nature portfolio

Peer Review File

Peripheral positioning of lysosomes supports melanoma aggressiveness

Corresponding Author: Professor Jacky G. Goetz

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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)
In this revised manuscript, Jerabkova-Roda and colleagues investigate the role of peripheral lysosomal positioning in metastatic and invasive potential of melanoma cells. The authors show that lysosomes become more peripherally localized in more invasive patient-derived melanoma cells. Re-localization of lysosomes to perinuclear positions, using a chemical-genetic system (Rapalog-inducible), decreases the invasion of cells in culture and in two in vivo models of melanoma invasion, a mouse ear/skin model and a zebrafish model. This study supports a direct link between lysosome position, peripheral lysosome-mediated matrix degradation, and increased melanoma invasion.

In response to reviewer comments, the authors added significant new data and clarifications to improve the rigor of their study. This includes studies of additional patient-derived melanoma cell lines, an analysis of what controls lysosome positioning (KIF1B and KIF5B), an analysis of patient samples at various stages of melanoma alongside benign nevi and normal tissue, an in vivo mouse model of melanoma invasion, and a further control (FKBP only + Rapalog with spread lysosomes) in their zebrafish melanoma invasion assay. I am satisfied with the response the authors made to my specific concerns. I particularly appreciated further clarifications that were made with the results from the zebrafish model (Fig. 5D-H), which included adding small diagrams to indicate which conditions were tested (e.g., clustered lysosomes in WM983 FKBP-FRB + 5nM Rapalog). I also appreciated the new results in Figure 4D-E, analyzing 3D invasion of spread versus clustered lysosomes. Overall, this study should be of broad interest to readers of Nature Communications, especially those who are interested in lysosome functions, general organelle positioning in cells (including in cancer), and researchers who study melanoma.

I have a couple minor suggestions.
1. In the response to reviewers, the authors showed that the Rapolog-inducible repositioning of lysosomes does not affect other aspects of melanoma cell biology, including organization of microtubules, F-actin, early endosomes, mitochondria, etc. (shown in reviewer figures Re-1; Re-8). These are very nice controls that support the specificity of the lysosomal clustering experiments. If possible, these data could be placed in the supplemental materials.
2. In Figure 1F legend, the authors could describe the colors and outlines in the EM images (e.g., what the green circles are indicating in the zoomed-in region of the WM1862 cell).
3. The data in Figure 2A-C is convincing. However, I found it hard to see the peripheral lysosomes in Figure 2E (bottom panels). This may be due to the resolution of the images. The authors could either outline the cells or add arrows to indicate the peripheral versus perinuclear lysosomes.

Reviewer #2

(Remarks to the Author)
This manuscript is improved from the original submission. In particular, the relationship between lysosome positioning and invasive/metastatic potential has been strengthened. The chemo-genetic approach remains elegant. I also note that it has been transferred from Nature Cell Biology to Nature Communications. With some modest additional work, it could be

suitable for publication.

Specific comments
1. It is pleasing to see the analysis of kinesins; however, the authors need to complete the analysis by investigating the effect of Kif1b & Kif5b depletion on invasion and metastasis. At minimum, in vitro collagen invasion and invadopodia assays should be performed.
2. The in vivo analysis in Figure 5 seems to use only two mice per condition (unless I misunderstood the figure legend). This is not sufficient. Further mice should be added to the groups to demonstrate the robustness of the finding.

Reviewer #3

(Remarks to the Author)
I have reviewed the authors' revised manuscript. While there have been improvements, I still have concerns, particularly with the chosen model system and the lack of proper controls
Major concerns:
1) The authors make substantial claims about lysosome positioning and melanoma invasive capacity, which necessitates a reliable model system with appropriate controls. As I previously mentioned, I find the choice of the model system problematic. Although not explicitly stated in the Methods section, the rapalog used appears to be AP21967, which has been reported to interfere with mTORC1 activity (PMID 17350953). Since mTORC1 inhibition can activate mTORC2 through feedback loops (PMID 25323681), the potential effects on mTORC1/mTORC2 signaling must be thoroughly examined. However, this has not been adequately addressed in the revision.
-In Fig S4H, there appears to be a slight decrease in pS6K T389 at the 1-hour mark. I find the authors' quantifications unclear. The figure legend states that the data are based on individual experiments (n=4), represented by four dots, yet only two dots are clearly visible. It seems unlikely that the remaining two experiments produced identical results. Total S6K levels are not expected to change significantly within 1h of rapamycin treatment. Altogether, I don't find this data convincing.
-Assessing mTORC2 signaling is crucial, and p-AKT S473 is a suitable target. However, measuring phosphorylation changes by flow cytometry is not ideal. See PMID 31130364 for examples on how to measure p-AKT.
-How are lysosomes distributed in rapalog treated cells after division – is the perinuclear effect conserved? If not, will it affect the authors conclusions?
2) Given that the rapalog-FKBP12 system drives an artificial clustering of lysosomes, I believe some of the authors' conclusions should be validated using at least one alternative approach, to justify the authors claims. See PMID 31130364 for alternative methods to disturb lysosome peripheral positioning.
3) The authors need to clearly demonstrate that lysosome degradative function is not significantly impaired by lysosome clustering using their Rapalog-FKBP system. If lysosome function is heavily affected, it will be difficult to attribute the observed effects solely to changes in positioning. Some relevant data on this issue appear in Fig Re-1; however, this figure lacks important details, such as treatment duration, and if I understand correctly, it is not included in the main manuscript. Control experiments in vitro should align with the time frames used in vivo. Testing lysosome degradative function using methods in addition to lysotracker and lysosensor (for example MagicRed like the authors use elsewhere) is important.

3) MITF amplifications occur in 5-20% of melanomas (PMID 37400313), and activation of TFEB-related factors is known to drive changes in lysosome positioning and secretion (PMID 29146937, 21889421). While I understand the space constraints, I believe the manuscript would significantly benefit from addressing this important research area in some way within the text.

There seems to be an increase in total LAMP1 levels observed in Fig. 2 from patient biopsies – how can the authors differentiate between changes in levels and positioning?

The authors suggest that changes in KIF expression can drive alterations in lysosome positioning independently of MiTF/TFE factors, which presents a very interesting model. However, MiTF/TFE factor activity is highly regulated post-translationally. Therefore, assessing the activity of their downstream target genes would be more appropriate than measuring only the mRNA levels of the factors themselves, as was done in a few cell lines in Fig. Re5.

Additionally, MITF activation in melanoma has been reported to increase non-degradative endosomes (PMID 25605940), which should be considered when interpreting results obtained from lysophilic dyes.

4) "We now demonstrate that lysosomal peripheral distribution can be used as a proxy for melanoma metastatic dissemination. We further show that deeply invading cells away from the epidermis tend to display increased lysosome spreading as if such repositioning increased with the invasive properties of cells. This suggests that probing lysosomal positioning could allow to refine classification criteria for malignant and non-malignant cutaneous lesions." Unless the authors' findings are validated with additional patient material, I believe these statements should be tuned down.

Reviewer #4

(Remarks to the Author)
In this considerably revised manuscript, the authors provide a detailed response to the prior reviews from NCB. They have done an admirable job of addressing all of our prior comments. the data on KIF5 is especially welcome, as this provides a new mechanistic link that others in the field can follow up on. Moreover, the quality of the images, and a much better

explanation of their methods, is welcome. the addition of new models as well as patient samples further augments the importance of the findings. Overall, I feel satisfied at the responses they have provided and the improvements to the manuscript.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have addressed my remaining concerns and I happily recommend publication of this interesting study.

