from HEK 293T cells treated with DMSO or MG132 (20 μM, 16 h). WB analysis confirmed that enhanced ubiquitination did not alter SUMO modification of CFL1 (n = 2 biological replicates).

(D) Neddylation inhibition does not impact CFL1 SUMOylation. HEK 293T cells with endogenous CFL1 knockdown were transfected with CFL1-HA or $CFL1^{K112R}$-HA, followed by treatment with MLN4924 (2 μM, 24 h). IP-WB analysis revealed that inhibition of neddylation or K112R mutation did not affect CFL1 SUMOylation (n = 2 biological replicates).

(E) CFL1 SUMOylation and subcellular localization are unaffected by neddylation modulation. HEK 293T cells were treated with DMSO or MLN4924 (2 μM, 24 h), or transfected with NEDD8-Myc plasmid, followed by IP using control IgG or anti-CFL1 antibody and WB with anti-NEDD8, anti-SUMO1, and anti-CFL1 antibodies. Mito, Cyto, and total fractions were analyzed by WB. Neither inhibition nor enhancement of neddylation altered CFL1 SUMOylation or mitochondrial localization, confirming that neddylation does not crosstalk with SUMO modification of CFL1 (n = 3 biological replicates).

Reference:

Vogl, A. M. et al. *Nat. Struct. Mol. Biol*. 27, 210–220 (2020).

C. The authors show that CFL1 interacts with the mitochondrial receptors TOM20 and TOM70, and that siRNA mediated knockdown of these proteins decreases mitochondrial translocation. TOM20 and TOM70 are highly abundant proteins at the outer mitochondrial membrane, and immunoprecipitations do not necessarily demonstrate that these proteins facilitate mitochondrial recruitment. (Notably the authors state "CFL1 translocates to mitochondria via direct interactions with Tom20 and Tom70" on lines 366-67, but immunoprecipitations do not demonstrate direct interactions.) Furthermore, the knockdown of Tom20 and Tom70

would be expected to have highly pleiotrophic effects, disrupting much of protein import and compromising mitochondrial function. Thus, these studies do not effectively demonstrate the recruitment mechanism of CFL1. I have similar comments for the Tim23 experiments.

Response: We appreciate this important critique. To address the specificity of CFL1 import, we performed additional experiments beyond Co-IP and knockdown, designed to test direct interaction and sequence-specific recruitment:

1. ***In vitro* binding assays**

   • Recombinant CFL1 bound directly to Tom20 and Tom70 in pull-down assays, both with and without HSP70 (Reviewer Fig. 8A).

   • Mutational mapping showed K13/73A lost Tom20 binding, while Δ9–18 lost Tom70 binding (Reviewer Fig. 8B).

   • Δ9–18 also abolished Tim23 binding *in vitro*, consistent with defective import in cells (Reviewer Fig. 8C).

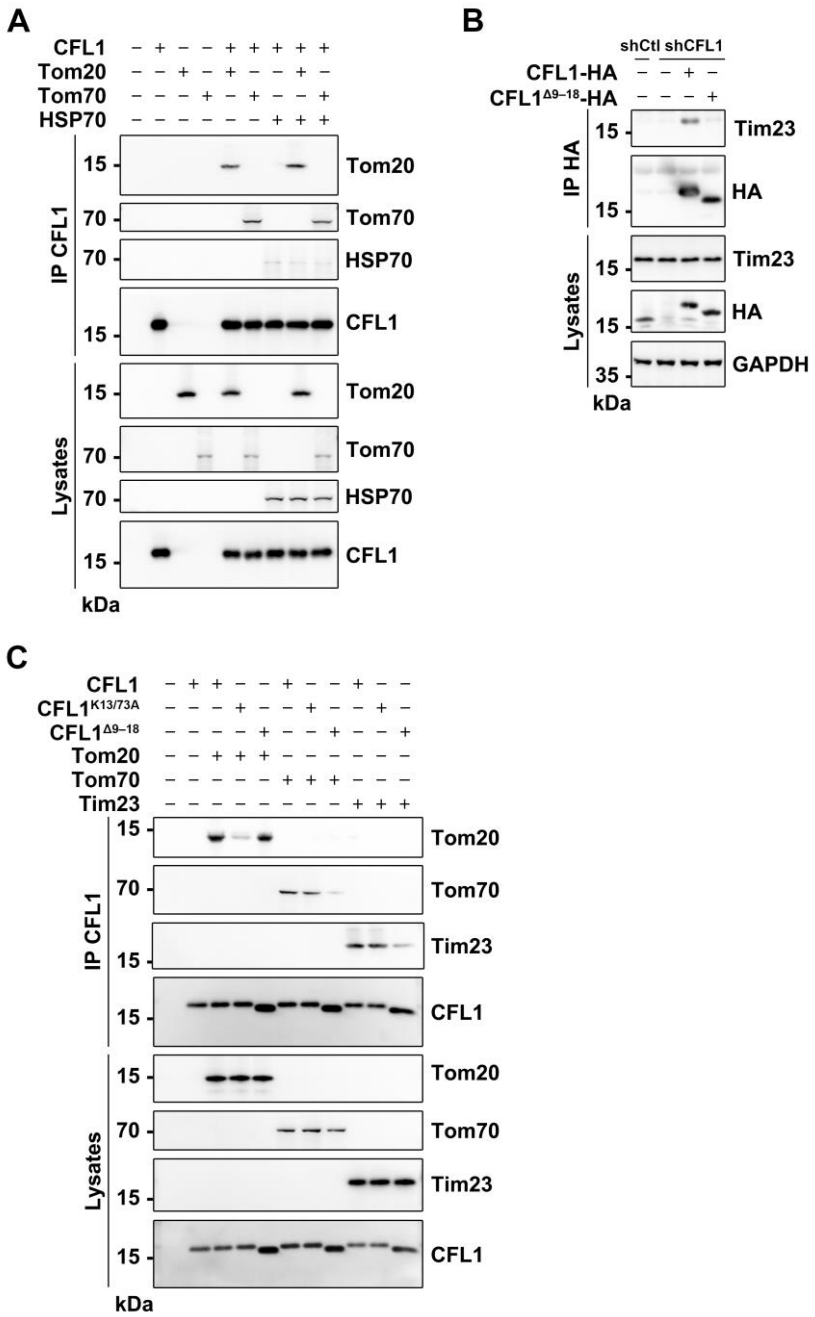
Due to the time-intensive nature of protein purification, the experiments presented in Reviewer Fig. 8A–C were conducted in two independent biological replicates. Although these data are preliminary, both replicates yielded consistent and reproducible trends, demonstrating the robustness of the observed effect. The results are provided below for the reviewers' evaluation and to support the mechanistic conclusions discussed in the manuscript. We would be pleased to incorporate Reviewer Fig. 8A–C into the main text, and will expand the dataset with additional replicates should the reviewers or editors consider it appropriate for inclusion.

