

pH-controlled histone acetylation amplifies melanocyte differentiation downstream of MITF

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1st Editorial Decision

26 April 2019

Thank you for the transfer of your research manuscript to EMBO reports. I have now read your paper and went through the referee reports from The EMBO Journal (which you will find attached at the end of this message).

All referees acknowledge the potential interest of the findings, and I would be happy to receive a revised version of the manuscript. Nevertheless, all referees have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn. As EMBO reports emphasizes novel functional over detailed mechanistic insight, we will not require to address points regarding more refined mechanistic details, e.g. to decipher the exact mechanism of nuclear shuttling of CA14 (if you already have data addressing such points, we would of course welcome their inclusion in a revised version).

Important, though, would be to provide additional data demonstrating that transcription of CA14 is indeed directly regulated by MITF, that CA14 is found in the nucleus, and that it regulates transcription of pigmentation genes via CBP/300. Thus, in particular the first 6 points of referee #1 need to be addressed experimentally. Moreover, all technical issues and suggestions regarding the experiments in the current version of the manuscript should be dealt with.

Given the constructive referee comments, we would like to invite you to revise your manuscript for EMBO reports with the understanding that the referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review (involving the same referees). It is our 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.

Revised manuscripts should be submitted within three months of a request for revision; they will

otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

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Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Important: All materials and methods should be included in the main manuscript file.

Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable. See:
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When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide#revision>). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)

Please note that we now mandate that the corresponding author provides an ORCID digital identifier that is linked to his/her EMBO reports profile.

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

REFeree REPORTS

Referee #1:

Summary: Acidic conditions have been shown to inhibit melanogenesis, however, this has not previously been known to involve epigenetic or transcriptional regulation. Raja et al present evidence that pH regulates melanogenesis at the transcriptional level by a feed forward mechanism

involving MITF regulation of CA14. MITF promotes CA14 expression at the RNA level and CA14 promotes melanogenesis by regulating intracellular pH. CA14 is found to be localized to the nucleus where it regulates transcription of pigmentation genes by modulating CBP/P300 activity and promoting H3k27ac at MITF target loci.

Critique: The finding that CA14 is regulated by MITF and promotes melanogenesis in vitro and in vivo through a transcriptional mechanism involving modulation of pH provides a significant advance of our understanding of the regulation of melanocyte differentiation. The data are strong support for CA14 as a regulator of melanogenesis. However, the conclusion that transcription of CA14 is directly regulated by MITF requires additional experiments. Furthermore, direct regulation of transcription by CA14 modulation of CBP/P300 activity in the nucleus should be further strengthened.

Major Concerns:

1. The studies are not sufficient to substantiate the conclusion that MITF directly regulates CA14 expression. CA14 expression at the mRNA level in shMITF cells should be evaluated by q-RT-PCR. In addition, several RNA-seq studies have depleted MITF by shRNA in melanocytes and melanoma cells. Do these studies support the conclusion that MITF regulates CA14 at the mRNA level?
2. Does MITF affect luciferase activity in reporter assays with wild-type putative E/M boxes and is that regulation abolished with mutations in the putative E/M boxes? A more comprehensive set of reporter assays should be performed investigating the role of MITF and the requirement for any or all of the putative MITF recognition sequences.
3. Direct binding of MITF to the CA14 locus is also not strongly supported by the data. ChIPs cannot determine direct binding. EMSAs (supershifts and competition EMSAs) with oligonucleotides containing putative MITF binding sites should be conducted.
4. The authors should review the database for ChIP-seq investigating MITF binding genome-wide. Do any of these studies reveal that MITF binds to the regions of the CA14 locus as shown in Fig. 2F?
5. Fig. 4E shows both nuclear and cytoplasmic localization of CA14. The nuclear signal of CA14 in Fig. 4F is very weak. It would be informative to determine what proportion of CA14 is nuclear and what proportion is cytoplasmic with additional Westerns looking at the cytoplasmic levels of CA14 and quantifying each relative to the WCL.
6. If CA14 is primarily cytoplasmic, then it will be crucial to show that nuclear CA14 is regulating melanogenesis. Does CA14 have a nuclear localization domain? If so, it would strengthen the conclusion that nuclear CA14 regulates expression of melanogenesis, if an intact CA14 but not CA14 lacking the nuclear localization domain rescues melanogenesis in shCA14 depleted cells.
7. The ChIP-experiments in Fig. 5F lack statistics. What are the standard deviations and p-values?
8. Additional H3K27ac ChIPs should be conducted with the CBP/P300 inhibitor to determine if CBP/P300 activity is involved. Also, the effects of pH on this histone modification should also be interrogated.

Minor Concerns:

1. The pigmentation models in Fig. 2S are not well explained. More details are needed regarding the passage of cells in mice and in culture and the effects on pigmentation. Furthermore, it is not clear which pigmentation model was used for the studies described on page 6 and shown in Fig. 1.
2. Although the CA14 is regulated by MITF, unlike other MITF target genes, its expression at the protein level is not affected by pH (shown in Fig. 4C). Is the CA14 mRNA level altered by pH? Is it regulated by CBP/p300? The authors should investigate why CA14 expression, if MITF dependent, is not regulated by pH.