Reviewer #3

(Remarks to the Author)
The authors have addressed my concerns thoroughly and have significantly enhanced the presentation of their important and interesting findings

Reviewer #5

(Remarks to the Author)
In this study, the authors performed bottom-up proteomics analysis on the supernatant of WM983B cells with and without Rapalog treatment. This part of the work has a reasonable experimental design and the data is complete. However, to enhance the reliability and reproducibility of the results, the following aspects should be addressed:
1. The authors cite the SP3 method for protein extraction and digestion but should briefly provide more details regarding the specific chemicals used, their concentrations, the time of protein digestion, and any additional steps in the protocol.".
2. Further details on the mass spectrometry (MS) and tandem mass spectrometry (MS/MS) parameters should be included. Specifically, the mass range for data acquisition, scan rate, and other relevant settings.
3. The manuscript should include a more detailed description of the protein database search parameters, such as peptide length restrictions, precursor and fragment mass tolerances.
4. The number of biological replicates used in this experiment should be clearly stated. Additionally, to assess data quality, the authors should consider including common quality control figures for bottom-up proteomics, such as Log2 Normalized LFQ distributions, and coefficient of variation (CV) plots.

Point-by-point response for our manuscript NCOMMS-24-57679-T and entitled "Peripheral positioning of lysosomes supports melanoma aggressiveness"

By Katerina Jerabkova-Roda[1-6✉], Marina Peralta[1-5$£], Kuang-Jing Huang[1-5£] , Clara Bourgeat Maudru[1-5£], Louis Bochler[1-5£], Antoine Mousson[3,7£], Ignacio Busnelli[1-5], Rabia Karali[3,7], Hélène Justiniano[3,7], Lucian-Mihai Lisii[3,7], Philippe Carl[3,7], Vincent Mittelheisser[1-5], Nandini Asokan[1-5], Annabel Larnicol[1-5], Olivier Lefebvre[1-5], Hugo Lachuer[6,8#], Angélique Pichot[2,3,4,9], Tristan Stemmelen[2,3,4,9], Anne Molitor[2,3,4,9,10], Léa Scheid[12], Quentin Frenger[2-4], Frédéric Gros[2-4], Aurélie Hirschler[11], François Delalande[11], Emilie Sick[3,7], Raphaël Carapito[2,3,4,9,10], Christine Carapito[11], Dan Lipsker[12], Kristine Schauer*[6,8✉], Philippe Rondé*[3,7✉], Vincent Hyenne*[1-5,13✉], Jacky G.Goetz*[1-5✉]

First of all, we would like to thank the reviewers for their positive appreciation and useful questions and suggestions upon revision of our manuscript to ***Nature Communications***. We provide here a **point-by-point response** to the additional comments the reviewers have raised. **Our responses appear in *blue*** in this document. Corresponding changes have been made accordingly in the revised manuscript and highlighted in yellow. These changes have led to new figure panels which are the following: **Fig.5C, Fig.S3H, Fig.S4H-J, Fig.S6A.**

Reviewer's Comments:

Reviewer #1 (Remarks to the Author)

In this revised manuscript, Jerabkova-Roda and colleagues investigate the role of peripheral lysosomal positioning in metastatic and invasive potential of melanoma cells. The authors show that lysosomes become more peripherally localized in more invasive patient-derived melanoma cells. Re-localization of lysosomes to perinuclear positions, using a chemical-genetic system (Rapalog-inducible), decreases the invasion of cells in culture and in two in vivo models of melanoma invasion, a mouse ear/skin model and a zebrafish model. This study supports a direct link between lysosome position, peripheral lysosome-mediated matrix degradation, and increased melanoma invasion.

In response to reviewer comments, the authors added significant new data and clarifications to improve the rigor of their study. This includes studies of additional patient-derived melanoma cell lines, an analysis of what controls lysosome positioning (KIF1B and KIF5B), an analysis of patient samples at various stages of melanoma alongside benign nevi and normal tissue, an in vivo mouse model of melanoma invasion, and a further control (FKBP only + Rapalog with spread lysosomes) in their zebrafish melanoma invasion assay. I am satisfied with the response the authors made to my specific concerns. I particularly appreciated further clarifications that were made with the results from the zebrafish model (Fig. 5D-H), which included adding small diagrams to indicate which conditions were tested (e.g., clustered lysosomes in WM983 FKBP-FRB + 5nM Rapalog). I also appreciated the new results in Figure 4D-E, analyzing 3D invasion of spread versus clustered lysosomes. Overall, this study should be of broad interest to readers of Nature Communications, especially those who

are interested in lysosome functions, general organelle positioning in cells (including in cancer), and researchers who study melanoma.

I have a couple minor suggestions.

1. In the response to reviewers, the authors showed that the Rapolog-inducible repositioning of lysosomes does not affect other aspects of melanoma cell biology, including organization of microtubules, F-actin, early endosomes, mitochondria, etc. (shown in reviewer figures Re-1; Re-8). These are very nice controls that support the specificity of the lysosomal clustering experiments. If possible, these data could be placed in the supplemental materials.

Re: We would like to thank the reviewer for their positive appreciation of our work and for their suggestions. Based on these, we have decided to include a deeper analysis of the consequences of lysosome re-positioning on lysosome function. We now show that lysosome mass and acidity are not significantly perturbed by Rapalog-induced clustering, or by Rapalog treatment alone (as an additional control). These results are now discussed in the results section “Forcing lysosome perinuclear clustering in metastatic melanoma cells” and included in Figure S4J. While we faced technical limitations (fluorochrome overlap between dyes and our FKBP constructs) that prevented us from assessing the degradative capacities of lysosomes in the context of rapalog-induced lysosome re-positioning (please see Response to Reviewer 3.3), we circumvented such issue by evaluating Magic Red levels in cells depleted for KIF1B or KIF5B. Doing so, we observed that the degradative capacities of lysosomes are maintained upon clustering (Fig Re1).

While we agree that these data (in addition to the description of additional compartments) further support the specificity of the lysosomal clustering methods, they have opened additional avenues of research and set the grounds of an independent and follow-up study. As a consequence, we beg the understanding of this reviewer (and the editor) as we wish to keep these data and thus not include them.

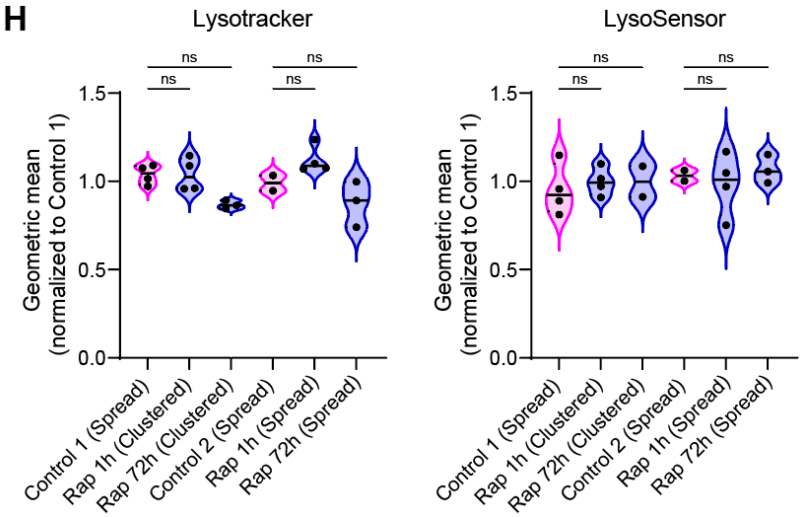


Fig S4, new panel H

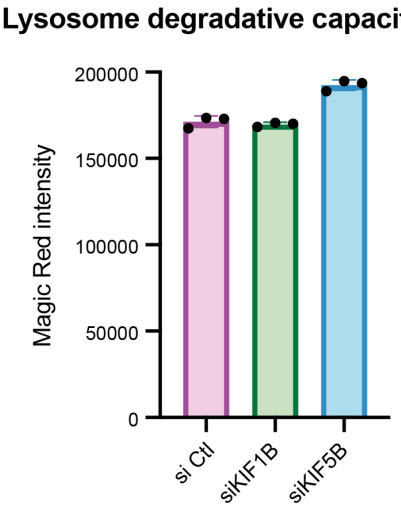


Fig Re-1 Lysosome degradative capacity (MagicRed) in WM983B cells is preserved upon lysosome clustering by KIF1B/5B silencing.

