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mTORC1 activity negatively regulates human hair follicle growth and pigmentation

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1st Editorial Decision **23rd Jan 2023**

Dear Dr. Paus

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, both referees consider the findings interesting but they also raise a number of concerns regarding missing control experiments, missing experimental details and clarity, or quantification, which need to be addressed.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (April 23rd). Please discuss the revision progress ahead of this time with me if you require more time to complete the revisions.

I am also happy to discuss the revision further via e-mail or a video call, if you wish.

Your article will be published as a short report (unless the revision has more than 5 figures). For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice.

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We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

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When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

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Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) At EMBO Press we ask authors to provide source data for the main figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

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10) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)

- If the data are obtained from n {less than or equal to} 5, show the individual data points in addition to the SD or SEM.
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- Please also include scale bars in all microscopy images.

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I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

You can use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In this manuscript, the authors report on the role of TORC1 as a regulator of hair pigmentation and even growth using a whole organ culture of human hair follicles.
In their findings the authors show that mTORC1 is significantly upregulated during hair greying. Notably, melanogenesis is increased when mTORC1 is inhibited with rapamycin- even in hair greying follicles that have not reached the point of irreversibility , whereas hair pigmentation decreased when mTORC1 is overexpressed. In an effort to find the mechanism behind the regulation of melanogenesis by mTORC1, the authors found that mTORC1 inhibition stimulated intrafollicular MSH production and activation of MC1R. Interestingly, this control of melanogenesis is linked to a prolongation of the hair cycle anagen phase and therefore hinting that rapamycin has potential as a hair growth stimulator by extending the anagen phase. Overall, this manuscript is a solid hypothesis-driven study where the authors make good use of their ex vivo human hair follicle culture model to uncover promising strategies to stimulate hair pigmentation during normal pigmented or hair greying follicles by inhibiting mTORC1. Therefore this work will be catching the attention and interest from general and specialized audiences.

Minor concerns/comments:

According to the authors, the increase of melanogenesis by rapamycin induced
mTORC1 inhibition is driven by stimulating intrafollicular tyrosinase activity and melanin transfer. And, this increase in melanogenesis is enhanced by -MSH production and activation of MC1R. Interestingly, this is not primarily mediated by MITF. Here the authors measure -MSH expression by (immuno-)histomorphometry and also block MC1R activity using ASP, however it would be interesting to measure MC1R expression as well. Could the authors provide quantification of MC1R expression before and mTORC1 inhibition?. In addition, this reviewer finds the sentence "Contrary to a widespread misconception, human hair greying does not primarily result from a dysfunction of HF melanocyte stem cells, but is initiated by a dysfunction of differentiated melanocytes in the HFPU" interesting and provocative but unsupported by the single reference provided (O'Sullivan et al, 2021). In fact, it opens the question whether mTORC1- or its inhibition- is also regulating the differentiation of HF melanocyte stem cells. Given that their ex vivo model may not include the HF stem cell compartment , the authors could try to the measure tyrosinase activity of the more undifferentiated melanocytes found in the upper ORS layers after mTORC1 inhibition?

Referee #2:

In this study, Suzuki et al. have revealed a role of mTORC1 on ex vivo hair follicle (HF) pigmentation combining elegant models of HF organ culture with molecular analyses. These are novel and important findings, especially in the context of human HF biology. Given that HF pigmentation is the result of multiple intracellular pathways in melanocytes together with highly regulated interactions between melanocytes and keratinocytes, the observations reported are of interest well beyond the fields of cutaneous biology. Indeed, many human biological and pathophysiological aspects are modulated or the result of complex cell-cell and cell-environment interactions, aspects of which can be modeled by the HF system. The body of work presented is extensive, and the data support the major conclusions of the paper. That being said, some details of the studies are lacking throughout the manuscript and, consequently, it is difficult to assess whether several conclusions drawn from some experiments are validated by the data shown, as detailed below.