2. **Cellular validation**

   • These binding defects were reproduced in cellular Co-IP experiments (Revised Fig. 3D, E).

3. **Structural modeling**

   • Motif analysis identified a Tom20-binding sequence at the CFL1 N-terminus and a positively charged iMTS-like element for Tom70 and Tim23 recognition (Bauer et al., 1996; Jain et al., 2025), consistent with the mutational data (Revised Supplementary Figs. 3, 4).



Reviewer Figure 8. Direct interaction of CFL1 with Tom20, Tom70, and Tim23.

(A) Direct interactions between recombinant CFL1 and Tom 20, Tom70, and HSP70. Purified CFL1, Tom20, and Tom70 were mixed in lysis buffer and incubated at 4°C for 1 hour with gentle rotation. Samples were subjected to IP using an anti-CFL1 antibody, followed by WB with anti-CFL1, anti-Tom20, anti-Tom70, and anti-HSP70 antibodies as indicated (n = 2 biological replicates).

(B) Deletion of the Tom70-binding sequence (CFL1$^{\Delta 9-18}$-HA) impaired the interaction between CFL1 and Tim23. HEK 293T cells with endogenous CFL1 knockdown were transfected with CFL1-HA and CFL1$^{\Delta 9-18}$-HA plasmids. 24 h later, cell lysates were subjected to Co-IP with anti-HA beads

and anti-Tim23 antibody, followed by WB using anti-HA and anti-Tim23 antibodies (n = 2 biological replicates).

(C) The CFL1 K13/73A mutation ($CFL1^{K13/73A}$) markedly reduced affinity for Tom20, while deletion of residues 9–18 ($CFL1^{\Delta 9–18}$) impaired binding to Tom70 and Tim23. Purified CFL1, $CFL1^{K13/73A}$, $CFL1^{\Delta 9–18}$, Tom20, Tim23 and Tom70 were mixed in lysis buffer and incubated at 4 °C for 1 hour with gentle rotation. Samples were subjected to IP with anti-CFL1 antibody, followed by WB with anti-CFL1, anti-Tom20, anti-Tim23, and anti-Tom70 antibodies (n = 2 biological replicates).

Collectively, the biochemical reconstitution, mutational mapping, cellular validation, and structural predictions converge to demonstrate that CFL1 directly and sequence-specifically interacts with Tom20, Tom70, and Tim23. These interactions mediate CFL1 mitochondrial import independently of secondary effects caused by receptor knockdown.

References:

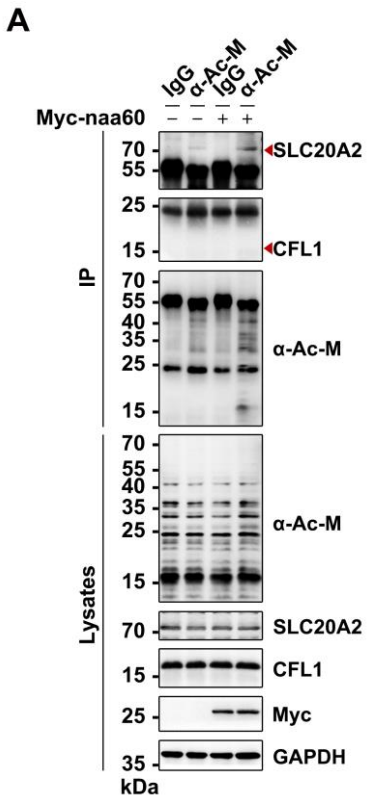
Bauer, M. F. et al. *Cell* 87, 33–41 (1996).

Jain, N. et al. *Trends Biochem. Sci*. 50, 585–595 (2025).

