

Decline of chaperone-mediated autophagy in aging impairs macrophage clearance of senescent cells

Corresponding Author: Professor Ana Maria Cuervo

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)

Sereda et al explore the role of chaperone mediated autophagy (CMA) in driving aging through alterations in different cell types including macrophages. Considerable evidence has been provided that CMA declines with both chronological and biological aging. Previously the authors have shown that CMA is upregulated in response to senescence-induction however the impact of CMA impairment on cellular senescence is still relatively underexplored. The authors sought to address this using mouse models of natural aging and CMA-deficient mice. Using mouse ear fibroblasts from young and old Dendra reporter mice, the authors found an age-related decline in CMA. Using fibroblasts knocked down for LAMP2, a key component of CMA, they observed an enhanced levels of nuclear abnormalities, SABGal, p21, and gH2AX staining, all features of senescent cells. Principal component analysis revealed that while CMA-deficient fibroblasts have features of senescence, they are still distinct from senescent cells. Analysis of the proteome showed alterations in multiple pathways of aged or CMA-deficient (LAMP2A KO) cells including altered Golgi trafficking and apoptosis regulation, both of which are relevant to senescence (SASP production and apoptotic escape). Beyond this, proteomic analysis demonstrated that CMA-deficient cells phenocopied many of the energetic changes that occur with natural aging. Innovative approaches like Turbo-ID labeling of the conventional versus unconventional secretome (EVs), showed CMA-driven changes in SASP profiles.

To determine the if the effects of CMA on senescence were regulated through SASP, the authors employed conditioned media experiments using conditioned media from control or CMA-deficient cells. These experiments clearly demonstrated that loss of CMA drives paracrine senescence in fibroblasts. Because of the relationship of senescent cells and their clearance by macrophages, the authors then investigated whether CMA-deficient fibroblasts could induce paracrine senescence in bone marrow-derived macrophages (BMDMs) and they indeed could. Furthermore, they showed that CMA declines in macrophages with aging and reduced CMA in macrophages (using a myeloid-specific model of LAMP2 deficiency) can cause intrinsic defects in macrophage activation and phagocytosis. Interestingly, the authors observed cytotoxicity when CMA-deficient macrophages were cultured with the conditioned media from senescent CMA-deficient fibroblasts. The authors then go on to show that mice with CMA-deficient macrophages develop robust features of senescence tissues and that this and tissue pathology can be alleviated by treatment with the CMA activator CA77.1. To assess the translational potential for restoring CMA, the authors investigated its use in idiopathic pulmonary fibrosis (IPF). They demonstrate that CMA declines in human lung fibroblasts with age and similar findings are present within the lungs of natural aged mice. Using a mouse model of idiopathic pulmonary fibrosis that relies upon bleomycin reduction, they observed that CA77.1 treatment reduced features of fibrosis, macrophage infiltration and senescence. These findings demonstrate the importance of CMA in limiting the spread of senescent cell burden and the use of CMA activators to treat age-related diseases.

Overall the study is well-designed and performed and the phenotypic data is robustly supported by the mechanistic studies that underly the role of CMA changes in macrophage senescence and tissue dysfunction. A few points should be addressed to improve the manuscript for clarity and implications. Therefore, I am recommending revisions.

Major Points

The authors robustly explore changes in CMA on macrophage phagocytosis and its role in aging, however macrophages play a very important role in the clearance of dead cells via efferocytosis. This process yields an anti-inflammatory response that creates a positive environment for tissue regeneration and repair. The effect of impaired CMA in efferocytosis should be explored to understand how much of role this contributes to the tissue effects in the constitutive or myeloid-specific LAMP2

KO mice.

The p16 antibody used is reported to be human specific and was generated on a sequence from the C-terminal of p16 which does not share significant homology with the mouse protein. Has this antibody been validated for mouse use, or could some preliminary testing with another mouse-specific antibody be used to reconfirm the data in Extended Data Figure 2A?

In Figure 5, the authors show increased senescence in adipose, liver and skin. Were other parenchymal (especially lung), lymphoid (spleen) or brain tissues examined for senescence in ML2A KO mice (either by SABGal, p21 protein levels or p16/p21 transcript)?

In Figure 6, CA77.1 treatment was capable of modestly reducing SABGal in WAT and liver but had a much stronger effect in the lungs of naturally aged mice. Similar changes were found for senescence and SASP transcripts. Would this be due to inherent pharmacology and tissue distribution of CA77.1 (if that data even exists) or does it speak to tissue-specific CMA levels and their importance?