2. In Figure 1F legend, the authors could describe the colors and outlines in the EM images (e.g., what the green circles are indicating in the zoomed-in region of the WM1862 cell).

Re: We have now improved the legend of Figure 1F: "endolysosome-rich regions in protrusions are circled in dashed lines, color-coded per melanoma stage".

3. The data in Figure 2A-C is convincing. However, I found it hard to see the peripheral lysosomes in Figure 2E (bottom panels). This may be due to the resolution of the images. The authors could either outline the cells or add arrows to indicate the peripheral versus perinuclear lysosomes.

Re: To help the readers and as recommended by this reviewer, we have now added red arrows pointing to clustered lysosomes in Figure 2E.

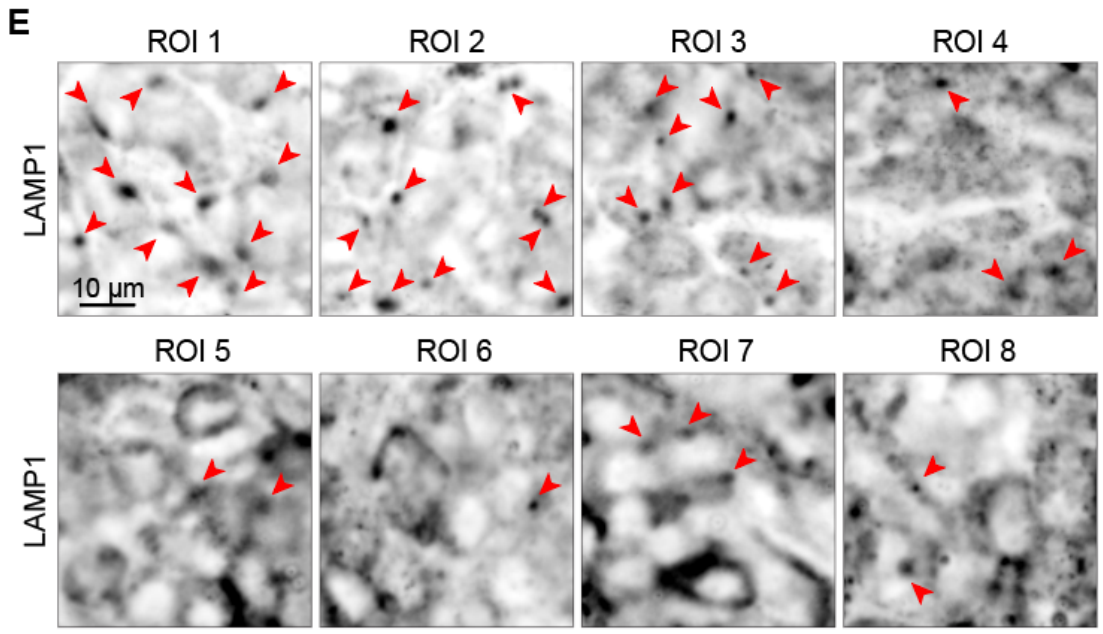


Fig 2, Updated panel E

Reviewer #2 (Remarks to the Author):

This manuscript is improved from the original submission. In particular, the relationship between lysosome positioning and invasive/metastatic potential has been strengthened. The chemo-genetic approach remains elegant. I also note that it has been transferred from Nature Cell Biology to Nature Communications. With some modest additional work, it could be suitable for publication.

We thank this reviewer for their positive feedback and we have now performed additional work based on their comments.

Specific comments

1. It is pleasing to see the analysis of kinesins; however, the authors need to complete the analysis by investigating the effect of Kif1b & Kif5b depletion on invasion and metastasis. At minimum, in vitro collagen invasion and invadopodia assays should be performed.

Re: We appreciate that this reviewer is now convinced by the additional data based on KIF1B and KIF5B depletion. We have opted for testing the functional impact of such depletion on invasion of melanoma cells (and thus opted for not testing invadopodia as these are linked) using our collagen invasion assay. As shown below (and now added to the manuscript), cells depleted for either KIF1B or KIF5B show significantly reduced invasion abilities. These results confirm the importance of lysosome positioning in tumor cell invasion and are now discussed in the results section "Forcing lysosomal clustering impairs invasion potential of melanoma cells" and are included in Supplementary Figure S3H. While we believe that these data firmly demonstrate the link between lysosome positioning and invasion of melanoma cells, we have further strengthened our *in vivo* data (described below) allowing us to consolidate the importance of lysosome positioning for melanoma cell invasion.

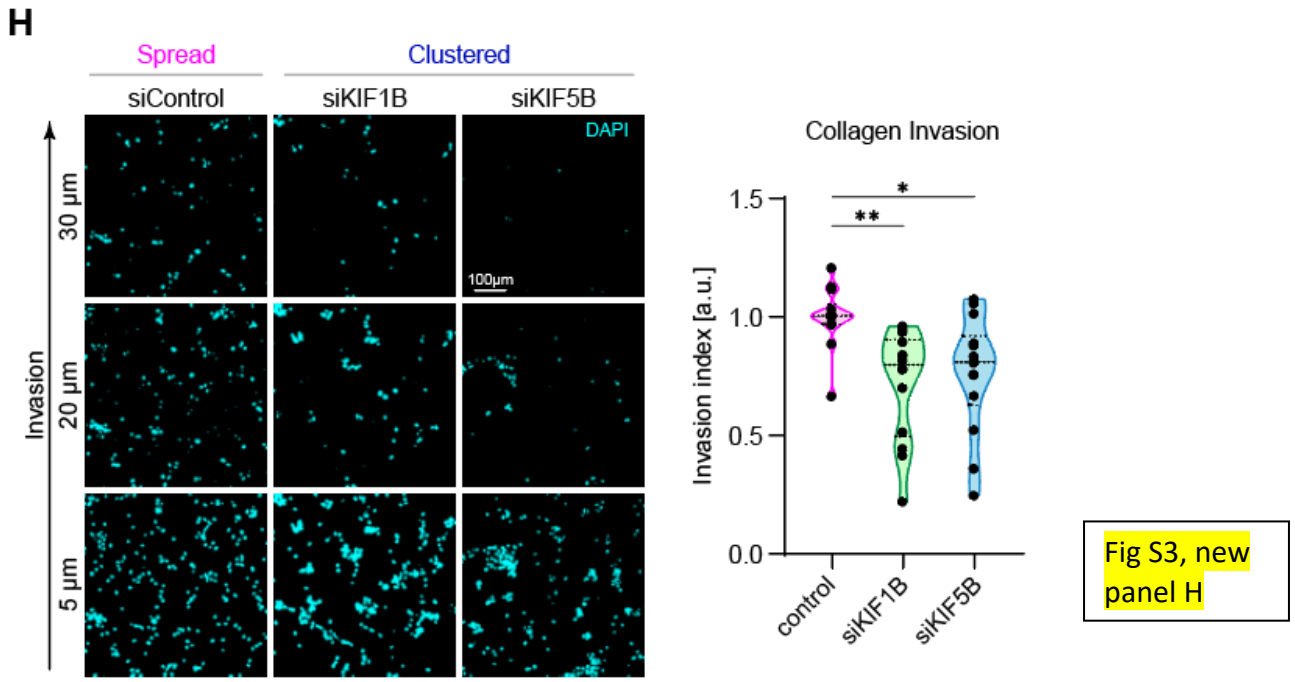


Fig S3, new panel H

2. The in vivo analysis in Figure 5 seems to use only two mice per condition (unless I misunderstood the figure legend). This is not sufficient. Further mice should be added to the groups to demonstrate the robustness of the finding.