Additional table and figures for the reviewers

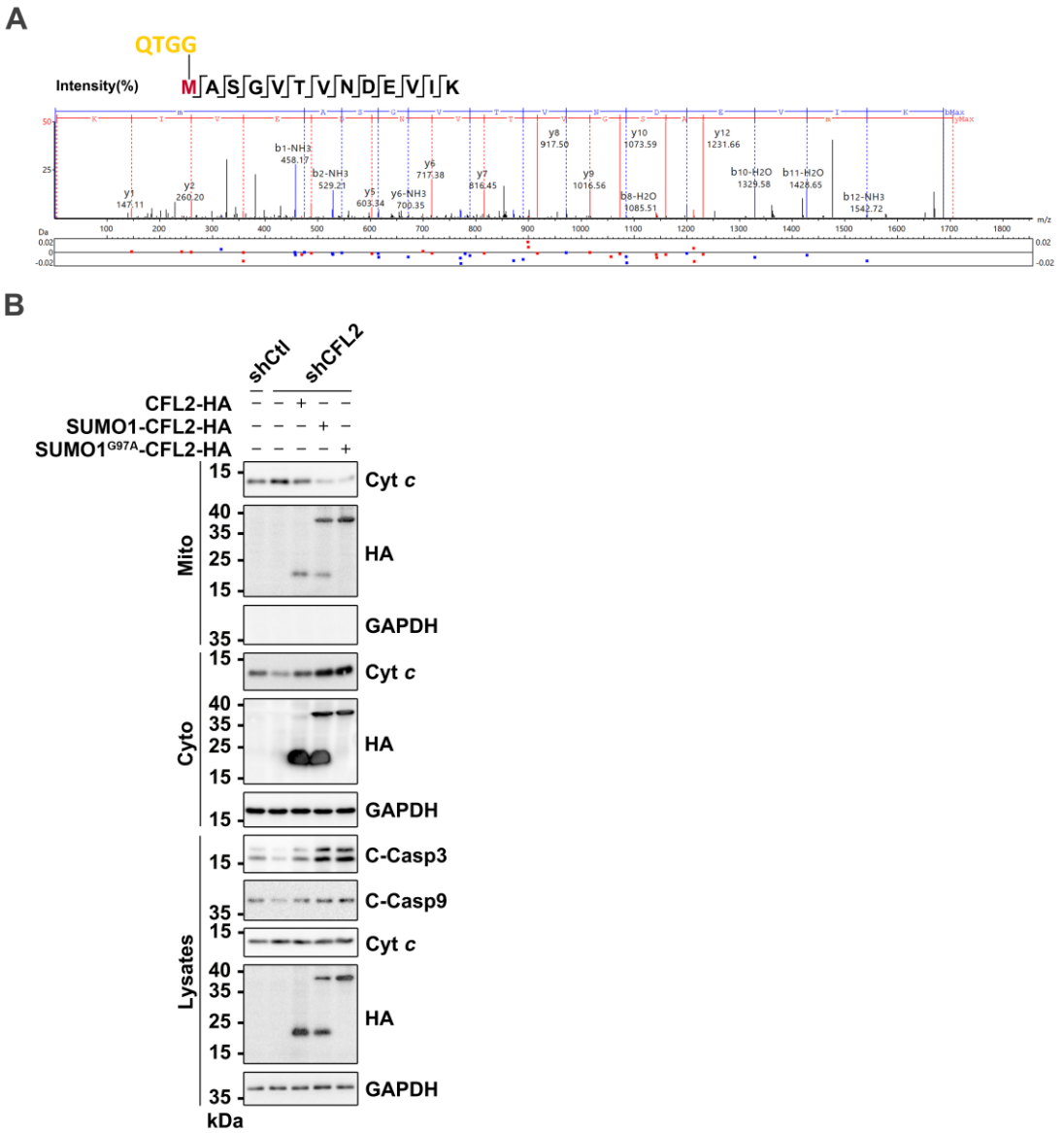
Protein Accession	Peptides
P23528 (CFL1)	A(+42.01)SGVAVSDGVIK
	A(+42.01)SGVAVSDGVIK
	ASGVAVSDGVIK
	M(+15.99)ASGVAVSDGVIK
	A(+42.01)SGVAVSDGVIK
	ASGVAVSDGVIK
	MASGVAVSDGVIK

Reviewer Table 1. Identification of CFL1 N-terminal processing forms by immunoprecipitation–mass spectrometry (IP–MS).
HEK 293T cell lysates were immunoprecipitated with an anti-CFL1 antibody. The immunoprecipitated proteins were digested with trypsin and analyzed by LC–MS/MS. Two distinct N-terminal peptide species of CFL1 were consistently detected in three independent experiments: the unprocessed methionine-retaining peptide (M-A-S-G-V-A-V-S-D-G-V-I-K) and the N-acetylated alanine peptide (Ac-A-S-G-V-A-V-S-D-G-V-I-K, the acetylation modification is characterized by a mass shift of +42.01 Da). These findings demonstrate that CFL1 exists in both Met-retaining and demethionylated N-acetylated forms, consistent with co-translational N-terminal processing in mammalian cells.



Reviewer Figure 1. N-terminal acetylation of methionine on CFL1 compared with SLC20A2.

(A) HEK 293T cells were transfected with or without Myc-Naa60 plasmid, which encodes the N-terminal acetyltransferase Naa60. Cell lysates were subjected to immunoprecipitation (IP) using either control IgG or anti-α-Ac-M (N-acetyl-methionine) antibody, followed by immunoblotting with anti–α-Ac-M, anti-SLC20A2, and anti-CFL1 antibodies. Input lysates were analyzed in parallel to confirm protein expression. The membrane regions containing CFL1 and SLC20A2 were excised from the same PVDF membrane and separately probed with anti-CFL1 and anti-SLC20A2 antibodies. The red arrows indicate the positions of the acetylated SLC20A2 and the putative acetylated CFL1 bands; however, CFL1 showed no detectable acetylation signal. Overexpression of Myc-Naa60 enhanced the α-Ac-M signal of SLC20A2 but not CFL1, indicating that CFL1's N-terminal methionine is not acetylated. GAPDH served as a loading control (n = 2 biological replicates).