In Figure 7, CA77.1 treatment reduced macrophage infiltration in the murine IPF model. Meanwhile treatment appeared to T cell infiltration. Is it possible to know if this is a generalized recruitment of T cells or potentially a more specific recruitment of Tregs to dampen or resolve the immune response? (CD3 vs FoxP3 staining or expression in lung tissue)

Minor points

The fat tissue is labeled as epididymal (eWAT) even in female mice (Extended Data Figure 6a). It may be more accurate to label as gonadal (gWAT).

Reviewer #2

(Remarks to the Author)

This is an important and well done study linking the age-related decline in chaperone-mediated autophagy (CMA) to the persistence of senescent cells in vivo. The study gets at the fundamental issue of why senescent cells persist in aged animals but are cleared in young animals. The findings demonstrate that reduced CMA in senescent cells alters their SASP, which then suppresses macrophage CMA and phagocytic function, leading to impaired clearance of the senescent cells. Extensive in vitro and in vivo data are presented to support the conclusions of the paper.

I have the following points for the authors to consider:

1. The impairment of phagocytosis by the secretome of senescent fibroblasts, and CMA-deficient fibroblasts in particular, is a crucial finding. However, it is unclear how much of the impairment in phagocytosis in macrophages is due to impaired CMA in the macrophages or due to other changes related to the secretome. The findings would be further supported if the defect in phagocytosis could be reversed by restoring CMA in the macrophages exposed to the secretome of senescent fibroblasts or CMA-deficient fibroblasts. Whether the restoration of phagocytosis in this setting is partial or complete would help clarify this issue.
2. In the senescence field, palbociclib is one of the lessor used agents to induce senescence. Although the authors present some data using other inducers, it is fairly limited. It would help if the key finding – i.e., impairment of macrophage phagocytosis by the secretome of senescent fibroblasts, could be replicated using etoposide or another senescence inducer.
3. The human data are mainly correlative and do not directly validate the proposed model described above. This should be clearly acknowledged in the Discussion.
4. The proteomic and metabolomic sections are quite dense. Is there a way to simplify these to some degree? That would make the paper easier to read.
5. Given that loss of CMA by itself does not cause senescence, the authors should probably not refer to their secretome as a SASP.
6. The section on paracrine senescence (Fig 3n, Ex Data Fig 6k) could be strengthened. A more rigorous assay beyond SABGal should be used (e.g., TAF) to establish a senescent phenotype of the cells exposed to the secretome of the senescent cells.

Reviewer #3

(Remarks to the Author)

This is a genuinely impressive and very substantial piece of work that addresses an important question at the intersection of ageing, autophagy and immune surveillance. The authors combine a wide range of complementary approaches, including primary cells from young and old animals, sophisticated proteomic and metabolomic analyses, genetically modified mouse models, macrophage-specific interventions, and pharmacological activation studies in vivo. Although the scope is very broad, there is a clear mechanistic thread throughout the manuscript, linking age-related decline in CMA to altered

senescent cell behaviour, impaired macrophage clearance, and increased tissue senescence burden. Particularly striking is the translational aspect of the paper showing that pharmacological CMA activation can reduce senescent cell load in aged mice and ameliorate fibrosis in a lung injury model. The study is ambitious, technically strong, and conceptually important, and it significantly advances our understanding of how proteostasis decline contributes to senescent cell persistence during ageing. Overall, I found this to be a highly compelling manuscript that should be of broad interest to the ageing and cell biology communities. Several modifications are requested to be clarified below in order to improve the manuscript and make it more accessible to the reader.