Re: We apologize for the low statistical power of the initial data as these were technically-challenging experiments. In order to strengthen our data, we have now added additional mice to this *in vivo* study, with results that include n=3 mice and 4 tumors per group. These new data are now merged with the previous ones in the Figure 5C and Figure S6A.

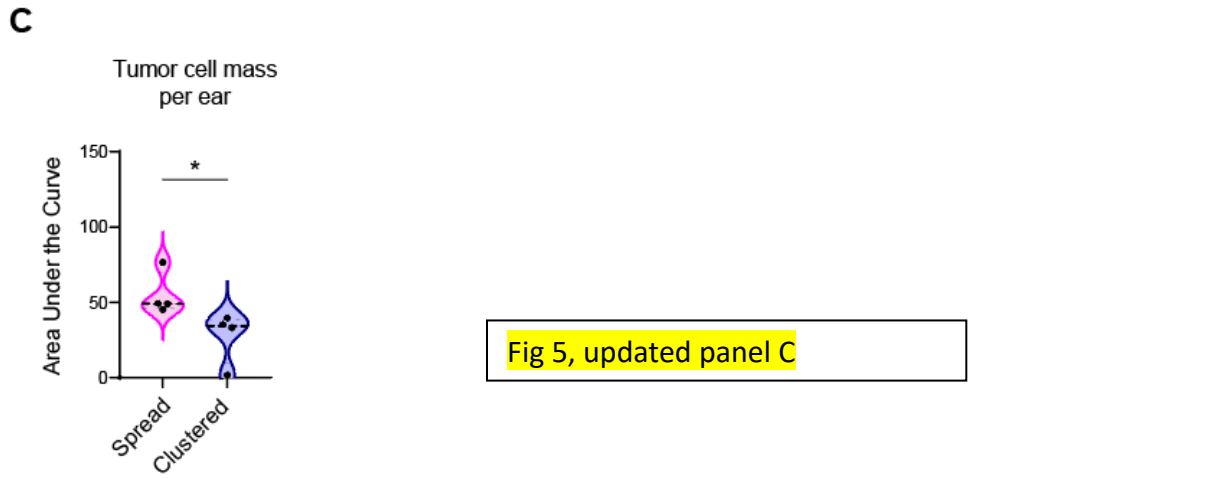


Fig 5, updated panel C

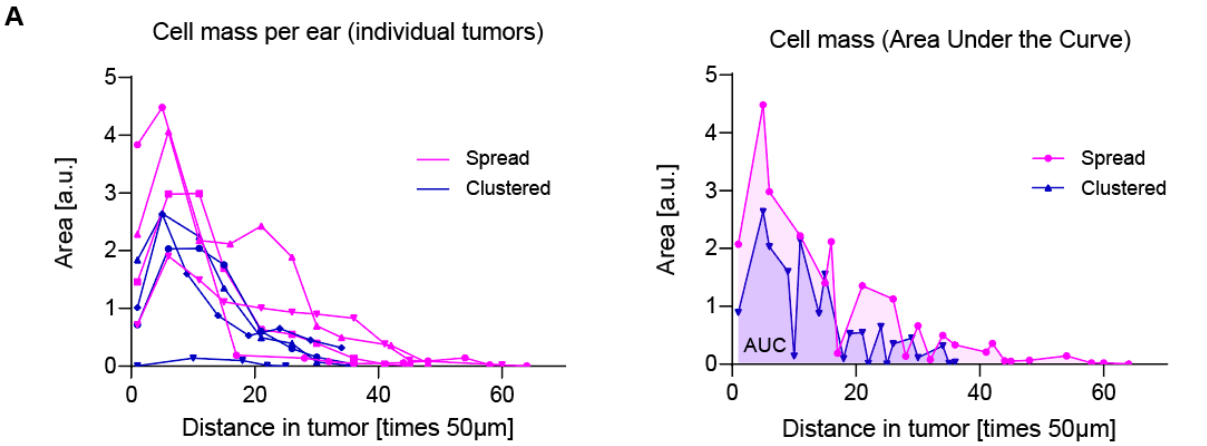


Fig S6, updated panel A

Reviewer #3 (Remarks to the Author)

I have reviewed the authors' revised manuscript. While there have been improvements, I still have concerns, particularly with the chosen model system and the lack of proper controls
Major concerns:

1) The authors make substantial claims about lysosome positioning and melanoma invasive capacity, which necessitates a reliable model system with appropriate controls. As I previously mentioned, I find the choice of the model system problematic. Although

not explicitly stated in the Methods section, the rapalog used appears to be AP21967, which has been reported to interfere with mTORC1 activity (PMID 17350953). Since mTORC1 inhibition can activate mTORC2 through feedback loops (PMID 25323681), the potential effects on mTORC1/mTORC2 signaling must be thoroughly examined. However, this has not been adequately addressed in the revision.
-In Fig S4H, there appears to be a slight decrease in pS6K T389 at the 1-hour mark. I find the authors' quantifications unclear. The figure legend states that the data are based on individual experiments (n=4), represented by four dots, yet only two dots are clearly visible. It seems unlikely that the remaining two experiments produced identical results. Total S6K levels are not expected to change significantly within 1h of rapamycin treatment. Altogether, I don't find this data convincing.

Re: To strengthen our results, we have added a supplementary experiment aiming at measuring mTORC1 activity. Consistently with previous experiments, we found that Rapalog does not affect the levels of p-4EBP1 nor those of p-P70S6K. We now have five independent experiments showing that Rapalog does not interfere with mTORC1 activity in our melanoma cells, by contrast to rapamycin (n=5 for Rapalog, n=4 for rapamycin). These new data are now merged with the previous ones in the Fig S4J. Earlier and current quantification had been performed by measuring the intensity of the band of the phosphorylated form over the total protein intensity, as it is classically performed. We have now improved the readability of the graphs, by changing their format as well as the dots' size to avoid any confusion with regards to the number of independent experiments that are reported as shown below. In the Methods section, we now explicitly state : Rapalog (A/C Heterodimerizer ref. n°635057, from Takara Bio Inc., also known as AP21967.

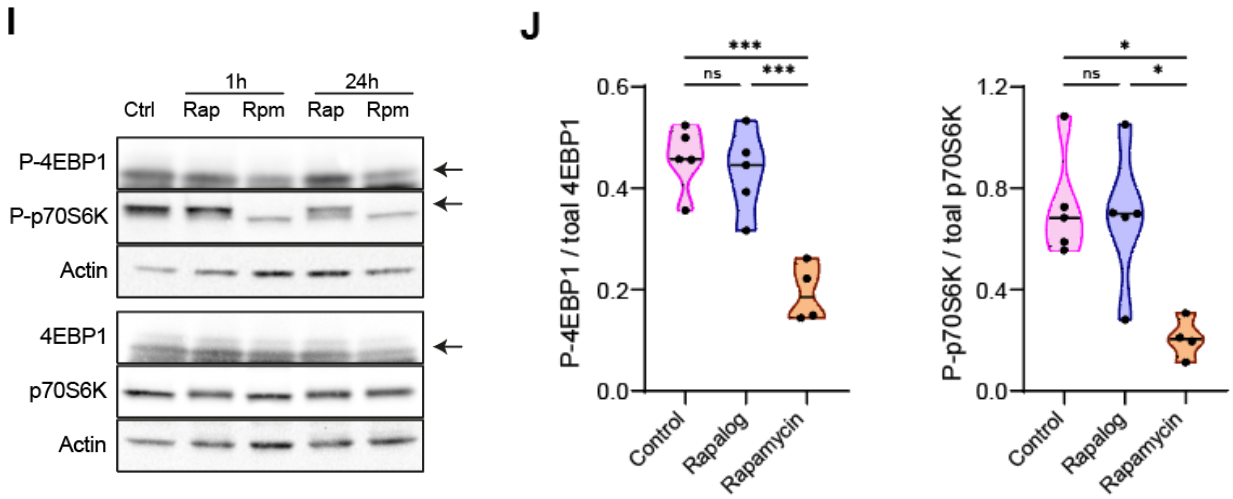


Fig S4, updated panel I-J

-Assessing mTORC2 signaling is crucial, and p-AKT S473 is a suitable target. However, measuring phosphorylation changes by flow cytometry is not ideal. See PMID 31130364 for examples on how to measure p-AKT.