Other experiments:

Investigate the effects of CBP/P300 inhibitor on melanogenesis in zebrafish.

Referee #2:

Here Raja et al. identified carbonic anhydrase 14 (CA14) as a new MITF target. Further, they observed that CA14 up-regulation causes an increase in the intracellular pH, which in turn activates p300/CBP histone acetyl transferase activity favoring through H3K27 acetylation MITF

transcriptional activity and the pigmentation process.

The paper is mostly well written and provides interesting new data.

However, there are a number of issues with the manuscript that need to be addressed.

Below are my comments to be considered by the authors.

FigS1A: What are the mechanisms behind pHi and pHe stability at 10% CO₂ compared to 5% CO₂? Can the authors comment on that?

Fig 1B and 1D: the authors should show pigmentation of cell pellets along with protein expression for the whole kinetics (days 4, 6 and 8). TYRP1 level should be shown as well.

Fig1E: MITF level should be shown.

FigS1B- text L130 p7: The authors state that cells in an acidic state of pHi loose pigmentation. This is an over interpretation of the results. What the results show is that changes in the CO₂ level (5>10%) delay the pigmentation process.

Fig2A: Is there any correlation between MITF and CA14 level in publicly available datasets?

Fig2B-C: As aMSH+IBMX treatment increases CA14 level, pHi changes in B16 cells would be expected. Is there any pH change when cells are exposed to aMSH+IBMX? Is MITF (forced expression) sufficient to induce CA14 expression and impact the pHi in B16 cells? The authors show that IBMX treatment stimulates CA14 promoter activity, however, MITF has not yet been assessed. Is the effect of MITF a direct transcriptional response? This should be demonstrated with a Luc reporter assay.

Fig2G-H: At least 2 different oligos against MITF should be used to ensure specificity and reduce off target possibilities.

What is the effect of MITF loss of function on pHi in B16 cells?

FigS3: what are the effects of CA14 gain and loss of function on pHi in B16 cells? On pigmentation?

Fig4A-FigS5: MITF level should be shown as control

Fig4C: the complete kinetics should be shown for 5% and 10% (D0, D4, D6, D8)

What is the effect of CA14 knock down on cell viability and proliferation?

Same for p300/CBP inhibitor?

Fig5A and C: The effect of shCA14 on TYR expression and promoter activity should be shown?

Fig5E: More convincing immunoblot for Ach3K27 decrease in CA14 si-treated cells should be shown.

Fig5F: The role of CBP/P300 in this network is poorly documented. The authors stated that reduced CBP/p300 activity is the cause of reduced Ach3K27, implying that CBP/p300 colocalize with Ach3K27 at the loci investigated.

ChIP-QPCR for CBP/p300 should be shown to support the authors conclusion.

How CA14 and pHi can exert their effect on CBP/P300 activity?

Fig5H: same experiments should be redone with siRNA specific to CBP/p300.

FigS6A: What is the mechanism involved in the nuclear shuttling of CA14 in pigmented NHEM? Is there any change in CA14 localisation in MBM-4 cells exposed to IBMX or aMSH compared to control cells?

FigS6B: Pigmentation of cell pellets for NHEM grown in MBM4 and M254 should be shown.

Minor:

Introduction section:

A number of references are missing

- L51 p3: the references Price et al JBC 1998 and Bertolotto et al JCB 1998 should be added for the regulation of MITF by the cAMP signaling pathway.
- L54 p3: Laurette et al, Elife 2015 should be added to document MITF interaction with SOX10.
- L55 p3: Laurette et al, Elife 2015 should be added to document MITF interaction with BRG1

Results section:

- Spell "wrt" used all throughout the text
- L128: the reference is not correctly cited" Hoashi et al"
- Fig1E: the legend indicated with filled-in box should be modified to be clearer. As indicated on the Y-axis, the results are expressed as 10% CO₂ compared to 5% CO₂.
- Fig2F: Trp1 should be changed into the official gene name Tyrp1 (Legend Fig2F, L176p8...)
- P10, L227: Fig 5E-G should read Fig3E-G
- P13, L295: Experiments using Promoter of kif1b is not referenced.
- Legend of figure 6: correct the numbering A, B, C.....

Referee #3:

This is a study exploring the role of carbonic anhydrase 14 as a critical actor of melanocyte maturation through a feed-forward MITF activation.

Although generally well designed, this study does not address how CA14 localized within the nucleus may so dramatically increase the intracellular pH further resulting into p300/CBP activation. Changes in histone acetylation upon intracellular pH change are proposed to be linked to the need of acetate to "buffer" protons and contribute to their release out of the cells by monocarboxylate transporters (MCT), preventing further reductions in pH_i. Conversely, if pH_i increases, the flow of acetate and protons is favored toward the inside of the cell leading to global histone hyperacetylation. The conventional extracellular location of CA14 (together with CA12) would certainly support this hypothesis by contributing to the formation of bicarbonate, thereby supporting pH_i regulation. In their study, the authors however focus on the nuclear location of CA14. The reasons for this specific location need to be clarified. Is another CA involved in the alkalinization of the intracellular medium while CA14 acts as a scaffold protein for TF stabilization? What are the effects of MCT inhibition? In figure 4 in particular, if CA14 plays such a key role in pH modulation, silencing CA14 in the 5% CO₂ condition and associated pH increase up to pH 7.9 (fig 4B) should document a more striking effect than the 0.15 pH unit change reported in fig. 4D. Stable transduction is required (see comment below). A knock-in experiment is also needed to support the author's claims.