1. Fig. 1b: as the reporter indicates recruitment to the lysosome and not translocation/degradation, can the authors exclude the possibility that translocation and therefore CMA flux is actually increased in aged cells? Similarly, can the authors provide evidence that the reporter is expressed at a similar level in aged cells?
2. None of the representative images in Fig. 1 except for panel h demonstrate increased nuclear size in L2AKD cells reducing confidence in this phenotype. Additionally, some redouts are normalised in the quantification graphs which makes it difficult to assess the effectiveness of Palbo and L2AKD to induce individual phenotypes. Can the authors provide an explanation for performing normalisation?
3. There is a clear discrepancy between increased nuclear p21 in L2AKD cells (Fig. 1e) and lack of a difference at mRNA and protein level. Considering that p21 contributes significantly to the perceived different senescent properties of L2AKD cells (visualised in Fig. 1j), this conclusion could be a mis-interpretation. mRNA/protein levels should therefore not be used as independent criteria (as these do not explain increased nuclear p21). The authors should evaluate the proliferation rates of Palbo and L2AKD cells, for example using EdU incorporation. Based on nuclear p21, both interventions could result in cell cycle arrest, and therefore conclusions about the state of senescence of L2AKD cells might need to be re-evaluated.
4. While the proteome, metabolome and secretome data Fig 2/3 identify differences between L2AKD and control cells induced to senescence, functionally the secretome of L2AKD cells appears to be indistinguishable from that of senescent cells (Fig 3l,m). In the essence, L2A KD induces senescence (which is unsurprising given the importance of the CMA) and which is consistent with other conventional markers of senescence in L2AKD cells (Fig. 1).
5. Given lack of evidence for functional differences, the extremely dense and information-heavy description of Fig 2/3 (it would be very unlikely that an average reader will be able to penetrate it) should be simplified, and majority of the data relegated to supplement where it can serve as a resource to the community. At the same time the authors should re-focus the narrative and concentrate on the key observations.
6. Page 22: "As in the case of CMA-deficient fibroblasts, although L2A-/- BMDM were not senescent, they displayed elevated p21 protein levels without transcriptional upregulation." What is the conclusion that FB and BMDM are not senescent based upon? There is no data for BMDMs and FB clearly show markers of senescence. The authors suggest these markers are more akin to aging than senescence, but the current data makes it difficult to clearly separate two phenomena.
7. On a more conceptual note, do authors propose that aging and senescence are completely different or only distinguished by a degree by which the same program is manifested? Perhaps clarifying this question would help with providing a clearer narrative.
8. Minor: Extended Data Fig 10 is discussed before 9i-k.
9. Minor: several instances of typographical and grammatical errors should be corrected.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In the revised manuscript, the authors have fully addressed all reviewer concerns and comments. The manuscript has been improved and is now suitable for publication.

Reviewer #2

(Remarks to the Author)

The authors have performed additional studies and fully addressed my concerns. Thank you.

Reviewer #3

(Remarks to the Author)

The authors addressed my criticisms in full and the manuscript can be recommended for publication.

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Response to reviewers' comments

Reviewer #1 (Remarks to the Author)

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Overall, the study is well-designed and performed and the phenotypic data is robustly supported by the mechanistic studies that underly the role of CMA changes in macrophage senescence and tissue dysfunction. A few points should be addressed to improve the manuscript for clarity and implications. Therefore, I am recommending revisions.

Authors' Response (AR): We thank the reviewer for their very positive comments on our work and for their helpful suggestions to strengthen our manuscript. We have incorporated all of the requested changes into the revised version of the manuscript.

Major comment:

1. The authors robustly explore changes in CMA on macrophage phagocytosis and its role in aging, however macrophages play a very important role in the clearance of dead cells via efferocytosis. This process yields an anti-inflammatory response that creates a positive environment for tissue regeneration and repair. The effect of impaired CMA in efferocytosis should be explored to understand how much of role this contributes to the tissue effects in the constitutive or myeloid-specific LAMP2 KO mice.

AR: We thank the reviewer for bringing up this very interesting point. In this revised version, we investigated the effect of CMA deficiency on macrophage efferocytosis by performing a flow cytometry-based efferocytosis assay. Following the induction of cell death in control and senescent fibroblasts with staurosporine, we observed a striking reduction in the efferocytic capacity of CMA-deficient macrophages compared with control macrophages. These results provide direct experimental evidence supporting the mechanism underlying the increased number of TUNEL-positive cells observed in vivo in the macrophage-specific LAMP2A knockout mouse.

- These new data are included in **Fig. 4e** and described on **page 23**.

2. The p16 antibody used is reported to be human specific and was generated on a sequence from the C-terminal of p16 which does not share significant homology with the mouse protein. Has this antibody been validated for mouse use, or could some preliminary testing with another mouse-specific antibody be used reconfirm the data in Extended Data Figure 2A?

AR: We apologize for the confusion. We listed the p16 antibody used for the human lung samples but inadvertently omitted the antibody used for the mouse fibroblasts. We have now corrected the Methods section to indicate that we used ThermoFisher Antibody (MA5-17142) which recognizes both human and mouse. However, we agree with the reviewer on the value of confirming these findings with a second antibody, and to that effect we have included an immunoblot using a different anti-p16 antibody from Cell Signaling (used in mouse samples in multiple publications) [1-5], which confirms the increase in p16 protein levels in the L2AKD cells.

- These new data are included in **Extended Data Fig. 2b** and described on **page 10**.

3. In Figure 5, the authors show increased senescence in adipose, liver and skin. Were other parenchymal (especially lung), lymphoid (spleen) or brain tissues examined for senescence in ML2A KO mice (either by SABGal, p21 protein levels of p16/p21 transcript)?