Re: To complement our cytometry analysis, we quantified the levels of p-AKT s473 by western blot (Figure **Re-2**). These new experiments show that Rapalog does not alter

p-AKT levels at 1h and 24h. Together, the cytometry and western blots approaches show that mTORC2 activity is not affected by Rapalog in our system.

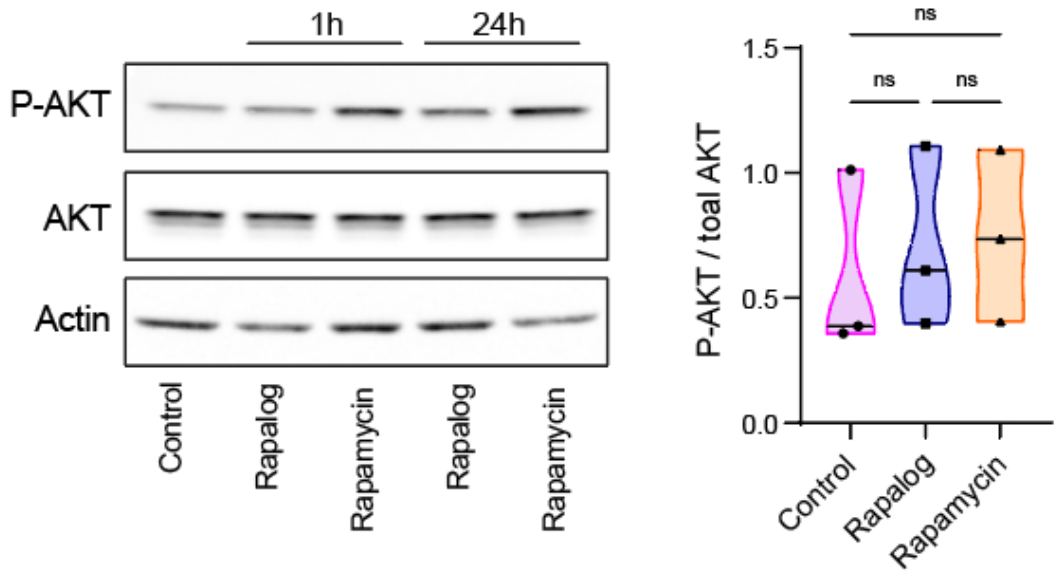
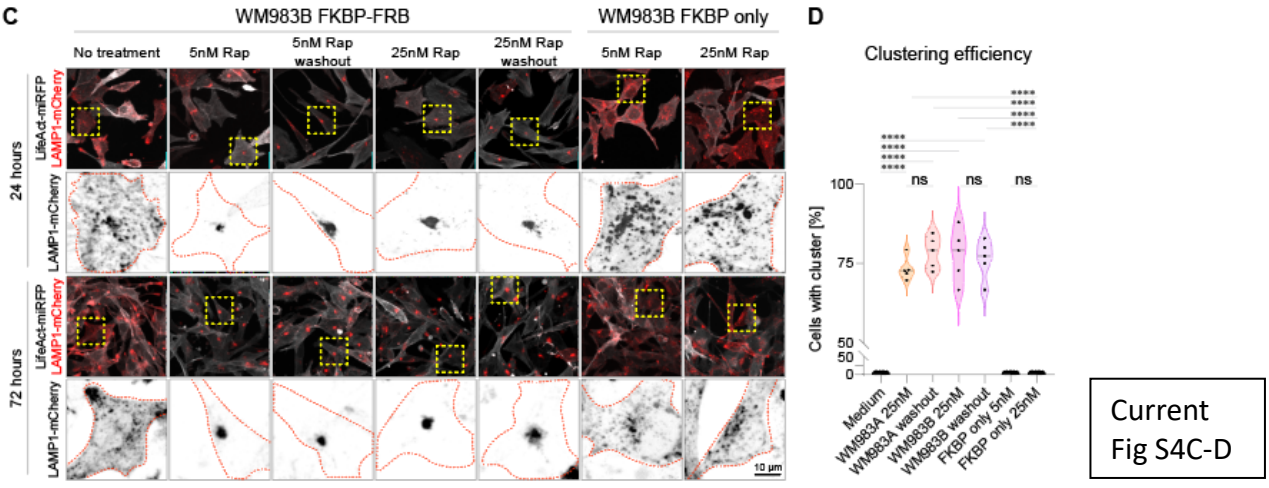


Fig Re-2 P-AKT S473 levels are not affected by Rapalog treatment.

-How are lysosomes distributed in rapalog treated cells after division – is the perinuclear effect conserved? If not, will it affect the authors conclusions?

Re: In the **Figure S4C-D**, we had initially shown that the clustering is maintained for 72h in culture. At this timing (72h), cells have divided at least two times, yet around 80% have visible lysosome clusters, demonstrating the stability of the clustering after cell division. We have specified the time-point in the figure legend. In addition, during the course of our experiments, we spotted several dividing cells in which we observed one lysosome cluster in prometaphase and two lysosome clusters from metaphase to telophase, suggesting that the lysosome cluster is divided and then segregated to the two daughter cells, see **Fig Re-3**.



Current Fig S4C-D

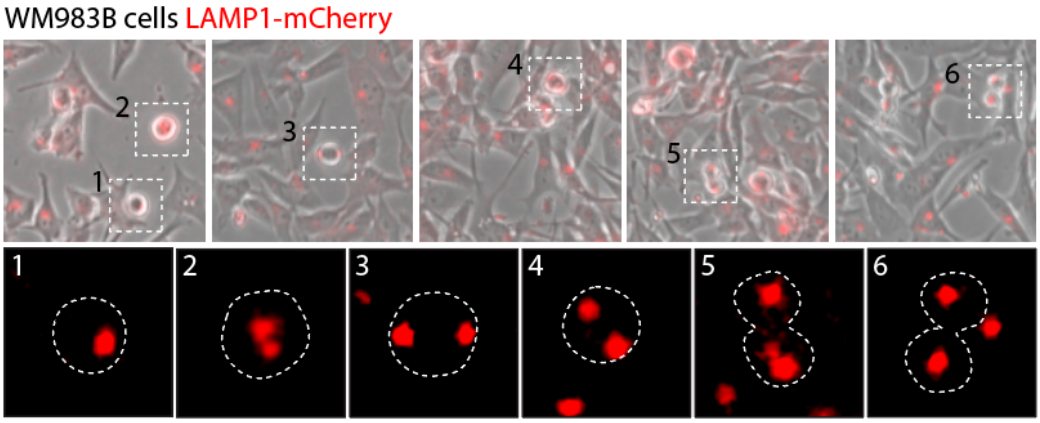
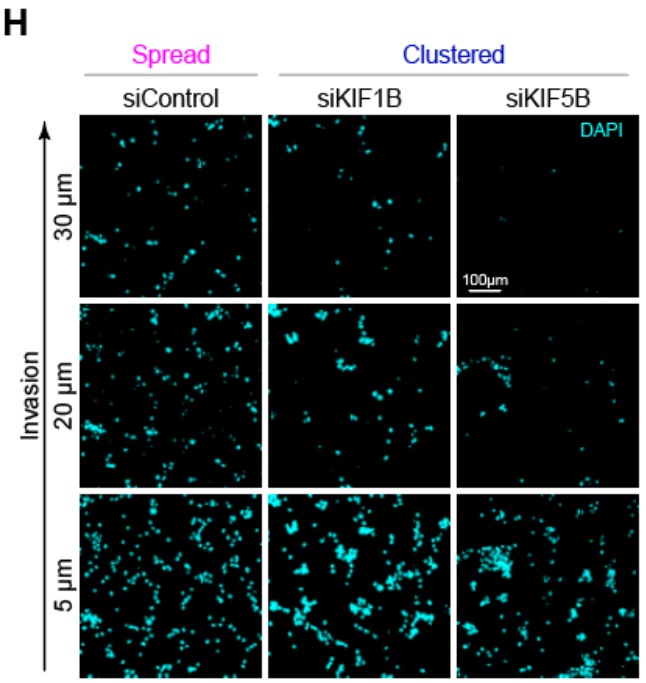