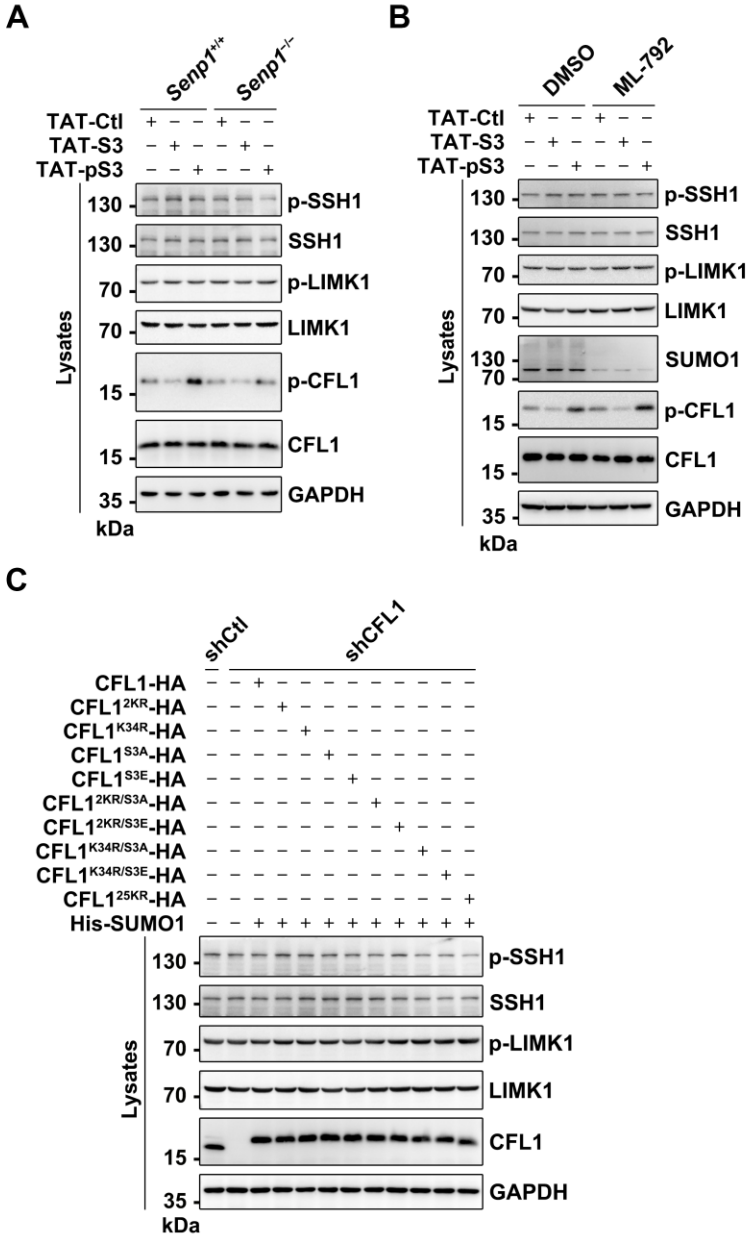


Reviewer Figure 2. N-terminal SUMOylation of CFL2 promotes mitochondrial-dependent apoptosis in avian cells.

(A) DF-1 cells were co-transfected with CFL2-HA and His-SUMO1^{E93R} plasmids. Cell lysates were subjected to immunoprecipitation using anti-HA beads, followed by LC–MS/MS analysis. The MS/MS spectrum shows a tryptic peptide containing the glutamine-threonine-glycine-glycine (QTGG) remnant attached to the N-terminal methionine of CFL2, confirming N-terminal SUMOylation.

(B) CFL2 SUMOylation enhances mitochondrial translocation and activation of apoptotic signaling. DF-1 cells were transfected with CFL2-HA, SUMO1-CFL2-HA, or SUMO1^{G97A}-CFL2-HA plasmids. Mitochondrial (Mito), cytoplasmic (Cyto), and total lysate fractions were analyzed by Western blotting (WB) using antibodies against HA (since the import and customs clearance process for a commercial chicken CFL2 antibody is still pending, HA-tag detection was used to monitor exogenous CFL2-HA expression),

cytochrome *c* (Cyt *c*), cleaved caspase-3 (C-Casp3), and cleaved caspase-9 (C-Casp9). SUMO-fused CFL2 promoted Cyt *c* release from mitochondria to cytosol and increased caspase activation, indicating that N-terminal SUMOylation facilitates CFL2-mediated mitochondrial apoptosis. GAPDH served as the loading control (n = 3 biological replicates).



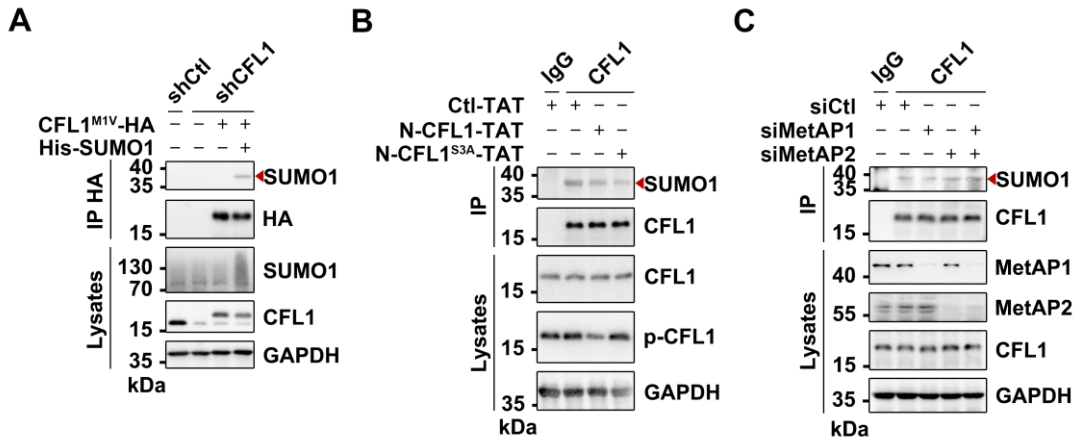
Reviewer Figure 3. LIMK and SSH activities are unaffected by SUMOylation manipulations in our experimental system.

(A) SENP1 deficiency does not alter LIMK/SSH activity. *Senp1*$^{+/+}$ and *Senp1*$^{-/-}$ MEF cells were treated with TAT-Ctl, TAT-S3, or TAT-pS3 peptides (10 μM, 16 h). Whole-cell lysates were analyzed by Western blotting (WB) using antibodies against phospho-SSH1 (Ser978), SSH1, phospho-LIMK1 (Thr508), LIMK1, phospho-CFL1 (Ser3) and CFL1. No significant differences were observed in the phosphorylation of SSH1 or LIMK1 (n = 2

biological replicates).

(B) Pharmacological inhibition of SUMOylation does not affect LIMK/SSH activity. HEK 293T cells were treated with TAT-Ctl, TAT-S3, or TAT-pS3 peptides (10 μM, 16 h), followed by DMSO or ML-792 (0.5 μM, 4 h). Cell lysates were subjected to WB with the indicated antibodies. ML-792 efficiently reduced global SUMOylation but did not alter SSH1 or LIMK1 phosphorylation, indicating that SUMO inhibition does not influence LIMK/SSH activity (n = 3 biological replicates).

(C) LIMK/SSH activities remain stable across CFL1 SUMOylation and phosphorylation mutants. HEK 293T cells with endogenous CFL1 knockdown were transfected with the indicated CFL1-HA constructs (WT, 2KR, K34R, S3A, S3E, 2KR/S3A, 2KR/S3E, K34R/S3A, K34R/S3E, or 25KR) together with His-SUMO1 plasmid. WB analysis revealed that neither LIMK1 nor SSH1 phosphorylation was affected by CFL1 mutations or SUMO manipulations, confirming that LIMK/SSH signaling remains unaltered across all conditions. GAPDH served as a loading control (n = 2 biological replicates).