AR: Thank you for this suggestion. We have now examined senescence markers in both the lungs and brains of ML2A^{KO} mice, and we agree with the reviewer that these new data strengthen our conclusion that these mice exhibit an increased senescence burden.

- The new data from the lung and brain, using the same assays performed in the other tissues, are presented in **Fig. 5d, e, h, i** and **Extended Data Fig. 9a, b** and are described on **page 26**.

4. In Figure 6, CA77.1 treatment was capable of modestly reducing SABgal in WAT and liver but had a much stronger effect in the lungs of naturally aged mice. Similar changes were found for senescence and SASP transcripts. Would this be due to inherent pharmacology and tissue distribution of CA77.1 (if that data even exists) or does it speak to tissue-specific CMA levels and their importance?

AR: We thank the reviewer for raising this interesting point, which we have expanded upon in the Discussion. Given the lipophilic properties of CA77.1, we would not expect reduced bioavailability in adipose tissue. Although accelerated hepatic clearance could contribute to the lower efficacy observed in the liver, our analysis of CA77.1-induced CMA activation across multiple organs supports comparable tissue biodistribution.

Following the reviewer's suggestion, we compared basal CMA activity and age-related changes in CMA activity in each tissue with the changes in senescence markers observed across these tissues

in response to drug treatment (Reviewer Fig. 1). Among the parameters examined, basal CMA activity emerged as the strongest predictor of CA77.1 efficacy in both sexes, although the correlation was stronger in females: the higher the basal CMA activity in a tissue, the more pronounced the effect of the drug in reducing senescence markers (Reviewer Fig. 1a). In contrast, tissue-specific age-related changes in CMA activity correlated with the effects of CA77.1 on senescence markers only in females: the more pronounced the age-related decrease in CMA activity in an organ, the greater the effect of the drug in reducing senescence markers (Reviewer Fig. 1b). Although these correlations are interesting and may provide a plausible explanation for the tissue-specific effects of CA77.1 on senescence, we believe that extensive additional experimentation would be required to support these conclusions. Therefore, we consider it more appropriate not to include this data in the current manuscript.

- We have added this interesting observation and plausible explanation on **page 36** of the discussion.

5. In Figure 7, CA77.1 treatment reduced macrophage infiltration in the murine IPF model. Meanwhile treatment appeared to T cell infiltration. Is it possible to know if this is a generalized recruitment or T cells or potentially a more specific recruitment of Tregs to dampen or resolve the immune response? (CD3 vs FoxP3 staining or expression in lung tissue)

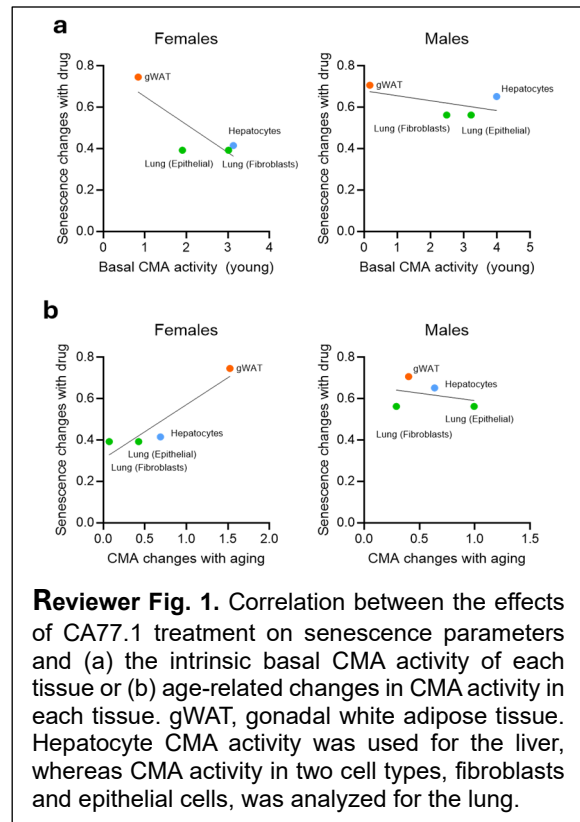
AR: Motivated by the reviewer's question, we performed additional immunohistochemical staining to identify the T cell subsets contributing to the changes previously observed using the pan-T cell marker CD3. Specifically, we stained lung sections for CD4, CD8, and Foxp3 (as a marker of regulatory T cells, Tregs) and found distinct recruitment kinetics for the different T cell subpopulations. These new data support an early CA77.1-mediated recruitment of Tregs following bleomycin injury (by day 4), whereas the increased T cell infiltration observed by day 7 was primarily attributable to CD4⁺ T cells.