Fig Re-3
Segregation of the lysosome cluster during mitosis

2) Given that the rapalog-FKBP12 system drives an artificial clustering of lysosomes, I believe some of the authors' conclusions should be validated using at least one alternative approach, to justify the authors claims. See PMID 31130364 for alternative methods to disturb lysosome peripheral positioning.

Re: In parallel to the Rapalog approach, we force lysosomal clustering by depleting KIF1B or KIF5B using siRNA. As shown in the new Figure S3H, lysosome clustering prevents melanoma cell invasion in a 3D collagen assay, similarly to the effect of the Rapalog system (See also Re2.1).



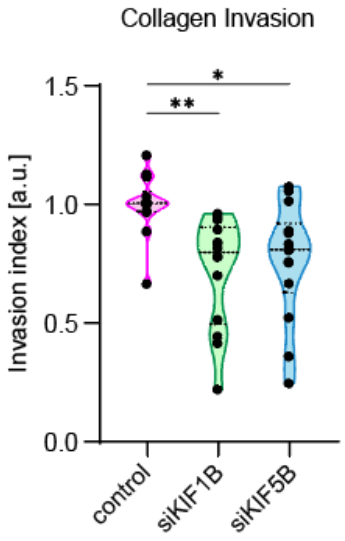


Fig S3, new panel H

3) The authors need to clearly demonstrate that lysosome degradative function is not significantly impaired by lysosome clustering using their Rapalog-FKBP system. If lysosome function is heavily affected, it will be difficult to attribute the observed effects solely to changes in positioning. Some relevant data on this issue appear in Fig Re-1; however, this figure lacks important details, such as treatment duration, and if I understand correctly, it is not included in the main manuscript. Control experiments in vitro should align with the time frames used in vivo. Testing lysosome degradative

function using methods in addition to lysotracker and lysosensor (for example MagicRed like the authors use elsewhere) is important.

Re: We appreciate this reviewer's comment and have now further characterized lysosomes upon Rapalog-induced clustering. The resulting data that are now added to the manuscript as a new Figure S4H show that neither lysosome mass and nor their acidity are perturbed upon rapalog treatment (see also Re 1.1). Unfortunately, the Rapalog- and FKBP-based system remain incompatible with the usage of the Magic Red dye due to fluorescence emission spectra overlap. To circumvent this problem, we used Magic Red labeling to probe lysosomes' degradative capacities upon clustering in cells depleted for KIF1B & KIF5B. As shown now in Fig.Re-1 in response to reviewer #1, we could not observe any significant changes in Magic red intensity upon either KIF1B or KIF5B depletion.

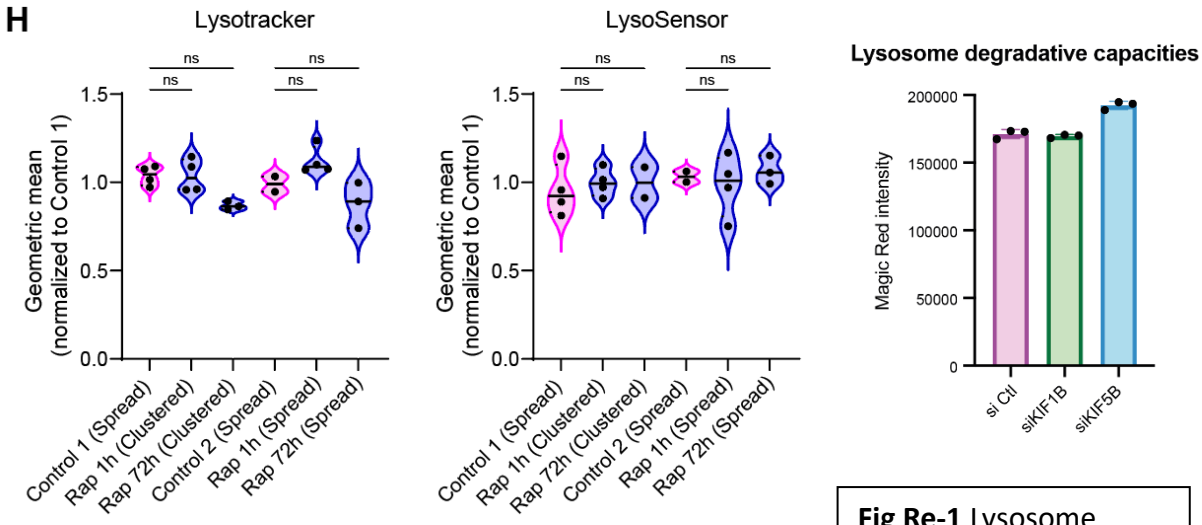


Fig S4, new panel H

Fig Re-1 Lysosome degradative capacity (MagicRed) in WM983B cells is preserved upon lysosome clustering by KIF1B/5B silencing.

3) MITF amplifications occur in 5-20% of melanomas (PMID 37400313), and activation of TFEB-related factors is known to drive changes in lysosome positioning and secretion (PMID 29146937, 21889421). While I understand the space constraints, I believe the manuscript would significantly benefit from addressing this important research area in some way within the text.

Re: We appreciate such advice and have now discussed this aspect (and associated references) in the revised version of the manuscript "Such programs could involve the MITF/TFE transcription factors, which are often amplified in melanoma (Zoncu and Perera, 2023) and control various catabolic functions of lysosomes, including their exocytosis (Medina et al., 2011)."

There seems to be an increase in total LAMP1 levels observed in Fig. 2 from patient biopsies – how can the authors differentiate between changes in levels and positioning?

Re: We acknowledge that there could be some confusion when evaluating these data.

As it is currently visible in Figure 2B and E, the clustering of lysosomes appears as a single dense perinuclear dot. While we agree that there could be some variation in LAMP1 intensities, this pattern is very different from a “spread” pattern where multiple dots of various intensities and relative sizes are visible. In the attached Fig.Re-4, we have now quantified the mean LAMP1 intensity in several patient’s samples and show that there is no statistical difference in the LAMP1 levels between the human-derived samples.

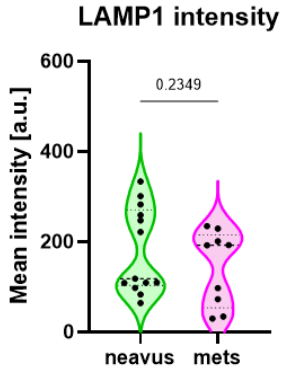


Fig Re-4 Mean LAMP1 intensity in tissue from nevus (n=2 patients) and from metastatic site (n=3 patients). One dot = 1 field of view.

The authors suggest that changes in KIF expression can drive alterations in lysosome positioning independently of MiTF/TFE factors, which presents a very interesting model. However, MiTF/TFE factor activity is highly regulated post-translationally. Therefore, assessing the activity of their downstream target genes would be more appropriate than measuring only the mRNA levels of the factors themselves, as was done in a few cell lines in Fig. Re5.