1. Although the differences in p-S6 fluorescence intensity are obvious in Fig. 1, there is no description included concerning how images were acquired and fluorescence signals were quantified to ensure accuracy of measurements and conclusions. This aspect of the work needs to be addressed. Similar considerations are relevant for other experiments involving quantification of fluorescence signals.

2. Figure 2D appears to be incomplete, as micrographs of Catagen HF treated with rapamycin and Anagen HF treated with vehicle have been omitted. The description of the experimental methods appears to indicate that all HF used in these studies were in Anagen VI.

3. Although it is well known that melanocytes develop dendrites, the micrographs included do not allow evaluation of the abundance of dendrites in said melanocytes. Please include clear micrographs that demonstrate melanocyte dendricity in the tissue sections.

4. The conclusions regarding the effect of rapamycin on melanosome transfer to HF keratinocytes (p.8) are not supported by the data shown, as the experiments presented are not designed to measure melanosome transfer from melanocytes to keratinocytes.

5. The mechanistic experiments that included TSC silencing are very important. It is somewhat unusual that a relatively minor approximately 20% decrease in TSC2 abundance (as per immunofluorescence quantification shown in Fig. 3B, C would be associated with a major downstream effect. This should be further discussed.

6. The description of drug treatments is not clear and needs to be revised (P. 18). Specifically, the drugs were administered several times. However, it is not clear if a stock solution of 20 ng/ml rapamycin was used, or if the final concentration in the culture medium was 20 ng/ml. Similar considerations apply to ASIP and DMSO. Further, it is not clear whether there were changes in the culture medium, so that rapamycin was always present at the same concentration, or whether there were simply additions to the existing culture medium, in which case the drug and vehicle concentrations varied from day 1 to day 7. The micrographs appear to indicate that the HF were cultured in the presence of 20 ng/ml rapamycin, but the description in the "Methods" section is not very clear.

7. Similarly, for siRNA experiments it is not clear whether a 1 M stock solution (non-specified volume) was used, or the follicles were culture for 48 h in the presence of 1 M siRNA.

Minor points:
1. P4: "...TSC loss-of-function in mouse tissues and cultured fibroblasts show upregulated..." should read "...TSC loss-of-function in mouse tissues and cultured fibroblasts shows upregulated..."

2. The micrographs in Fig. 2H are not of very good quality. Please replace.

Editor comments:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

We have now explained in the data availability section that we have not deposited any data:
"*This study includes no data deposited in external repositories.*"

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.
Thanks, done as requested.
We have now exchanged the graph in questions to show scatter blots (see new Fig 3D)

Referee #1:

Overall, this manuscript is a solid hypothesis-driven study where the authors make good use of their ex vivo human hair follicle culture model to uncover promising strategies to stimulate hair pigmentation during normal pigmented or hair greying follicles by inhibiting mTORC1. Therefore this work will be catching the attention and interest from general and specialized audiences.
We thank the reviewer for these very encouraging comments.

Minor concerns/comments:

According to the authors, the increase of melanogenesis by rapamycin induced mTORC1 inhibition is driven by stimulating intrafollicular tyrosinase activity and melanin transfer. And, this increase in melanogenesis is enhanced by a-MSH production and activation of MC1R. Interestingly, this is not primarily mediated by MITF. Here the authors measure a-MSH expression by (immuno-)histomorphometry and also block MC1R activity using ASIP, however it would be interesting to measure MC1R expression as well. Could the authors provide quantification of MC1R expression before and after mTORC1 inhibition?