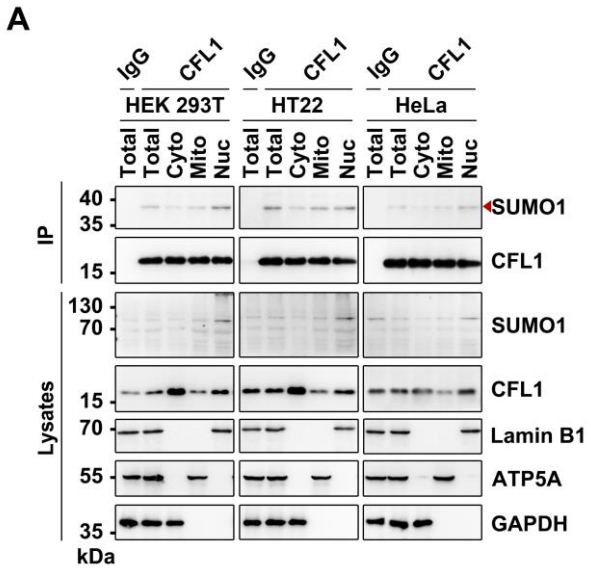
Reviewer Figure 4. Regulation of CFL1 N-terminal α-amino SUMOylation.

(A) CFL1 retains SUMOylation in the absence of its N-terminal methionine. HEK 293T cells with endogenous CFL1 knockdown were transfected with CFL1^{M1V}-HA (Met→Val substitution at the initiator codon) and His-SUMO1 plasmids. Immunoprecipitation (IP) with anti-HA beads followed by WB using anti-SUMO1, ant-HA, and anti-CFL1 antibodies demonstrated that CFL1^{M1V} still undergoes SUMO conjugation at its N-terminal α-amino group (n = 3 biological replicates).

(B) N-terminal CFL1 peptides competitively inhibit endogenous SUMOylation. HEK 293T cells were treated with Ctl-TAT, N-CFL1-TAT, or phosphorylation-deficient N-CFL1^{S3A}-TAT peptides (20 μM, 16 h). IP with control IgG or anti-CFL1 antibody followed by WB using anti-SUMO1, anti-phospho-CFL1 (Ser3), and anti-CFL1 antibodies revealed that both N-CFL1-TAT and N-CFL1^{S3A}-TAT peptides reduced endogenous CFL1 SUMOylation without

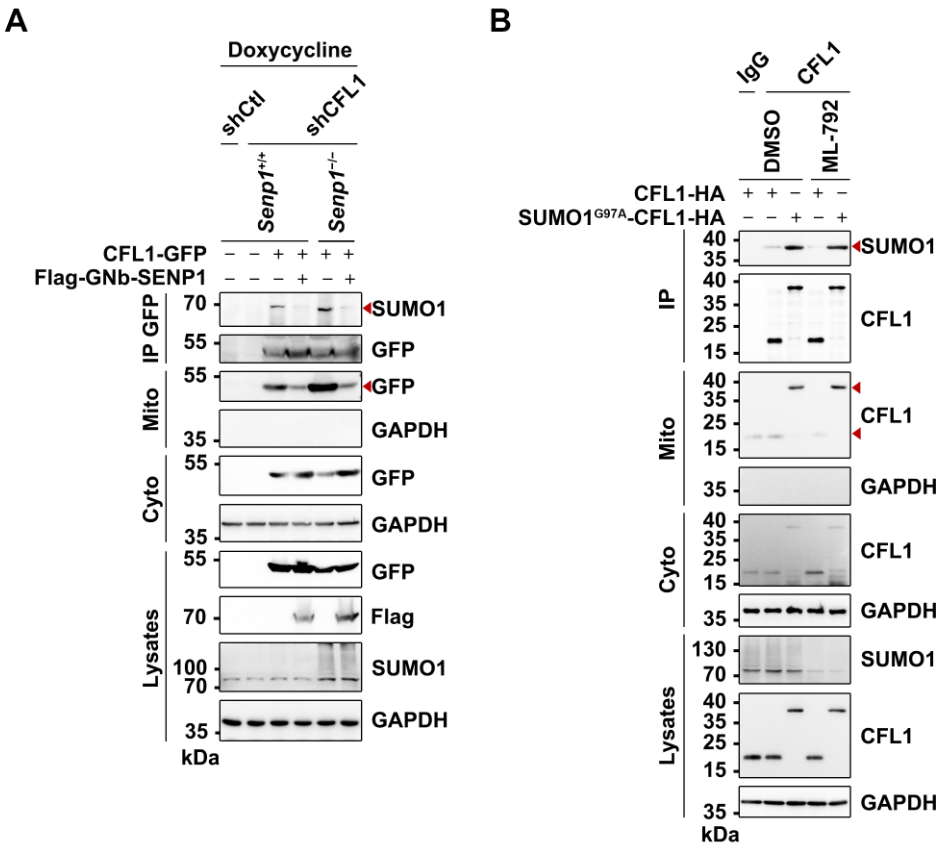
altering phosphorylation (n = 3 biological replicates).

(C) N-terminal methionine excision modulates CFL1 SUMOylation. Knockdown of MetAP1 or MetAP2 in HEK 293T cells by siRNA increased the SUMOylation of CFL1, indicating that reduced N-terminal methionine removal enhances CFL1 N-terminal SUMO conjugation (n = 2 biological replicates).



Reviewer Figure 5. Subcellular distribution of CFL1 SUMOylation.

(A) CFL1 SUMOylation levels were examined in total, cytoplasmic (Cyto), mitochondrial (Mito), and nuclear (Nuc) fractions of HEK 293T, HT22, and HeLa cells by IP-WB using anti-CFL1 and anti-SUMO1 antibodies. Fractionation purity was verified with ATP5A (mitochondrial marker), Lamin B1 (nuclear marker), and GAPDH (cytoplasmic marker) (n = 2 biological replicates).