Re: We thank this reviewer for their insights. Yet, we have not and do not claim that lysosome positioning in melanoma cells is independent of MITF/TFE and further believe that such question goes beyond the scope of our study. Yet, we have now analyzed the expression levels of known MITF/TFE targets in our three major human melanoma cells based on our initial transcriptomic analysis. As shown in Fig Re-5 below, no clear gene expression changes in MITF/TFE targets can be observed despite the slight yet expectable variations among the identified genes.

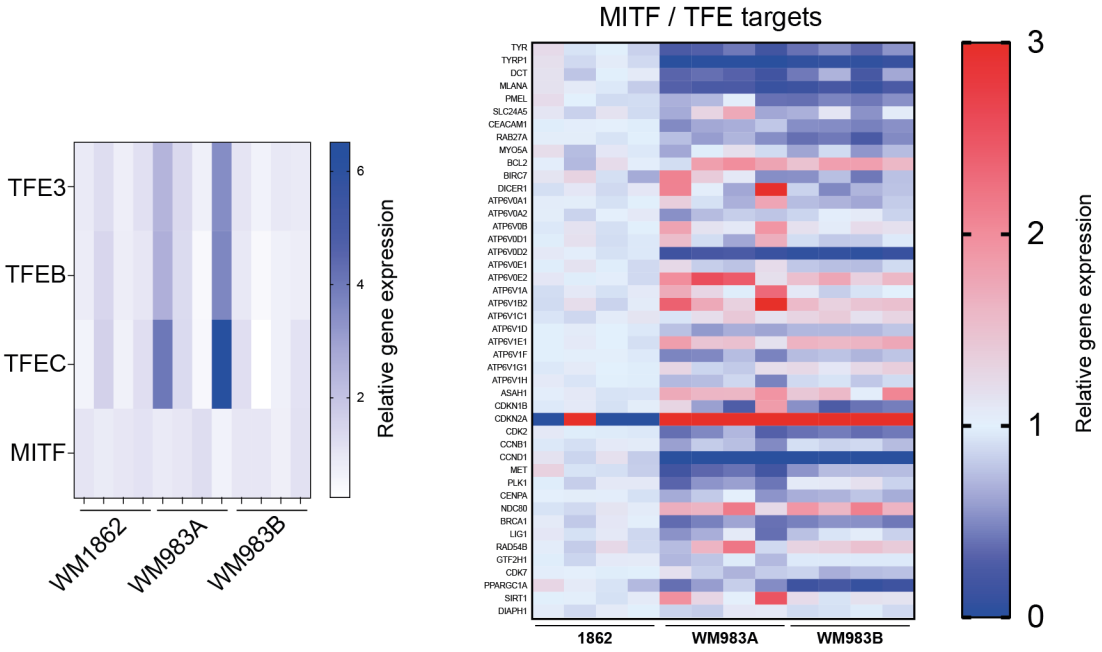


Fig Re-5 Expression levels of MITF/TFE and their targets in human melanoma cells.

Additionally, MITF activation in melanoma has been reported to increase non-degradative endosomes (PMID 25605940), which should be considered when interpreting results obtained from lysophilic dyes.

Re: Although we appreciate this reviewer's insights, our data do not suggest any activation of MITF in our cells.

4) "We now demonstrate that lysosomal peripheral distribution can be used as a proxy for melanoma metastatic dissemination. We further show that deeply invading cells away from the epidermis tend to display increased lysosome spreading as if such repositioning increased with the invasive properties of cells. This suggests that probing lysosomal positioning could allow to refine classification criteria for malignant and non-malignant cutaneous lesions." Unless the authors' findings are validated with additional patient material, I believe these statements should be tuned down.

Re: We have now toned-down our initial claim although we humbly believe that our data firmly demonstrate such link "Our results suggest that lysosomal peripheral distribution could be used as a proxy for melanoma metastatic dissemination. In melanoma biopsies, deeply invading cells away from the epidermis tend to display increased lysosome spreading as if such repositioning increased with the invasive properties of cells. This suggests that probing lysosomal positioning could allow to refine classification criteria for malignant and non-malignant cutaneous lesions."

Reviewer #4 (Remarks to the Author)

In this considerably revised manuscript, the authors provide a detailed response to the prior reviews from NCB. They have done an admirable job of addressing all of our prior comments. the data on KIF5 is especially welcome, as this provides a new mechanistic link that others in the field can follow up on. Moreover, the quality of the images, and a much better explanation of their methods, is welcome. the addition of new models as well as patient samples further augments the importance of the findings. Overall, I feel satisfied at the responses they have provided and the improvements to the manuscript.

Re: We are grateful to this reviewer for their positive feedback.

Reviewer #5 (Remarks to the Author)

In this study, the authors performed bottom-up proteomics analysis on the supernatant of WM983B cells with and without Rapalog treatment. This part of the work has a reasonable experimental design and the data is complete. However, to enhance the reliability and reproducibility of the results, the following aspects should be addressed:

1. The authors cite the SP3 method for protein extraction and digestion but should briefly provide more details regarding the specific chemicals used, their concentrations, the time of protein digestion, and any additional steps in the protocol."

We extended the method description and included an additional paragraph:

"In brief, each protein extract was reduced with 12 mM dithiothreitol (DTT) and alkylated using 40 mM iodoacetamic acid (IAM) at 37°C and 25°C, respectively. A mixture of hydrophilic and hydrophobic magnetic beads was used to clean up the proteins at a ratio of 125:1 beads:proteins (Sera-Mag Speed beads, Thermo Fisher Scientific). After acetonitrile (ACN) was added to a final concentration of 50%, the beads were allowed to bind to the proteins for 18 minutes. Protein-bead mixtures were washed twice with 80% ethanol and once with 100% ACN. The protein-bead complexes were digested with trypsin:LysC (Promega, Madison) at a 1:20 ratio overnight at 37°C. Extracted peptides were cleaned-up using automated C18 solid phase extraction with the AssayMAP 5µL C18 cartridges (ref. n°5190-6532) on the same platform. After elution with 70% ACN, the resulting peptide mixture was evaporated using a speed-vac (Thermo Fisher Scientific), reconstituted in 2% ACN and 0.1% FA, and analysed by nanoLC-MS/MS on a nanoUPLC system (nanoAcquityUPLC, Waters) coupled to a quadrupole-Orbitrap mass spectrometer (Q-Exactive HF-X, Thermo Scientific). Peptide separation was performed on an ACQUITY UPLC® Peptide BEH C18 Column (250 mm x 75 µm with 1.7 µm diameter particles) and an ACQUITY UPLC® M-Class Symmetry® C18 Trap Column (20 mm x 180 µm with 5 µm diameter particles; Waters). The solvent system consisted of 0.1% formic acid (FA) in water (solvent A) and 0.1% FA in ACN (solvent B). Samples were loaded into the enrichment column over 3 min at 5 µL/min with 99% of solvent A and 1% of solvent B. Peptides were eluted at 350 nL/min with the following gradient of solvent B: from 2% to 25% over 53 min, from 25% to 40% over 10 min and from 40% to 90% over 2 min."

2. Further details on the mass spectrometry (MS) and tandem mass spectrometry (MS/MS) parameters should be included. Specifically, the mass range for data acquisition, scan rate, and other relevant settings.

We now provide extended description of these parameters:

"The system was operated in a data-dependent acquisition mode with automatic switching between MS and MS/MS. Survey full scan MS spectra (mass range 300–1,800) were acquired in the Orbitrap at a resolution of 60 K at 200 m/z with an automatic gain control fixed at 3 × 106 and a maximal injection time set to 50 ms. The ten most intense peptide ions in each survey scan with a charge state ≥2 were selected for fragmentation. MS/MS spectra were acquired at a resolution of 15 K at 200 m/z, automatic gain control was set to 1 × 105, and the maximal injection time was set to 50 ms. Peptides were fragmented by higher-energy collisional dissociation with a normalized collision energy set to 27. Peaks selected for fragmentation were automatically included in a dynamic exclusion list for 60 s."