More generally, the use of a Dharmacon pool of siRNA against CA14 is certainly a strength for the efficacy of silencing but further increases the risk of off-targets. Distinct sequences for RNAi strategies should be considered to separately transduce cells and validate the most salient results; possible compensatory mechanisms by other CA should be evaluated. Also the extent of CA14 silencing presented in Suppl. Fig3 is limited, barely reaches 50%. A CRISPR-Cas9 approach should be implemented. This could translate in more striking effects in the AcH3K27 experiments (fig. 5F).

Minor:

- not sure why activity of the unrelated promoter kif1b is increased (instead of unchanged)

1st Revision - authors' response

3 September 2019

Referee 1

Comments for authors

Major concerns:

1. The studies are not sufficient to substantiate the conclusion that MITF directly regulates CA14 expression. CA14 expression at the mRNA level in shMITF cells should be evaluated by q-RT-PCR. In addition, several RNA-seq studies have depleted MITF by shRNA in melanocytes and melanoma cells. Do these studies support the conclusion that MITF regulates CA14 at the mRNA level?

Our Response: We thank the reviewer for constructive suggestions. We have now performed these additional experiments and arrive at the direct regulation of Ca14 by Mitf.

CA14 expression at the mRNA level in shMITF cells is evaluated by q-RT-PCR, and was found to be reduced (**Fig 2D**).

Meta-analysis of shMITF RNA seq studies performed on 501Mel & H3A cells by Laurette P et al; *Elife*, 2015 (PMID-25803486) was carried out. In this data there are two replicates wherein 58% and 52% Mitf knockdown of MITF was observed. In this we observe a 15% and 53% reduction of CA14 RNA raw read counts respectively.

Furthermore, we have analyzed the TCGA melanoma data and find a high correlation in MITF and CA14 expression across several different melanoma samples. Pearson's correlation coefficient for the expression values of CA14 and MITF was found out to be 0.61, comparable to other bona fide Mitf targets (**EV2A**).

These publicly available data substantiate our observations. Wherein we observe decreased mRNA levels of Ca14 in MITF silenced state and a corresponding increase upon MITF overexpression (**Fig 2D**).

These strongly support our conclusions that the regulation is at the mRNA level.

2. Does MITF affect luciferase activity in reporter assays with wild-type putative E/M boxes and is that regulation abolished with mutations in the putative E/M boxes? A more comprehensive set of reporter assays should be performed investigating the role of MITF and the requirement for any or all of the putative MITF recognition sequences.

Our Response: Thank you for the suggestion. We predicted two putative E/M boxes in 1 kb and 3 kb regions of the CA14 promoter. The promoter assays with mutations in E/M box 1 & 2 demonstrate that both the binding sites contribute to CA14 induction by MITF additively. These new data have been included in **Fig 2G** and **EV2F**.

3. Direct binding of MITF to the CA14 locus is also not strongly supported by the data. ChIPs cannot determine direct binding. EMSAs (supershifts and competition EMSAs) with oligonucleotides containing putative MITF binding sites should be conducted.

Our Response: Thank you for the suggestion. We now substantiate the binding of MITF to CA14 promoter by *in vitro* binding experiments.

- Using a comprehensive set of EMSA studies we demonstrate that the encompassing regions of CA14 promoter (in 1 kb and 3 kb upstream of TSS) show a shift. Using a cold competition with cognate wild type (E/M box probe) but not with a mutant E/M box sequence probe these shifts are abrogated (**Fig 2H**).
- Further the biotinylated promoter sequence that demonstrated shift, binds MITF in a promoter pull-down experiment. This is competed by the non-biotinylated cognate E/M box sequence (**Fig 2I**).

Both these experiments establish the direct binding of MITF to CA14 locus.

4. The authors should review the database for ChIP-seq investigating MITF binding genome-wide. Do any of these studies reveal that MITF binds to the regions of the CA14 locus as shown in Fig. 2F?

Our Response: Thank you for the suggestion. We have now analyzed the data.

- In study by Strub et al; *Oncogene* 2011 (PMID: 21258399) we identify MITF occupancy in the proximal promoter regions of human CA14 locus.
- In study by Laurette P et al; *Elife*, 2015 (PMID-25803486), we identify significant enrichment of MITF on human CA14 promoter within 1.5 Kb from the TSS.

In all both of these studies MITF binds to Ca14 locus. MITF ChIP-seq track on human Ca14 promoter is provided in **Appendix** and these points discussed in the revised text. As our studies are in mouse cells, the regions are not identical but the same trend is observed.