- The new data is shown in **Fig. 7p** and **Extended Data Fig. 12l, m** and described on **pages 33 and 34**.

Minor comment:

1. The fat tissue is labeled as epididymal (eWAT) even in female mice (Extended Data Figure 6a). It may be more accurate to label as gonadal (gWAT).

AR: Thank you for pointing out this error. We have corrected all instances throughout the manuscript in which eWAT was incorrectly labeled as gWAT.



Reviewer #2 (Remarks to the Author)

This is an important and well done study linking the age-related decline in chaperone-mediated autophagy (CMA) to the persistence of senescent cells in vivo. The study gets at the fundamental issue of why senescent cells persist in aged animals but are cleared in young animals. The findings demonstrate that reduced CMA in senescent cells alters their SASP, which then suppresses macrophage CMA and phagocytic function, leading to impaired clearance of the senescent cells. Extensive in vitro and in vivo data are presented to support the conclusions of the paper.

I have the following points for the authors to consider:

Authors' Response (AR): We thank the reviewer for highlighting the importance of our study. We have incorporated all of the reviewer's suggestions into the revised manuscript, including additional experiments that we believe address the points raised.

1. The impairment of phagocytosis by the secretome of senescent fibroblasts, and CMA-deficient fibroblasts in particular, is a crucial finding. However, it is unclear how much of the impairment in phagocytosis in macrophages is due to impaired CMA in the macrophages or due to other changes related to the secretome. The findings would be further supported if the defect in phagocytosis could be reversed by restoring CMA in the macrophages exposed to the secretome of senescent fibroblasts or CMA-deficient fibroblasts. Whether the restoration of phagocytosis in this setting is partial or complete would help clarify this issue.

AR: This was an excellent idea. We have performed the proposed experiment by restoring CMA activity in macrophages exposed to the senescent secretome using the CMA activator. We observed a partial restoration of phagocytic activity, supporting the hypothesis that the observed effect is, at least in part, due to impaired CMA in macrophages.

- The new data are presented in **Fig. 4h** and described on **pages 23 and 24**.

2. In the senescence field, palbociclib is one of the lessor used agents to induce senescence. Although the authors present some data using other inducers, it is fairly limited. It would help if the key finding – i.e., impairment of macrophage phagocytosis by the secretome of senescent fibroblasts, could be replicated using etoposide or another senescence inducer.

AR: We agree with the reviewer that Palbociclib is less commonly used than other senescence-inducing agents. Our primary rationale for using Palbociclib was to avoid the transient upregulation of CMA that occurs in response to DNA damage or oxidative stress induced by other senescence-inducing stimuli. However, this concern does not apply to the phagocytosis experiments requested by the reviewer, as these experiments required only the secretome collected after senescence had been established.

Accordingly, we have now included data using the secretome from Etoposide-induced senescent cells, which confirms a similar reduction in macrophage phagocytic activity (Fig. 4g). In addition, we found that the secretome from Etoposide-induced, CMA-deficient senescent cells had an even stronger effect on macrophage cell death than the secretome from Palbociclib-induced senescent cells (Extended Data Fig. 8d).

- These new data are presented in **Fig. 4g** and **Extended Data Fig. 8d** and are described on **pages 23 and 24**. We have also included a rationale for the preference for palbociclib as the starting experiment on **page 10**, in addition to noting this limitation in the “limitations of the study” section on **page 39**.

3. The human data are mainly correlative and do not directly validate the proposed model described above. This should be clearly acknowledged in the Discussion.

AR: We appreciate this comment and agree that the human data showing age-related changes in the CMA score are correlative in nature. We have now acknowledged this as a limitation in the revised manuscript. However, we remain encouraged by the changes in lysosomal LAMP2A levels observed in human lung samples, as they reveal a previously unrecognized instability of the CMA receptor in the context of idiopathic pulmonary fibrosis. We hope these findings will stimulate future studies aimed at elucidating the mechanisms underlying this instability.

- We have included the current limitation of the human data in the “limitations of the study” section on **page 39**).

4. The proteomic and metabolomic sections are quite dense. Is there a way to simplify these to some degree? That would make the paper easier to read.

AR: We thank the reviewer for this comment. We consider the proteomic and metabolomic datasets to be valuable resources for the senescence research community. However, we agree that this section was overly dense. We have therefore simplified the main figures to highlight the key findings and improve readability. Briefly, Fig. 2 contains now the key data regarding the basal and senescence-induced changes in total proteome and lysosomal degradation (panels a-g) and all the additional analysis have been moved to Extended Data Figs. 3 and 4. Similarly, we have reduced the number of panels dedicated to the proteomic analysis of the secretomes in Fig. 3a-g, moving the additional analysis for those readers interested in more detail, to Extended Data Figs. 6 and 7.