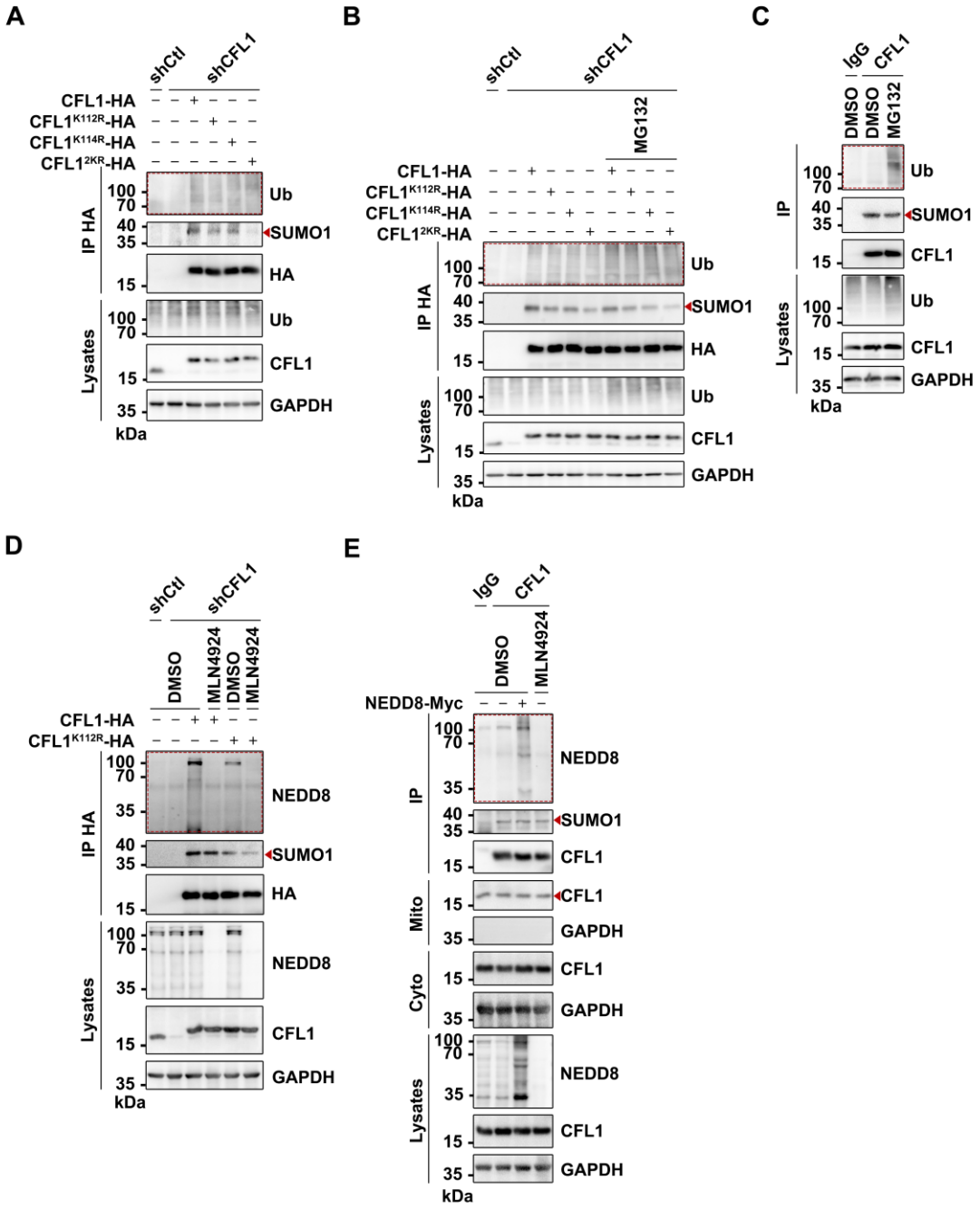


Reviewer Figure 6. Rescue of CFL1 SUMOylation and mitochondrial localization under SENP1 deficiency or ML-792 inhibition.

(A) Selective removal of CFL1 SUMOylation in SENP1-deficient (*Senp1*$^{-/-}$) cells. *Senp1*$^{+/+}$ and *Senp1*$^{-/-}$ MEF cells were treated with doxycycline (2 μg/ml, 24 h) and then transfected with CFL1-GFP and Flag-Gnb-SENP1 plasmids. After 24 h, immunoprecipitation (IP) was performed using anti-GFP beads, followed by Western blotting (WB) with anti-SUMO1, anti-GFP, and anti-Flag antibodies. Mitochondrial (Mito), cytoplasmic (Cyto), and total lysate fractions from the same cells were analyzed by WB with anti-GFP antibody. Expression of Gnb-SENP1 selectively removed SUMO from CFL1 and reduced its mitochondrial localization, confirming that CFL1 SUMOylation mediates its translocation in *Senp1*$^{-/-}$ cells (n = 2 biological replicates).

(B) SUMO-fused CFL1 rescues ML-792–induced inhibition of mitochondrial localization. HEK 293T cells with endogenous CFL1 knockdown were transfected with CFL1-HA or SUMO1^{G97A}-CFL1-HA plasmids and treated with DMSO or ML-792 (0.5 μM, 4 h). Cell lysates were subjected to IP using control IgG or anti-CFL1 antibody, followed by WB with anti-SUMO1 and anti-CFL1 antibodies. Mitochondrial, cytoplasmic, and total lysate fractions were analyzed by WB with anti-CFL1 antibody. Expression of SUMO-fused CFL1 restored mitochondrial localization suppressed by ML-792, demonstrating that the inhibitory effect of ML-792 on CFL1 translocation

arises from blockade of CFL1 N-terminal SUMOylation. GAPDH served as the loading control (n = 3 biological replicates).



Reviewer Figure 7. Relationship between ubiquitination/neddylation and SUMOylation of CFL1.

(A) CFL1 K112R and K114R mutants do not alter ubiquitination. HEK 293T cells with endogenous CFL1 knockdown were transfected with CFL1-HA, CFL1^{K112R}-HA, CFL1^{K114R}-HA, or CFL1^{2KR}-HA plasmids. After 24 h, cell lysates were IP with anti-HA beads and analyzed by WB using anti-Ub, anti-SUMO1, anti-HA, and anti-CFL1 antibodies (n = 2 biological replicates).