Thank you for this suggestion. Looking at MC1R expression before and after rapamycin treatment would be interesting to investigate. Unfortunately, due to the limited amount of sections one can obtain from each hair follicle for immunohistology (5-6 sections), we cannot perform this additional analysis since we have exhausted all available sections for the different markers presented in our manuscript.
However, the expression level of MC1R in human hair follicles has been extensively characterized before by us and other (e.g., Ito et al 2005; Kauser et al, 2005, Böhm et al 2005). Since this additional analysis would not add substantial new insights into the mechanistic data already presented in the context of our study, this would not justify having to run new HF organ cultures. However, we now explicitly acknowledge in the revised Discussion (p. 14) that *"It would be intriguing to assess in a follow-up study whether rapamycin or TSC2 siRNA also impact on the levels of MC1R protein expression and/or signaling activity."*

In addition, this reviewer finds the sentence "Contrary to a widespread misconception, human hair greying does not primarily result from a dysfunction of HF melanocyte stem cells, but is initiated by a dysfunction of differentiated melanocytes in the HFPU" interesting and provocative but unsupported by the single reference provided (O'Sullivan et al, 2021). In fact, it opens the question whether mTORC1- or its inhibition- is also regulating the differentiation of HF melanocyte stem cells.

The authoritative review cited here (O'sullivan et al. 2021) explains extensively why human HF greying begins in and is driven by defective melanogenesis and melanin transfer in the HF pigmentary unit, with a dysfunction of HF melanocyte stem cells coming into play only fairly late during the hair greying process, namely when greying ultimately becomes irreversible. This had already been explained in detail in Paus, Pigm Cell Melanoma Res 2011, and has recently been underscored by our finding that even greying HFs can undergo extensive cycles of repigmentation and depigmentation (Rosenberg et al. Elife 2021). We now point the interested reader to all three references (p. 16).

Unfortunately, the reviewer's very intriguing question whether mTORC1- or its inhibition - also regulates the differentiation of human HF melanocyte stem cells, cannot currently not be satisfactorily answered in the human system, since no specific marker for HF melanocyte stem cells exists to date (in "fact, so-called "melanocyte stem cell markers" such as MITF, TRP1/2, or tyrosinase are by no means stem cell-specific). However, the interesting alternative hypothesis raised by this expert referee has encouraged us to now mention in the revised Discussion (p. 14): *"Once – as yet unavailable – truly selective markers for human HF melanocyte stem cells become available, it will deserve systematic scrutiny if mTORC1 inhibition by rapamycin also promotes the intrafollicular differentiation of melanocyte stem cells located in the bulge and/or of amelanotic melanoblasts located in the periphery of the proximal hair bulb epithelium (Tobin et al. 2011, O'Sullivan et al. 2021)."*

Given that their ex vivo model may not include the HF stem cell compartment, the authors could try to the measure tyrosinase activity of the more undifferentiated melanocytes found in the upper ORS layers after mTORC1 inhibition?

Tyrosinase activity is exclusively found in mature melanocytes where melanin production is active and even though ectopic pigmentation can be found in particular cases (e.g. stress) in ORS, tyrosinase activity is found in the HF pigmentary unit only (Tobin 2011, O'Sullivan et al. 2021). However, we have now specifically searched for evidence of tyrosinase activity outside of the HF pigmentary unit in HFs treated rapamycin: *No tyrosinase activity could be observed outside of the hair follicle pigmentary unit*. This has now been clarified in the revised Results section (p. 8)

Referee #2:

The body of work presented is extensive, and the data support the major conclusions of the paper. That being said, some details of the studies are lacking throughout the manuscript and, consequently, it is difficult to assess whether several conclusions drawn from some experiments are validated by the data shown, as detailed below.

1. Although the differences in p-S6 fluorescence intensity are obvious in Fig. 1, there is no description included concerning how images were acquired and fluorescence signals were quantified to ensure accuracy of measurements and conclusions. This aspect of the work needs to be addressed. Similar considerations are relevant for other experiments involving quantification of fluorescence signals.