- Changes have been introduced in the organization of **Figs. 2 and 3** and **Extended Data Figs. 3, 4, 6 and 7**. We have also shortened the corresponding description in the main text.

5. Given that loss of CMA by itself does not cause senescence, the authors should probably not refer to their secretome as a SASP.

AR: This is a very reasonable point, and we agree that we should have used the term "secretome" instead of "SASP" in this context.

- We have carefully revised the manuscript accordingly and now use the term "SASP" only when referring specifically to senescent cells.

6. The section on paracrine senescence (Fig 3n, Ex Data Fig 6k) could be strengthened. A more rigorous assay beyond SA-bGal should be used (e.g., TAF) to establish a senescent phenotype of the cells exposed to the secretome of the senescent cells.

AR: We are pleased by the reviewer's interest in the paracrine senescence phenotype, which we also consider an important contribution of this work. To further strengthen this aspect of the study, we performed two additional assays demonstrating that the secretome from CMA-deficient cells increases nuclear p21 levels (Fig. 3k) and decreases nuclear HMGB1 levels (Fig. 3l).

- These new data are presented in **Fig. 3k,l** and described on **page 20**.

Reviewer #3 (Remarks to the Author):

This is a genuinely impressive and very substantial piece of work that addresses an important question at the intersection of ageing, autophagy and immune surveillance. The authors combine a wide range of complementary approaches, including primary cells from young and old animals, sophisticated proteomic and metabolomic analyses, genetically modified mouse models, macrophage-specific interventions, and pharmacological activation studies in vivo. Although the scope is very broad, there is a clear mechanistic thread throughout the manuscript, linking age-related decline in CMA to altered senescent cell behaviour, impaired macrophage clearance, and increased tissue senescence burden. Particularly striking is the translational aspect of the paper

showing that pharmacological CMA activation can reduce senescent cell load in aged mice and ameliorate fibrosis in a lung injury model. The study is ambitious, technically strong, and conceptually important, and it significantly advances our understanding of how proteostasis decline contributes to senescent cell persistence during ageing. Overall, I found this to be a highly compelling manuscript that should be of broad interest to the ageing and cell biology communities. Several modifications are requested to be clarified below in order to improve the manuscript and make it more accessible to the reader.

There are some issues that need to be addressed

Authors' Response (AR): We greatly appreciate the reviewer's positive remarks regarding our study and the quality of the data and their presentation. We also thank the reviewer for their valuable suggestions to make the manuscript more accessible to readers. We have fully implemented these suggestions in the revised version of the manuscript.

1. Fig. 1b: as the reporter indicates recruitment to the lysosome and not translocation/degradation, can the authors exclude the possibility that translocation and therefore CMA flux is actually increased in aged cells? Similarly, can the authors provide evidence that the reporter is expressed at a similar level in aged cells?

AR: We thank the reviewer for bringing up this important point. To directly assess CMA-mediated internalization and degradation of the reporter in aged cells, we incubated cells in the presence or absence of lysosomal proteolysis inhibitors (ammonium chloride and leupeptin (N/L)) and quantified Dendra-positive puncta following senescence induction. These experiments demonstrate reduced degradation of the Dendra reporter in aged cells under both basal conditions and following senescence induction. Furthermore, to determine whether differences in reporter expression could account for these findings, we measured Dendra mRNA levels by qPCR and found no significant differences in Dendra expression between cells from young and old mice.

- The new data analyzing reporter degradation are presented in **Extended Data Fig. 1e**, and the analysis of Dendra expression is shown in **Extended Data Fig. 1d**. These results are described on **page 8**.

2. None of the representative images in Fig. 1 except for panel h demonstrate increased nuclear size in L2AKD cells reducing confidence in this phenotype. Additionally, some readouts are normalised in the quantification graphs which makes it difficult to assess the effectiveness of Palbo and L2AKD to induce individual phenotypes. Can the authors provide an explanation for performing normalisation?

AR: We have included more representative images of the changes in nuclear size that we observe in L2AKD cells, but agree with the reviewer on the heterogeneity of the nuclear size phenotype. Consequently, in the revised version, we have focused primarily on nuclear indentation to complement the other senescence readouts. We have also clarified the rationale for the normalization of the data. Briefly, because the parameters analyzed are expressed in different units, normalization to the non-senescent control cells provided a more appropriate way to compare the magnitude of the changes observed in the L2A^{KD} cells across the different senescence markers.

- This explanation has now been included on **page 9**.