5. Fig. 4E shows both nuclear and cytoplasmic localization of CA14. The nuclear signal of CA14 in Fig. 4F is very weak. It would be informative to determine what proportion of CA14 is nuclear and

what proportion is cytoplasmic with additional Westerns looking at the cytoplasmic levels of CA14 and quantifying each relative to the WCL.

Our Response: We have now performed cellular fractionation studies and estimated the nuclear fraction to contain around 45% of CA14 and the rest is cytoplasmic (in post-nuclear supernatant)(**Fig 4E**). Nuclear localization of CA14 is further validated by a Ca14 overexpression and Immunofluorescence (**Fig 4F**).

6. If CA14 is primarily cytoplasmic, then it will be crucial to show that nuclear CA14 is regulating melanogenesis. Does CA14 have a nuclear localization domain? If so, it would strengthen the conclusion that nuclear CA14 regulates expression of melanogenesis, if an intact CA14 but not CA14 lacking the nuclear localization domain rescues melanogenesis in shCA14 depleted cells.

Our Response: Thank you for the suggestion. CA14 has a predicted nuclear localization motif. However, our attempts to trace the dynamic trafficking of Ca14 using tagged constructs suggest unconventional trafficking. We agree there are important and interesting aspects to look into and are working on this currently.

In this work we establish the nuclear localized fraction to be around 45% using fractionation and localize the protein to nucleus by Immunofluorescence studies (**Fig 2F**). Further studies on trafficking and dynamic localization would form a part of our future submissions.

7. The ChIP-experiments in Fig. 5F lack statistics. What are the standard deviations and pvalues?

Our Response: ChIP experiments have been performed in replicates and the mean fold change calculated wrt shNT (non-targeting control) \pm SEM. This has been performed in two independent biological replicates with three technical replicates each, in every experiment (averaged). This data is now provided in the **Appendix**.

8. Additional H3K27ac ChIPs should be conducted with the CBP/P300 inhibitor to determine if CBP/300 activity is involved. Also, the effects of pH on this histone modification should also be interrogated.

Our Response: Thank you for the suggestion. With revised experiments as suggested by you:

- we now demonstrate occupancy of P300 on the pigmentation promoters using ChIP experiments (**Fig 5G**).
- Upon p300/CBP inhibition by C646 a decrease in the H3K27 acetylation is observed in pigmentation gene promoters of Tyr and Dct. This Data is now included in (**Fig 5H**).
- We silenced Ca14 and analyzed H3K27 acetylation levels and found them to be decreased in Dct and Tyr loci (**Fig 5E**).
-

Referee 2:

Here Raja et al. identified carbonic anhydrase 14 (CA14) as a new MITF target. Further, they observed that CA14 up-regulation causes an increase in the intracellular pH_i , which in turn activates p300/CBP histone acetyl transferase activity favoring through H3K27 acetylation MITF transcriptional activity and the pigmentation process.

The paper is mostly well written and provides interesting new data. However, there are a number of issues with the manuscript that need to be addressed. Below are my comments to be considered by the authors.

Our Response: We thank the reviewer for finding the paper well written and the data interesting. Indeed your comments have been constructive and have helped us to substantially modify the manuscript and state our conclusions more emphatically. We are positive that in the revised version you would find most of the comments to be duly addressed.

FigS1A: What are the mechanisms behind pH_i and pH_e stability at 10% CO_2 compared to 5% CO_2 ? Can the authors comment on that?

Our Response: 10% CO_2 levels shifts the equilibrium $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^-$ to an acidic pH_e by promoting the reaction in the forward direction. pH_e then influences pH_i , this is observed in other cell types as well (Schneider D et. al., 2004) (PMID: 15017383) and (McBrian MA et. al., 2013) (PMID: 23201122).

Fig 1B and 1D: the authors should show pigmentation of cell pellets along with protein expression for the whole kinetics (days 4, 6 and 8).

Our Response:

- We provide the cell pellet data for Day 4, 6 and 8 in **Fig 1B**.

- We provide the protein data for whole kinetics for CA14 and MITF in this system (**EV1D**).

Fig1E: MITF level should be shown.

Our Response: MITF protein levels are included in Expanded View (**EV1D**) and the RNA levels in Fig 1E.

FigS1B- text L130 p7: The authors state that cells in an acidic state of pH_i lose pigmentation. This is an over interpretation of the results. What the results show is that changes in the CO_2 level ($5 > 10\%$) delay the pigmentation process.

Our Response: Thank you for this suggestion. We agree, and have modified the text to this effect. “We therefore identified that retaining the cells in an acidic state of pH_i , delays pigmentation by the decreased expression of pigmentation genes despite comparable levels of MITF.”

Fig2A: Is there any correlation between MITF and CA14 level in publicly available datasets?

Our Response: Thank you for raising this question. Yes, we do observe positive correlation between MITF and CA14 expression levels:

- as demonstrated by Laurette P et al (2015) by MITF downregulation in RNA Seq and MITF overexpression by Hoek et al (2008) (PMID:19067971).
- We have also observed that in the melanoma data from TCGA database the correlation of MITF and CA14 expression is very high (Pearson’s correlation coefficient of 0.61) and comparable to other bona fide MITF targets. This data is provided in Expanded View (**EV2A**).