In the original version of the manuscript, we had already indicated in the vehicle/control image for each marker the standardized reference area in which quantitative immunohistomophometry was performed. We have also clarified in the Material & Methods section how images were acquired and fluorescent signals were quantified (including corresponding references that refer to our standardized, well-published protocols): *"Images were acquired using the BZ-X800 All-in-one Fluorescence Microscope (Keyence Corporation, Osaka, Japan) maintaining a constant set exposure time throughout imaging. Images were taken at 200x magnifications. Image analysis and measurement of staining intensity were evaluated in well-defined reference areas (see vehicle pictures (dotted area)) using NIH ImageJ software (NIH, Bethesda, Maryland, USA), as previously described (Hardman et al, 2015; Tiede et al, 2021; Chéret et al, 2020; Gáspár et al, 2011). In brief, for measuring the staining intensity of our different markers, we first draw the reference area (see the dotted area on each figure) and measure the mean intensity. For analyzing the number of single and double positive cells, we have used the cell counter functions of Image J and individually pointed the positive (single and/or double)."* (p. 24)

2. Figure 2D appears to be incomplete, as micrographs of Catagen HF treated with rapamycin and Anagen HF treated with vehicle have been omitted. The description of the experimental methods appears to indicate that all HF used in these studies were in Anagen VI.

We had previously omitted micrographs of catagen HFs treated with rapamycin and anagen HF treated with vehicle for space constraints, since we considered these dispensable. However, as requested, we have now added the missing anagen and catagen HF images in the revised Fig 2D.

3. Although it is well known that melanocytes develop dendrites, the micrographs included do not allow evaluation of the abundance of dendrites in said melanocytes. Please include clear micrographs that demonstrate melanocyte dendricity in the tissue sections.

We thank the reviewer for this helpful suggestion. We have now replaced the corresponding images to demonstrate melanocyte dendricity more clearly in the tissue sections (see new Fig 2L).

4. The conclusions regarding the effect of rapamycin on melanosome transfer to HF keratinocytes (p.8) are not supported by the data shown, as the experiments presented are not designed to measure melanosome transfer from melanocytes to keratinocytes.

Her, we respectfully disagree with the referee. While transmission electron microscopy would be the optimal method of choice for visualizing melanosome transfer, gp100 IF (=demarcates premelanomes) is a well-recognized and sensitive tracker of melanosome transfer between melanocytes and keratinocytes (Singh et al 2008). We have now underscored this in the revised M+M (p. 23) and Results sections (p. 8), but acknowledge in the revised Discussion that

"*independent confirmation of this melanosome transfer effect by transmission electron microscopy would be desirable*" (p. 14).

5. The mechanistic experiments that included TSC silencing are very important. It is somewhat unusual that a relatively minor approximately 20% decrease in TSC2 abundance (as per immunofluorescence quantification shown in Fig. 3B, C would be associated with a major downstream effect. This should be further discussed.

Yes, this is indeed unusual and has intrigued us for a long time. In fact, in our extensive, published experience with gene silencing in organ-cultured scalp HFs (a method that we have pioneered in human hair research), a knockdown efficiency of 20-25%, and even of as little as 15%, can suffice to generate significant downstream functional effects in this highly sensitive *ex vivo* assay (Samuelov et al. JID 2012, Al-Nuaimi et al. JID 2014, Hardman et al. JID 2015, Cheret et al Nat Comm 2018, Mardaryev et al JID 2021). We have now clarified this in the revised Discussion, along with citing these references; here we now also emphasize that "*the major functional downstream effect observed despite the relatively modest knock-down efficiency highlights how tightly mTORC1 activity is regulated by TSC2 in human scalp HFs*" (p. 13).

6. The description of drug treatments is not clear and needs to be revised (P. 18). Specifically, the drugs were administered several times. However, it is not clear if a stock solution of 20 ng/ml rapamycin was used, or if the final concentration in the culture medium was 20 ng/ml. Similar considerations apply to ASIP and DMSO.