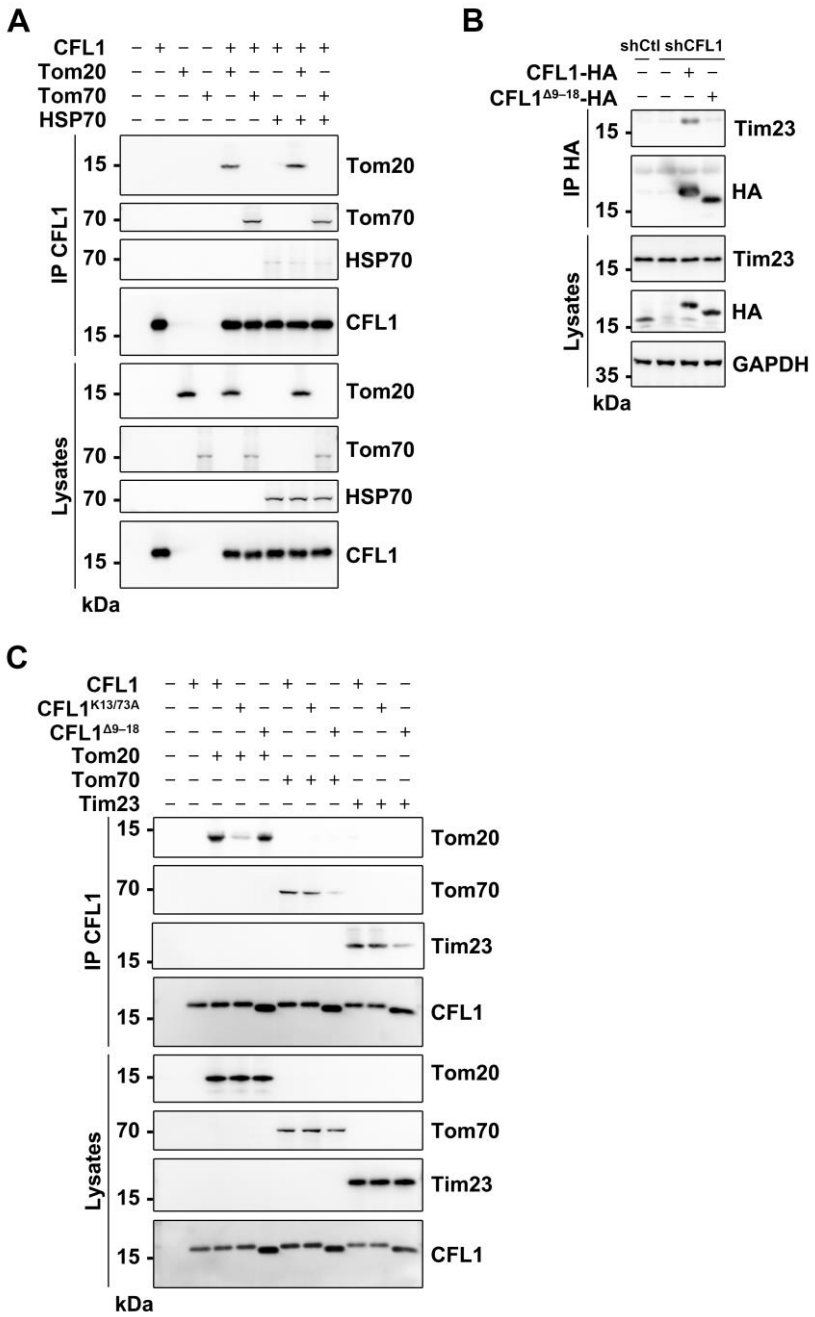
(B) Proteasome inhibition does not affect CFL1 SUMOylation. HEK 293T cells transfected with the same CFL1 constructs were treated with MG132 (20

μM, 16 h) or vehicle. IP-WB analysis showed that although MG132 increased global ubiquitination, CFL1 SUMOylation remained unchanged across all mutants, suggesting no regulatory interplay between ubiquitination and SUMOylation (n = 2 biological replicates).

(C) Endogenous validation of the independence between ubiquitination and SUMOylation. Endogenous CFL1 was IP from HEK 293T cells treated with DMSO or MG132 (20 μM, 16 h). WB analysis confirmed that enhanced ubiquitination did not alter SUMO modification of CFL1 (n = 2 biological replicates).

(D) Neddylation inhibition does not impact CFL1 SUMOylation. HEK 293T cells with endogenous CFL1 knockdown were transfected with CFL1-HA or $CFL1^{K112R}$-HA, followed by treatment with MLN4924 (2 μM, 24 h). IP-WB analysis revealed that inhibition of neddylation or K112R mutation did not affect CFL1 SUMOylation (n = 2 biological replicates).

(E) CFL1 SUMOylation and subcellular localization are unaffected by neddylation modulation. HEK 293T cells were treated with DMSO or MLN4924 (2 μM, 24 h), or transfected with NEDD8-Myc plasmid, followed by IP using control IgG or anti-CFL1 antibody and WB with anti-NEDD8, anti-SUMO1, and anti-CFL1 antibodies. Mito, Cyto, and total fractions were analyzed by WB. Neither inhibition nor enhancement of neddylation altered CFL1 SUMOylation or mitochondrial localization, confirming that neddylation does not crosstalk with SUMO modification of CFL1 (n = 3 biological replicates).



Reviewer Figure 8. Direct interaction of CFL1 with Tom20, Tom70, and Tim23.

(A) Direct interactions between recombinant CFL1 and Tom 20, Tom70, and HSP70. Purified CFL1, Tom20, and Tom70 were mixed in lysis buffer and incubated at 4°C for 1 hour with gentle rotation. Samples were subjected to IP using an anti-CFL1 antibody, followed by WB with anti-CFL1, anti-Tom20, anti-Tom70, and anti-HSP70 antibodies as indicated (n = 2 biological replicates).