3. There is a clear discrepancy between increased nuclear p21 in L2AKD cells (Fig. 1e) and lack of a difference at mRNA and protein level. Considering that p21 contributes significantly to the perceived different senescent properties of L2AKD cells (visualised in Fig. 1j), this conclusion could be a mis-interpretation. mRNA/protein levels should therefore not be used as independent criteria (as these do not explain increased nuclear p21). The authors should evaluate the proliferation rates of Palbo and L2AKD cells, for example using EdU incorporation. Based on nuclear p21, both interventions could result in cell cycle arrest, and therefore conclusions about the state of senescence of L2AKD

cells might need to be re-evaluated.

AR: We thank the reviewer for these insightful comments and for the opportunity to clarify this point. We have modified the main figure to better illustrate the discrepancy between the changes in p16 and p53 mRNA and protein levels in the L2A^{KD} cells (new Fig. 1j shows now side-by-side mRNA and protein levels). In the case of p21, as pointed out by the reviewer, we did not observe differences in either mRNA or total protein levels between the two genotypes. However, we detected a significant re-localization of p21 from the cytosol to the nucleus. To remain agnostic regarding the contribution of the different markers to the PCA analysis, we believe it was important to include both mRNA and protein measurements for all the markers analyzed. This is particularly relevant because, although p21 mRNA and protein levels were comparable between control and L2A^{KD} cells, p16 and p53 exhibited marked genotype-dependent differences. As predicted by the reviewer, the PCA results did not change when p21 levels data were included or excluded, since p21 levels did not differ between Ctrl and L2A^{KD} cells. We hope that the reorganization of the data between the main and supplementary figures will further clarify this point.

Regarding the proliferation analysis, we performed the requested EdU incorporation experiment and found no significant differences in the basal proliferation rates of L2A^{KD} cells compared with control cells. As expected, induction of senescence resulted in a significant reduction in the percentage of EdU-positive cells in both genotypes. We thank the reviewer for suggesting this experiment, as it further strengthens our conclusion that the cells used in these studies had indeed undergone senescence.

- The new data with the EdU incorporation is shown in **Fig. 1i** and described in **page 9**. We have also updated the PCA data in **Fig. 1k** to incorporate the new proliferation data.

4. While the proteome, metabolome and secretome data Fig 2/3 identify differences between L2AKD and control cells induced to senescence, functionally the secretome of L2AKD cells appears to be indistinguishable from that of senescent cells (Fig 3l,m). In the essence, L2A KD induces senescence (which is unsurprising given the importance of the CMA) and which is consistent with other conventional markers of senescence in L2AKD cells (Fig. 1).

AR: We apologize for the confusion caused by the presentation of the secretome data in Fig. 3h (now Fig. 3f), which may have given the impression that the SASP and the secretome of CMA-deficient cells are identical. In that heat map (now on the right side of Fig. 3f), we plotted only the changes in the secretome of senescent control cells (SASP) that were recapitulated in the basal secretome of L2AKD cells. To better illustrate that this overlap is only partial, we have now included a complete heat map comparing the full secretome of senescent control cells (SASP) with the basal secretome of L2AKD cells.

- The new heat map is presented in **Fig. 3f** and described on **page 18**.

5. Given lack of evidence for functional differences, the extremely dense and information-heavy description of Fig 2/3 (it would be very unlikely that an average reader will be able to penetrate it) should be simplified, and majority of the data relegated to supplement where it can serve as a resource to the community. At the same time the authors should re-focus the narrative and concentrate on the key observations.

AR: We thank the reviewer for this comment. We consider the proteomic and metabolomic datasets to be valuable resources for the senescence research community. However, we agree that this section was overly dense. We have therefore simplified the main figures to highlight the key findings and improve readability. Briefly, Fig. 2 now contains the key data regarding basal and senescence-induced changes in the total proteome and lysosomal degradation (panels a-g), and all additional analyses have been moved to Extended Data Figs. 3 and 4. Similarly, we have reduced the number of panels dedicated to the proteomic analysis of the secretomes in Fig. 3a-g, moving the additional

analyses to Extended Data Figs. 6 and 7 for readers interested in further detail.

- Changes have been introduced in the organization of **Figs. 2 and 3** and **Extended Data Figs. 3, 4, 6 and 7**. We have also shortened the corresponding description in the main text.

6. Page 22: “As in the case of CMA-deficient fibroblasts, although L2A^{-/-} BMDM were not senescent, they displayed elevated p21 protein levels without transcriptional upregulation.” What is the conclusion that FB and BMDM are not senescent based upon? There is no data for BMDMs and FB clearly show markers of senescence. The authors suggest these markers are more akin to aging than senescence, but the current data makes it difficult to clearly separate two phenomena.