Fig2B-C: As alpha MSH+IBMX treatment increases CA14 level, pH_i changes in B16 cells would be expected. Is there any pH change when cells are exposed to alpha MSH+IBMX?

Our Response: Thank you for the suggestion. We have now performed MSH mediated activation and MITF overexpression as well as knockdown, and observed changes in pH_i . This data is provided in (**EV3B & C**).

Is MITF (forced expression) sufficient to induce CA14 expression and impact the pH_i in B16 cells?

Our Response: Yes. MITF forced expression resulted in increase in CA14 (RNA Fig 2D, protein Fig 2E) and also an increase in pH_i (**EV3C**).

The authors show that IBMX treatment stimulates CA14 promoter activity; however, MITF has not yet been assessed. Is the effect of MITF a direct transcriptional response? This should be demonstrated with a Luc reporter assay.

Our Response: Thank you for the suggestion. We now demonstrate an increase in CA14 promoter activity upon MITF Over expression (**Fig2F**), and its abrogation upon abolishing M/E box sequences (**Fig 2G**).

Fig2G-H: At least 2 different oligos against MITF should be used to ensure specificity and reduce off target possibilities.

Our Response: We have performed MITF knockdown in mouse Melan-A as well as Human primary melanocytes using two different sequence independent siRNAs (**Appendix Table 3**), and observe similar outcomes on CA14 expression.

What is the effect of MITF loss of function on pH_i in B16 cells?

Our Response: Mitf siRNA followed by pH measurement indicates a decrease in pH_i of B16 cells. This data is now included in **EV3C**.

FigS3: What are the effects of CA14 gain and loss of function on pH_i in B16 cells? On pigmentation?

Our Response:

- Ca14 over-expression and silencing increases and decreases the pH_i respectively. This data is included in **Fig 4C**.
- To assess pigmentation alteration by pH, cells require constant and sustained decrease in pH for 8 days. However, to maintain such sustained alteration in pH_i over and above the extraneous CO_2 for 7 to 8 days by silencing Ca14 is challenging. Hence we resort to investigate the effect of CA14 on pigmentation in zebrafish embryos wherein the CO_2 effects are not compounded (**Fig 3A**).

Fig4A-FigS5: MITF level should be shown as control

Our Response: This data is now included in **EV1D**.

Fig4C: the complete kinetics should be shown for 5% and 10% (D0, D4, D6, D8)

Our Response: We have provided the complete kinetic data for the cell pellets (**Fig 1B**), as well western blot for CA14 and MITF (**EV1D**).

What is the effect of CA14 knock down on cell viability and proliferation? Same for p300/CBP inhibitor?

Our Response:

- Silencing of CA14 only mildly reduces the viability/proliferation as detected by MTT assay (<10%).
- Silencing of CA14 using morpholino only mildly reduces embryo survival at 2 days post fertilization (around 15-20%).
- 10 μ M C646 used in this study in Zebrafish (treatment time between 20h to 48 hpf) did not affect embryo viability.
- 10 μ M C646 treatment in B16 cells for 24h reduced the viability/proliferation by around 10-12% and shCa14 marginally around 5-10%.

This information is now provided in **Appendix S4**.

Fig5A and C: The effect of shCA14 on TYR expression and promoter activity should be shown?

Our Response:

- We demonstrate the mRNA levels of Tyr upon shCA14 (**Fig 5B**),
- Tyr protein levels by Western blot analysis upon shCA14 (**Fig 5A**)
- Tyr promoter activity upon Ca14 silencing is included in (**Fig 5D**).

Fig5E: More convincing immunoblot for AcH3K27 decrease in CA14 si-treated cells should be shown.

Our Response: We have now provided the western blot data for AcH3K27 from isolated histone preparation for better quality data upon CA14 silencing. This is included in **Fig 5E**.

Fig5F: The role of CBP/P300 in this network is poorly documented. The authors stated that reduced CBP/p300 activity is the cause of reduced AcH3K27, implying that CBP/p300 colocalize with AcH3K27 at the loci investigated.

ChIP-qPCR for CBP/p300 should be shown to support the authors conclusion.

How CA14 and pHi can exert their effect on CBP/P300 activity?

Our Response: Thank you for this suggestion. We have now performed these experiments.

- We now provide the ChIP-qPCR data for P300 occupancy in the relevant promoters (**Fig 5G**), implicating the binding of P300 in pigmentation gene promoters.
- Ac-H3K27 levels in these promoters upon C646 treatment is shown in (**Fig 5H**), demonstrates p300/CBP mediated local acetylation of H3K27 in pigmentation gene promoters.
- We demonstrate the effect of pH on the *in vitro* acetylation activity of P300 HAT domain, and observe that the enzyme is highly active in alkaline pH and relatively less active (around 8 to 10-fold) under acidic conditions in (**Fig 5I**). This is likely due to the proton abstraction capacity at the active site as suggested by (Zang et al 2014, PMID: 24521098).