(B) Deletion of the Tom70-binding sequence (CFL1$^{\Delta 9-18}$-HA) impaired the interaction between CFL1 and Tim23. HEK 293T cells with endogenous CFL1 knockdown were transfected with CFL1-HA and CFL1$^{\Delta 9-18}$-HA plasmids. 24 h later, cell lysates were subjected to Co-IP with anti-HA beads

and anti-Tim23 antibody, followed by WB using anti-HA and anti-Tim23 antibodies (n = 2 biological replicates).

(C) The CFL1 K13/73A mutation ($CFL1^{K13/73A}$) markedly reduced affinity for Tom20, while deletion of residues 9–18 ($CFL1^{\Delta 9-18}$) impaired binding to Tom70 and Tim23. Purified CFL1, $CFL1^{K13/73A}$, $CFL1^{\Delta 9-18}$, Tom20, Tim23 and Tom70 were mixed in lysis buffer and incubated at 4 °C for 1 hour with gentle rotation. Samples were subjected to IP with anti-CFL1 antibody, followed by WB with anti-CFL1, anti-Tom20, anti-Tim23, and anti-Tom70 antibodies (n = 2 biological replicates).

We thank all reviewers and editors for the constructive comments on our work. We have addressed all the comments and suggestions. We believe that the new data added in our revised manuscript provide additional strong support for our conclusions and substantially strengthen the paper. We hope that our manuscript is now acceptable for publication in *Nature Communications*.

Thank you very much for your consideration.

Yours sincerely,

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Below are our responses to the second-round comments.

Authors' Responses to the Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

The authors have properly addressed my comments.

Response: We thank the reviewer for the positive assessment and helpful feedback.

Reviewer #2 (Remarks to the Author):

The authors have done an outstanding job in responding to each comment of every reviewer, considerably strengthening what was already an excellent paper. In my 55 years of reviewing manuscripts, I have never seen such a complete revision of figures to include everything asked for by the reviewers. Figures were vastly improved, new IP controls were added, and writing was improved to eliminate tense

errors. Furthermore. The authors responded to reviewers' questions that they found both intellectually and functionally significant by performing new sets of experiments that added significantly to the impact of this manuscript.

One significant set of new experiments provided in the authors' response to reviewers demonstrated that in chicken DF-1 cells, which express ADF and cofilin-2 but NOT cofilin-1, only cofilin-2 undergoes N-terminal SUMOylation and mitochondrial targeting. This is significant in at least two respects: 1) ADF and Cofilin 1 and Cofilin 2 from both mammals and birds contain the identical N-terminal 5 amino acids (MASGV) and the new results show that the specificity for N-terminal SUMOylation requires additional recognition sites; and 2) that in avians where ADF substitutes for cofilin-1 for most general cell functions involving actin dynamics in motility and cytokinesis and cofilin-2 is mainly involved in muscle development as it is in mammals, it is cofilin-2 that carries out the mitochondrial targeted responses involved in triggering apoptosis. It appears that authors had planned to include these new data into this manuscript because the use of the DF-1 cells was added to the Materials and Methods sections (lines 606-607, 610, 676-679 and 744-746) but they eventually settled on just adding: "other functional comparisons between ADF/cofilin family members will be essential to determine whether similar SUMO-mediated pathways operate in other contexts" as stated in lines 528-531. Perhaps the lack of adding the additional results is due to the authors' desire to include experiments using a chicken CFL2 antibody which "was held up by customs" and thus their results rely only on the HA tag for SUMO detection? Because these findings are significant in showing a very specific difference between ADF and cofilin in terms of a cell function that does not require actin-interaction, I hope that they will include these results provided the authors are comfortable with the veracity of their findings, and that a paragraph in the discussion be devoted to this finding. Otherwise, I suggest the additions to the methods that reference the experiments using DF-1 cells be removed.

Regardless of the inclusion or removal of the ADF/cofilin-2 comparison in chick cells, I strongly support the publication of this manuscript.

Response: We sincerely thank Reviewer #2 for the very encouraging and generous comments, which have been highly motivating for our team. We deeply appreciate the insightful perspective regarding the DF-1 findings.

Although the preliminary DF-1 data consistently suggested that cofilin-2—but not ADF—undergoes N-terminal SUMOylation and mitochondrial targeting, we chose a conservative and rigorous approach and **removed all DF-1-related content from the manuscript and Methods** for the following reasons:

- SUMOylation is highly dynamic, and low-abundance ADF modification cannot yet be excluded.

- Proteomic workflows may under-detect rare SUMOylation events; targeted enrichment is warranted.

- Validation with a chicken-specific CFL2 antibody (currently delayed in customs) is pending.

- ADF contains predicted TOM20/70 motifs, suggesting potential mitochondrial functions requiring systematic study.

We agree this represents a conceptually important and biologically compelling direction. We have retained a brief statement in the Discussion noting that distinct SUMOylation and mitochondrial functions across ADF/cofilin isoforms will be explored in future work.

We are sincerely grateful to the reviewer for recognizing the significance of this observation and for the exceptionally positive evaluation.

Reviewer #3 (Remarks to the Author):

After a thorough review of this revised manuscript, it is apparent that the authors went through great length in their revision. Authors addressed all the suggestions raised in depth and with great rigor. Large portions of the manuscript were completely rewritten such as the discussion and their results put into the larger context of the cofilin reserach. Many figures were revised with additional data targeting very accurately the 'concerns' raised initially. Analysis of the data and interpretation provides a substantial and solid foundation of their conclusions. This manuscript does add a substantial degree of new and important knowledge of the role of cofilin independent of its crucial action on actin dynamics.

Response: We thank the reviewer for the thoughtful and generous comments. We greatly appreciate the guidance, which helped refine the manuscript and clarify the broader biological significance of this work.

Reviewer #4 (Remarks to the Author):

The authors have adequately addressed my concerns from the initial manuscript. This, in combination with substantial work to address the other reviewer's critiques, render this manuscript acceptable for publication in Nature Communications.

Response: We thank the reviewer for the encouraging comments and constructive suggestions, which improved the clarity and rigor of the revised

manuscript.

Thank you again for your consideration. We hope this final revised version will be suitable for publication in *Nature Communications*.

Yours sincerely,

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