AR: Thank you for your comment, and we apologize that our previous description did not clearly convey this important point. Our data support the conclusion that loss of CMA in either fibroblasts or macrophages is not sufficient to drive cells into a fully senescent state. Indeed, only a small fraction of cells in the tissues of the L2AKO mouse models display a complete senescent phenotype. In fibroblasts, as shown in Fig. 1, CMA deficiency induces changes in SA-β-gal activity and HMGB1 localization that are comparable to those observed in senescent cells. However, these cells do not reach the very high levels of nuclear p21 or γH2AX staining characteristic of fully senescent cells and retain normal proliferative activity, rather than undergoing the proliferative arrest observed in senescent cells (Fig. 1i). Consistent with these observations, our proteomic analyses demonstrate that CMA-deficient cells recapitulate only a subset of the changes observed during senescence, explaining the intermediate phenotype identified by PCA (Fig. 1k).

We propose that this intermediate phenotype underlies the distinct behavior of CMA-deficient cells when they are subsequently induced to undergo senescence, a response that more closely resembles that of cells from aged organisms. Notably, when L2A^{KD} cells are induced to undergo senescence, they exhibit transcriptional activation of senescence-associated regulators comparable to that observed in control cells, suggesting that the transcriptional program driving senescence is largely preserved. In contrast, these cells display marked differences in their proteome, secretome, and metabolome. Our data suggest that the impaired protein homeostasis resulting from defective CMA, as further characterized by our proteomic analyses, creates a disconnect between transcriptional and protein-level changes. We propose that this dissociation underlies the distinct properties of CMA-competent and CMA-deficient cells as they engage the senescence program.

In the case of CMA-deficient macrophages, we have now performed a more comprehensive analysis of senescence markers (Extended Data Fig. 8g–k). Although L2A^{-/-} BMDMs showed trends toward increased SA-β-gal activity and reduced nuclear HMGB1 levels, they did not exhibit differences in p16 or p21 expression. However, as observed in fibroblasts, we detected changes at the protein level that we attribute to defective protein turnover. Studies of CMA-deficient macrophages in vivo also support the conclusion that CMA blockade does not induce full senescence in macrophages. For example, these cells did not display a transcriptional senescence signature (Fig. 5j) but instead acquired a partial senescence-like state, as indicated by the PCA in Fig. 5m.

- We have included analysis of additional senescent markers in **Fig. 1i** (for fibroblasts) and **Extended Data Fig. 8g–k** (for macrophages). We have updated the PCA analysis in **Fig. 1k** (for fibroblasts) and **Fig. 5m** (for macrophages) with the new additional data to better illustrate the differences between L2AKO cells and senescent cells. We have included an extended explanation of the findings on **page 9** (for fibroblasts) and **page 24** (for macrophages)

7. On a more conceptual note, do authors propose that aging and senescence are completely differing or only distinguished by a degree by which the same program is manifested? Perhaps clarifying this question would help with providing a clearer narrative.

AR: We thank the reviewer for giving us the opportunity to discuss this important point, which we feel is strongly supported by our data. Although the terms *aging* and *senescence* have sometimes been used interchangeably in the literature, they represent distinct biological processes. While the abundance of senescent cells increases with age, only a small fraction of cells in aged tissues are senescent in vivo. Our study highlights that the proteomic and metabolomic remodeling induced by pro-senescent stimuli differs markedly between young and aged cells.

Similarly, CMA deficiency does not recapitulate all of the changes observed during aging, as aging is a multifactorial process, and impaired autophagy and cellular senescence represent only two of its hallmarks. To better convey these distinctions, we have revised the text throughout the manuscript and expanded the Discussion to clarify these important concepts.

- The new discussion of this topic has been included on **page 36**.

Minor points

1. Extended Data Fig 10 is discussed before 9i-k.

AR: We opted for this figure organization to keep all of the single-cell RNA-seq data together within a single Extended Data Figure. However, in the flow of the Results section, the proliferation and cell death data are more naturally discussed after describing the RNA-seq findings. Because we have already reached the maximum number of Extended Data Figures permitted by the journal, and the editorial guidelines allow different panels of the same figure to be referenced at different points in the text, we have retained the current organization. If the manuscript is accepted, we will work with the editorial team to determine the most appropriate presentation of these data and, if possible, request an exception to the limit on the number of Extended Data Figures.

2. Several instances of typographical and grammatical errors should be corrected.

AR: We apologize for these errors. We have carefully revised the manuscript to correct typographical and grammatical errors throughout.

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