Fig5H: same experiments should be redone with siRNA specific to CBP/p300.

Our Response: Thank you for the suggestion. In this study we have performed ChIP experiments with P300 antibody and H3K27 acetylation in the presence of C646 inhibitor on select MITF target promoters. There are two genes P300 and CBP that contribute to the HAT activity that are inhibited by C646. Further p300/CBP have transcriptional activator role in addition to their role as an acetyl transferase, making them difficult to silence in cells due to pleiotropic effects such as on cell cycle. Hence, we used the inhibition by C646 as a handle to modulate H3K27 acetylation.

FigS6A: What is the mechanism involved in the nuclear shuttling of CA14 in pigmented NHEM?

Is there any change in CA14 localization in MBM-4 cells exposed to IBMX or aMSH compared to control cells?

Our Response: We observe the presence of nuclear localized CA14 in primary human melanocytes (EV3H). Dynamic nuclear shuttling has been difficult to capture in Ca14 and it seems to follow an unconventional trafficking. However, we understand that this is an important aspect. Hence we are currently working on the mechanism Ca14 dynamic nuclear shuttling and would become the part of the next manuscript.

FigS6B: Pigmentation of cell pellets for NHEM grown in MBM4 and M254 should be shown.

Our Response: The cell pellet pictures have been included in the EV3I.

Referee 3

This is a study exploring the role of carbonic anhydrase 14 as a critical actor of melanocyte maturation through feed-forward MITF activation.

Although generally well designed, this study does not address how CA14 localized within the nucleus may so dramatically increase the intracellular pH further resulting into p300/CBP activation. Changes in histone acetylation upon intracellular pH change are proposed to be linked to the need of acetate to "buffer" protons and contribute to their release out of the cells by monocarboxylate transporters (MCT), preventing further reductions in pH_i . Conversely, if pH_i increases, the flow of acetate and protons is favored toward the inside of the cell leading to global histone hyperacetylation. The conventional extracellular location of CA14 (together with CA12) would certainly support this hypothesis by contributing to the formation of bicarbonate, thereby supporting pH_i regulation. In their study, the authors however focus on the nuclear location of CA14. The reasons for this specific location need to be clarified. Is another CA involved in the alkalization of the intracellular medium while CA14 acts as a scaffold protein for TF stabilization?

Our response: We thank the reviewer for finding the study well designed and for the constructive suggestions. We gather that the reviewer is referring to the work by McBrien MA et. al., Mol Cell 2013 (PMID:23201122) that we have cited in the manuscript. Our hypothesis was in the same lines, however the effect size of pH mediated acetylation activity of P300/CBP is very high in melanocytic cells and therefore we have followed this lead in the current study. In melanocytes around 50% of Ca14 is localized to the nucleus and may contribute to the pH mediated effects observed. It is likely that pH is buffered by other Carbonic anhydrases as well, but we do not observe them to be positively regulated with pigmentation genes like Ca14. Direct role of CA14 and locus specificity are interesting aspects currently being investigated in the laboratory and would form the part of the next manuscript. Thank you for the suggestion, we will investigate these aspects as well.

What are the effects of MCT inhibition? In figure 4 in particular, if CA14 plays such a key role in pH modulation, silencing CA14 in the 5% CO₂ condition and associated pH increase up to pH 7.9 (fig 4B) should document a more striking effect than the 0.15 pH unit change reported in fig. 4D. Stable transduction is required (see comment below). A knock-in experiment is also needed to support the author's claims.

Our response: Thank you for the suggestion. Consistent but modest pH_i change upon Ca14 silencing and forced-expression, is a result of extraneous CO₂ buffering in cultured cells that confounds pH_i stabilizations.

To circumvent the CO₂ effects we resort to zebrafish system wherein we report:

- Decrease in pH upon silencing
- Decrease in pigmentation upon silencing
- Rescue of pigmentation with increased pH
- Rescue of Pigmentation with catalytically active CA14

Hence, we establish the CA14-pH-pigmentation link better in the zebrafish system. This is now discussed clearly in the revised text.

More generally, the use of a Dharmacon pool of siRNA against CA14 is certainly a strength for the efficacy of silencing but further increases the risk of off-targets. Distinct sequences for RNAi strategies should be considered to separately transduce cells and validate the most salient results; possible compensatory mechanisms by other CA should be evaluated. Also the extent of CA14 silencing presented in Suppl. Fig3 is limited, barely reaches 50%. A CRISPR-Cas9 approach should be implemented. This could translate in more striking effects in the AcH3K27 experiments (fig. 5F).

Our Response: Thank you for the suggestions. Yes, we agree that silencing could have off-target effects.

- We employ the use of Ca14 siRNA and a sequence independent ShRNA to result in similar outcomes in B16 cells.
- Mitf silencing is achieved in mouse and human melanocytes using different sequence independent siRNA pools and we observe concordant changes in Ca14 levels.
- We show that forced-expression of Mitf or Ca14 has the opposite molecular phenotype to that of siRNA silencing
- Use of CRISPR-Cas9 approach is used in zebrafish system along with the rescue to establish the role of CA14.
- More striking changes in H3K27 acetylation is observed when we perform histone isolation followed by western analysis (**Fig 5E**). Also we substantiate the H3K27 changes upon C646 treatment in a promoter specific manner (**Fig 5H**).