3. The manuscript should include a more detailed description of the protein database search parameters, such as peptide length restrictions, precursor and fragment mass tolerances.

We have added the necessary details:

"Spectra were searched with a mass tolerance of 5 ppm in MS mode and 0.05 Da in MS/MS mode. One trypsin missed cleavage was tolerated and the minimum peptide length was set to 7 amino acids."

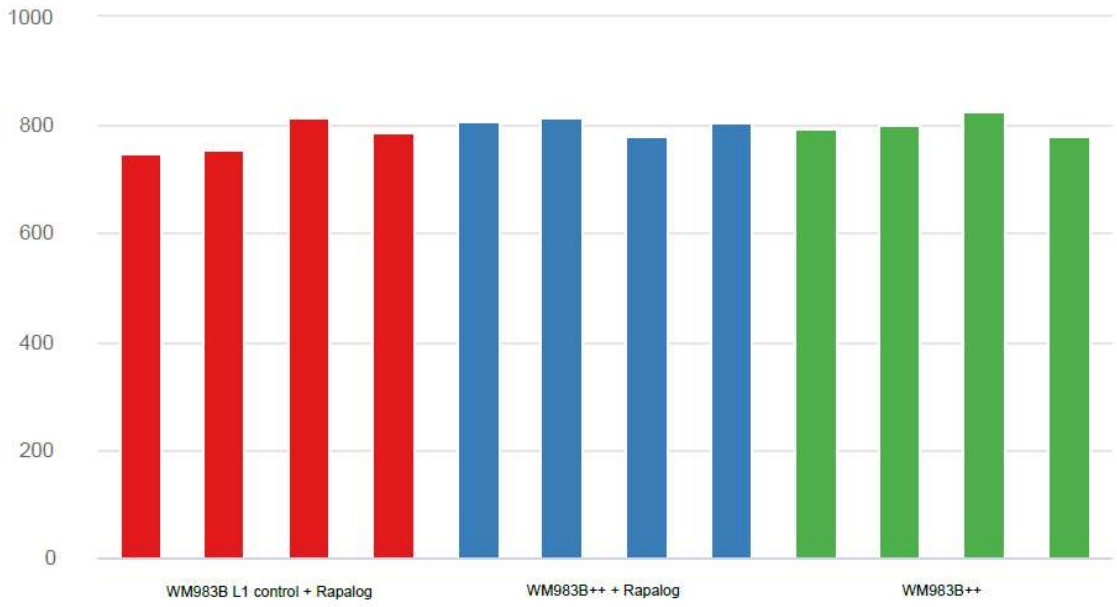
4. The number of biological replicates used in this experiment should be clearly stated.

The number of replicates is now clearly stated:

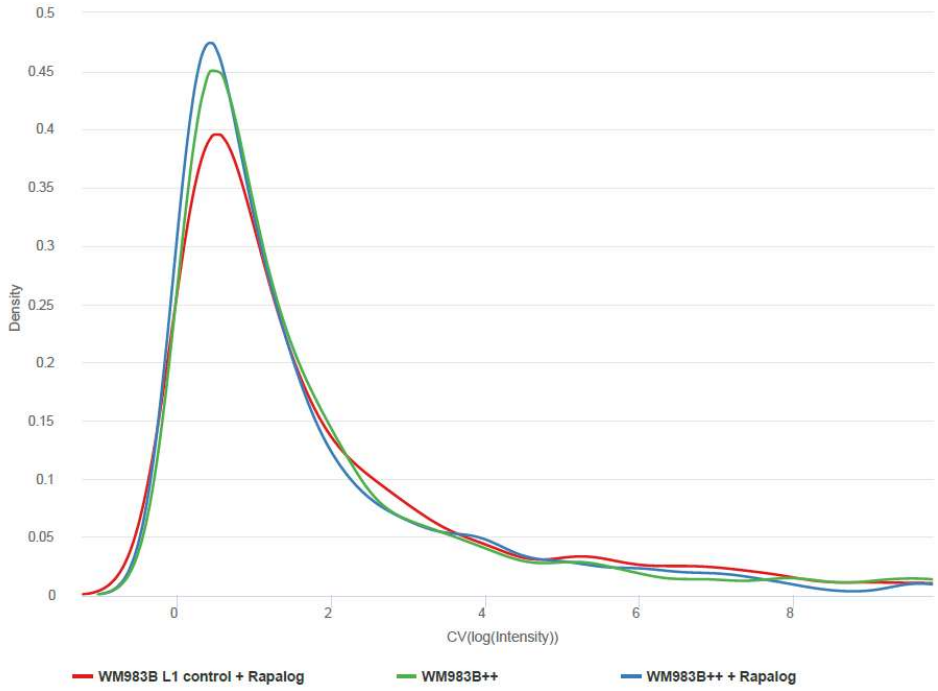
"For each of the three conditions, four biological replicates were prepared."

Additionally, to assess data quality, the authors should consider including common quality control figures for bottom-up proteomics, such as Log2 Normalized LFQ distributions, and coefficient of variation (CV) plots.

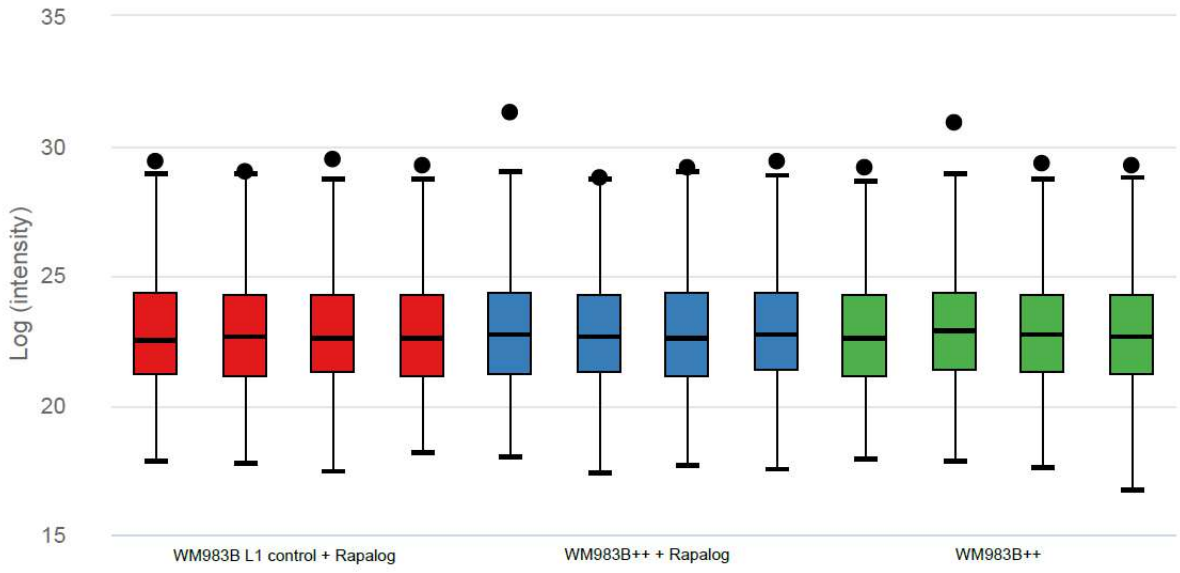
Quality control figures



Total number of unique protein groups quantified in each of the 4 biological replicates over the three conditions



Coefficient of variation distributions of the abundances (log(intensity)) of all quantified proteins



Log2 Normalized protein intensity distributions in the 4 biological replicates over the three conditions