Thanks for urging us to provide greater clarity on this. We have now clarified this in the revised Materials and Methods section as follows: "*Rapamycin (Final concentration in each rapamycin-treated well: 20 ng/mL, Santa Cruz, sc-3504), Agouti Signaling Protein (ASIP)/Agouti protein (Final concentration in each agouti-treated well: 2 ug/ml, R&D system, 9094-AG) or vehicle (Final concentration in each vehicle-treated well: 0.1% DMSO) (Sigma-Aldrich) was administrated on day 1, 3 and 5 during medium change.*" (p. 19)

Further, it is not clear whether there were changes in the culture medium, so that rapamycin was always present at the same concentration, or whether there were simply additions to the existing culture medium, in which case the drug and vehicle concentrations varied from day 1 to day 7. The micrographs appear to indicate that the HF were cultured in the presence of 20 ng/ml rapamycin, but the description in the "Methods" section is not very clear.

The medium was completely replaced every other day (at day 1, 3 and 5). We have now clarified this in the revised Material and Methods section (see also comment above), where we have also pointed out that 20 ng/ml rapamycin corresponds to the final concentration: "*Rapamycin (Final concentration in each rapamycin-treated well: 20 ng/mL, Santa Cruz, sc-3504)…"* (p. 19)

7. Similarly, for siRNA experiments it is not clear whether a 1 uM stock solution (non-specified volume) was used, or the follicles were culture for 48 h in the presence of 1 uM siRNA.

Hair follicles were each cultured in the presence of 1 uM siRNA or non-targeting oligos for 48 hrs. We have now clarified this in the revised Material and methods: "*Final concentration of 1 µM*

of siTSC2 or siRNA control (NTO) was added 24 h after microdissection for 48 h in each of the corresponding wells." (p.20)

Minor points:

1. P4: "...TSC loss-of-function in mouse tissues and cultured fibroblasts show upregulated..." should read "...TSC loss-of-function in mouse tissues and cultured fibroblasts shows upregulated..."

Thank you for spotting this typo, which has been corrected as suggested (p. 4).

2. The micrographs in Fig. 2H are not of very good quality. Please replace.

Agreed. We have now replaced the corresponding images as requested (see new Fig 2H).

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Tobin DJ. The cell biology of human hair follicle pigmentation. Pigment Cell Melanoma Res. 2011 Feb;24(1):75-88.

Dear Dr. Paus,

Thank you for the submission of your revised manuscript. I have now evaluated your response to the minor concerns raised by the referees and I have also gone through your manuscript to see what we still require from the editorial side before your manuscript can be officially accepted.

- Your manuscript will be published in our Reports section. To make this possible, please combine the Results and Discussion sections.

- Please add callouts to Fig 1C&D, EV3A-L and EV4A-E.

- Please move the Data availability section to the end of the Materials & Methods section.

- Please update the 'Conflict of interest' paragraph to our new 'Disclosure and competing interests statement'. For more information see
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- Please move Table 1 to after the figure legends.

- Please remove the section "The Paper Explained".

- Please remove "the section Funding sources" or merge it with the information in the Acknowledgment section.

- During our routine image analysis, which we perform on all revised manuscripts before publication, we noticed that the same images are shown in Figure 2J and Figure EV1D. Since these are representative images for the staining you did, please choose a different set of images for Fig EV1D.

- At EMBO Press we now ask authors to provide source data for the main figures. On January 25th, 2023 our source data coordinator Hannah Sonntag contacted you regarding the deposition of source data, i.e., the minimally processed data that was used to generate the figures. She attached a checklist detailing which data were required. I hope this e-mail was not filtered by your spam filter. Please submit the source data alongside your revised manuscript. If you need any further information, please let me know.

Additional information on source data and instruction on how to label the files are available <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

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We look forward to seeing a final version of your manuscript as soon as possible.

Kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

The authors have addressed all minor editorial requests.

Jérémy Chéret
University of Miami Miller School of Medicine
Dr. Phillip Frost Dept. of Dermatology & Cutaneous Surgery
United States

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The data shown in figures should satisfy the following conditions:

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- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
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- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
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