Minor Comments:

Referee 1:

1. The pigmentation models in Fig. 2S are not well explained. More details are needed regarding the passage of cells in mice and in culture and the effects on pigmentation. Furthermore, it is not clear which pigmentation model was used for the studies described on page 6 and shown in Fig. 1.

Our Response: Thank you for this suggestion. In this study we employ the *in vitro* pigment oscillator model for the studying pigmentation. We apologise for not being clear on this. Now we have written details in the text as well as elaborated this in **Appendix S1**.

2. Although the CA14 is regulated by MITF, unlike other MITF target genes, its expression at the protein level is not affected by pH (shown in Fig. 4C). Is the CA14 mRNA level altered by pH? Is it regulated by CBP/p300? The authors should investigate why CA14 expression, if MITF dependent, is not regulated by pH.

Our Response: Thank you for raising this question. In this study, we report that the pH mediated effect is specific to Tyr and Dct promoters, clearly this is promoter selective. As rightly pointed out, we observed from (**Fig 5G**) that Ca14 promoter is not occupied by P300. As you have rightly pointed out Ca14 and other MITF effectors such as Cdk2 behave differently. The epigenetic code alterations and their locus selection is indeed interesting and is being currently investigated.

In this study we report this observation and the players involved. Our future work would address the detailed molecular basis of promoter selectivity.

Other experiments:

Investigate the effects of CBP/P300 inhibitor on melanogenesis in zebrafish

Our Response: Thank you for this suggestion. We have now performed treatment of zebrafish embryos with C646 and observe a consistent decrease in pigmentation (**Fig 5K**).

Referee 2:

Introduction section:

A number of references are missing

- L51 p3: the references Price et al JCB 1998 and Bertolotto et al JCB 1998 should be added for the regulation of MITF by the cAMP signaling pathway.

- L54 p3: Laurette et al, Elife 2015 should be added to document MITF interaction with SOX10.

-L55 p3: Laurette et al, Elife 2015 should be added to document MITF

interaction with BRG1

Our Response: We apologise for these inadvertent omissions, we have rectified them all in the current submission.

Results section:

- Spell "wrt" used all throughout the text
- L128: the reference is not correctly cited" Hoashi et al"
- Fig1E: the legend indicated with filled-in box should be modified to be clearer. As indicated on the Y-axis, the results are expressed as 10% CO₂ compared to 5% CO₂.
- Fig2F: Trp1 should be changed into the official gene name Tyrp1 (Legend Fig2F, L176p8...)
- P10, L227: Fig 5E-G should read Fig3E-G
- P13, L295: Experiments using Promoter of kif1b is not referenced.
- Legend of figure 6: correct the numbering A, B, C...

Our Response: We apologize for these mistakes. These have now been rectified.

2nd Editorial Decision

1 October 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. Original referee #3 was not responsive to our invitations to re-assess the study. But, as you will see, both remaining referees now support the publication of your study in EMBO reports. However, referee #1 has remaining concerns and further suggestions to improve the study, we ask you to address in a final revised version of the manuscript. Please also provide a point-by-point-response addressing these remaining points.

Further, I have these editorial requests:

- I suggest a slightly shortened title:
pH-controlled histone acetylation amplifies melanocyte differentiation downstream of MITF
- Please format the references according to our journal style. Please use 'et al' if there are more than ten authors. See:
<http://www.embopress.org/page/journal/14693178/authorguide#referencesformat>
- It seems there is no call out for Fig. 1D. Please check.
- Fig. 4F is called out in the text, but there is no such panel in the figure. Please check.
- Please check the nomenclature used for EV and Appendix items. EV figures should be named and called out as 'Figure EVX', and items from the Appendix as 'Appendix Figure Sx, Appendix Table Sx'. Please correct the call outs in the manuscript text and also the naming in the Appendix.
- Please provide the Appendix file as pdf.
- Please add uniform and thicker scale bars to all microscopic images (also in the Appendix). Currently, Figures 1C, 6B/C and S5B seem not to have no scale bars, and the current scale bars in Figs. 4D, EV3H and S5C are too thin. Please provide all scale bars without any writing indicating their size on them. Please indicate the size only in the respective figure legend.
- The upper left panel in Figure S1 looks partly out of focus, and in both upper panels in this figure are a lot of question marks (?). Please fix this.
- There are also question marks in the upper labels of Fig. EV2A. Please fix this.
- The panels in Fig. 4A (tubulin), Fig. EV1C, Fig. EV2C (CA14 and Tubulin) are overcontrasted.

Please provide the Western blot data as unmodified as possible, showing similar brightness and contrast as in the source data.

- Thank you for providing the source data for the Western blots. Please upload these in the final version as pdf files. Please also provide the source data for the Western blots in the Appendix (as one pdf file).
- The source data for the first column in Fig. 2E is missing. Please provide this. In the paper the loading is labelled as GAPDH, in the source data as Tubulin. Please check.
- The source data for Fig. EV3 got mixed up. Data for Fig. EV3G is shown twice (as data for EV3D), and the source data for EV3I is labelled as EV3G. Please check.
- In the source data for Fig. EV4A the panel for acH3K27 is missing. Please add this.
- Please carefully go through the final source data files and check that for all manuscript figures panels are shown, and that they are labelled correctly.
- Statistical testing and error bars do not make much sense if only two replicate experiments are shown (see Fig. 5K, EV2E). Please add a third replicate, or remove statistics and error bars. Please show the two replicates as separate bars in these cases.
- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

In addition I would need from you:

- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

REFEREE REPORTS

Referee #1:

Most of my concerns have been met. There are just a few minor issues:

Fig. EVD2 shows reporter assays with IBMX only. The text on page 9, lines 189 to 190 state that EV2D show reporter activity with IBMX and alpha MSH. The text should be corrected or data with alpha MSH should be presented.

Fig. 5F, G, H: It is not clear which if any of the ChIP enrichments is significant. The authors should state whether the numbers reflect significant differences in ChIP signal compared to IgG. Was a control region assayed? Page 15, lines 349-352 suggest that the findings confirm a previous study that showed high HK24ac levels at pigmentation genes compared to the global average. However, this statement is valid only if the H3K27ac levels shown in Fig. 5F are compared to non-pigmentation loci. Also, it is difficult to see the numbers with the current color scheme. This data would really best be presented as graphs with error bars and standard deviations.

Referee #2:

The revised manuscript has been substantially improved by the new experiments provided. The

authors answered satisfactorily to my concerns and they provided some clarifications on the points highlighted. I have no further comments.

2nd Revision - authors' response

15 October 2019

Referee #1:

Most of my concerns have been met. There are just a few minor issues:

Our Response: Thank you for the constructive suggestions during the review process. In this revised version these minor issues are also addressed.

Fig. EVD2 shows reporter assays with IBMX only. The text on page 9, lines 189 to 190 state that EV2D show reporter activity with IBMX and alpha MSH. The text should be corrected or data with alpha MSH should be presented.

Our Response: Thank you for pointing out. We now provide the data with IBMX only and the text is suitably modified.

Fig. 5F, G, H: It is not clear which if any of the ChIP enrichments is significant. The authors should state whether the numbers reflect significant differences in ChIP signal compared to IgG. Was a control region assayed? Page 15, lines 349-352 suggest that the findings confirm a previous study that showed high HK24ac levels at pigmentation genes compared to the global average. However, this statement is valid only if the H3K27ac levels shown in Fig. 5F are compared to non-pigmentation loci. Also, it is difficult to see the numbers with the current color scheme. This data would really best be presented as graphs with error bars and standard deviations.

Our Response: We have performed students t test to compare the IgG and ChIP enrichment and the data is provided in Appendix Table S5, S6 and S7 for Fig 5F, 5G and 5H respectively. The same is also indicated now in the figure legend. We assayed a non-pigmentation MITF locus Cdk2 and observe no change in acH3K27 occupancy. The data is also provided in Appendix Table S5-7 for better clarity.

Referee #2:

The revised manuscript has been substantially improved by the new experiments provided. The authors answered satisfactorily to my concerns and they provided some clarifications on the points highlighted. I have no further comments.

Our Response: Thank you for your inputs.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Vivek T Natarajan

Journal Submitted to: EMBO reports

Manuscript Number: EMBOR-2019-48333-T

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures**1. Data****The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if $n < 5$, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions**Each figure caption should contain the following information, for each panel where they are relevant:**

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

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We employed technical replicates at least three per condition and the experiment was carried out biological triplicates in most of the data presented in this manuscript.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For Zebrafish experiments we performed experiments with a minimum of 100 animals per condition for morpholino injections. As the number of animals in the mutant were limited comparison between wild type and Ca14fs003 were carried out with 50 animals per condition.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We excluded embryos that were deformed or developmentally delayed to avoid secondary effects. This criteria was pre established.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Once we performed scoring of phenotype, the genotype of the animal was verified by PCR.
For animal studies, include a statement about randomization even if no randomization was used.	None
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	sufficient group size was included (50-100 embryos) derived from the same cross were used.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding was not done in these experiments. Phenotypic outcomes were verified by another independent assessor.
5. For every figure, are statistical tests justified as appropriate?	Yes to the best of our knowledge and understanding.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.

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Is there an estimate of variation within each group of data?	yes. Across independent biological experiments we have analyzed and estimates of variation are available.
Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), IDegreeBio (see link list at top right).	These are provided in Appendix.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	B16 cells were checked routinely checked for mycoplasma using MycoAlert kit from Lonza.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	These are provided in the text and elaborated in materials and methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	institutional Animal Ethics committee approved the protocol and the detail is provided Methods section.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	None. Not applicable
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Additional data such as full western blot images have been provided in Appendix.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomedels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	None applicable
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