THE EMBO JOURNAL

STIM1 activation of adenylyl cyclase 6 connects Ca2+ and cAMP signaling during melanogenesis

Rajender Motiani, Jyoti Tanwar, Desingu Ayyappa Raja, Ayushi Vashisht, Shivangi Khanna, Sachin Sharma, Sonali Srivastava, Sridhar Sivasubbu, Vivek T. Natarajan and Rajesh S. Gokhale

Review timeline:

Submission date:	15 June 2017
Editorial Decision:	12 July 2017
Revision received:	12 November 2017
Editorial Decision:	22 November 2017
Revision received:	29 November 2017
Accepted:	06 December 2017

Editor: Ieva Gailite

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 July 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received two referee reports on your manuscript, which I have included below for your information.

As you can see from the comments, both reviewers express interest in the presented mechanism of STIM1/ADCY6 interplay. However, they also raise substantive concerns with the analysis that would need to be addressed before they can support publication here. From my side, I judge the referee comments to be generally reasonable, therefore I would like to invite you to submit your revised manuscript while addressing the comments of both reviewers. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. Please contact us in advance if you would need an additional extension. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work to discuss how to proceed.

Referee #1:

These are interesting studies reporting several new findings related to melanocytes and

melanogenesis and for signaling by STIM1. The authors screens for genes and proteins that regulate melanogenesis and discovered an unexpected but separate roles for STIM1-Orai1 mediated Ca2+ influx and for STIM1 in melanogenesis. They continue to use biochemical and molecular assays together with a zebrafish model to show that Ca2+ influx by the STIM1-Orai1 complex regulates melanocytes proliferation, while STIM1 acts independent of Ca2+ to regulate the Ca2+-independent AC6 and cellular cAMP levels stimulated by aMSH. STIM1 interacts with AC6 through its S/P domain. This is the first study to show a specific and direct role of the S/P domain in STIM1 function.

Although most of the experiments are well done and for the most part the main conclusions are supported by the data, several controls are missing and how aMSH may regulate Ca2+ signaling may not be by cAMP-mediated Ca2+ release as proposed. Several experiments suggested below should strengthen the manuscript.

Major comments:

1. The authors should generate STIM1(delta S/P domain) and test whether it is capable of mediating Ca2+ release, supporting cAMP generation, pigmentation and, most importantly, interaction with AC6. The zSTIM1b differs in several sequences from zSTIM1a and the results with these constructs although strongly suggestive, will strengthen the conclusion concerning the role of the S/P domain.

2. Does overexpression of STIM1 and constitutively active STIM1 (like STIM1(D76A)) affect the activities above? Does expression of STIM1(D76A)+AC6 is sufficient to cause pigmentation? This will further reveal the role of activation of AC6 by STIM1 in melanocytes pigmentation and support the results in Figure 6.

3. In the rescue experiments, does STIM1(delta K) that does not target to the ER/PM junctions rescues cAMP generation and melanogenesis? This should reveal the specific targeting of STIM1 to the junctions in the regulation of AC6.

4. Figure 5: It is not clear why the authors attribute the aMSH-mediated Ca2+ release to cAMP. aMSH stimulation generates IP3 and this cannot be by cAMP. All the reference cited show that the increased Ca2+ release is due to activation of PLCbeta1 by Gβγ and this is likely the case here. The scheme is Figure 5 is not correct or supported by the experiments provided. To attribute the release to cAMP the authors needs to show that scavenging cAMP (not inhibition of production) in aMSH-stimulated cells prevents the Ca2+ release. Otherwise, the scheme in the Figure should be removed. Again, the important part is to show IP3 production and Ca2+ release from store. All the rest is not relevant or supported by the data.

5. To complete the studies with the ACs, the authors should test whether STIM1 interacts with AC4, 5 and 7 since knockdown of these ACs had an effect in one of the assays that are used all along the manuscript. If interaction is found, its role in STIM1-mediated cAMP generation and pigmentation studied and reported.

Minor comments:

Figure 4: The authors should clarify if pigmentation in zebrafish is regulated hormonally by an aMSH-like mechanism and provide quantification of the results in Figure 4E.

Figure 7b,c: Inputs should be shown for all conditions, including AC6 alone and STIM1 alone.

Page 3: Ref 9 did not show direct effect of STIM1 on AC as shown here. This study only showed STIM1 effect that is independent of Orai1.

Page 13, line 6: references should be given for the effect of 2ABP on STIM1 oligomerization.

Referee #2:

The manuscript entitled "STIM1 activation of Adenylyl cyclase 6 connects Ca2+ and cAMP signaling through a positive feedback loop during melanogenesis" is an intriguing study describing multi-level crosstalk mechanisms between SOCE and cAMP for control of melanin synthesis. Hence, the authors provide circumstantial evidence that cAMP mediates PLC activation by aMSH, leading to ER Ca2+ depletion. This then causes STIM1 oligomerization and direct activation of adenylate cyclase 6. The later point is the primary source of novelty for this study; although STIM1 was shown to induce cAMP production 8 years ago, the identity of the AC mediating this effect has not been published. Further, subsequent studies by other groups have focused on the role of Orai1 in cAMP production. These papers combined with the lack of prior identification of the AC targeted by STIM1 despite continued efforts to do so by the laboratory that made this finding provide both impact and controversy to these observations. With that in mind, I think that these findings could be high impact, but that the work needs to be more convincing and complete than in its current form. This and others concerns described below:
Comments:
1. In figure 5f, the authors show that forskolin stimulates ER Ca2+ release. The stated purpose of this experiment was to show that Gs mediates PLC activation through AC, however, this experiment is insufficient to demonstrate this. An inhibitor approach should have been used to show that AC mediates PLC activation by MSH. Assuming that this experiment will, in fact, show that AC mediates PLC activation, further effort is needed to delineate the relationship between AC and Ca2+ signaling, since AC is required both for ER Ca2+ depletion and subsequent AC activation. Indeed, considering that a screen of all ACs was performed in this study, it is surprising that the contribution of AC to MSH-induced ER Ca2+ depletion was not assessed. This would be a strong and seemingly achievable addition to the study. The implications of this apparent feedback loop between AC and ER Ca2+ depletion also requires discussion that was not provided.
2. In figure S6, the authors use 2-APB to disrupt STIM1 oligomerization. 2-APB has complex concentration-dependent effects on STIM-Orai function. As such, the author's interpretation of this data as demonstrating that STIM1 oligomerization has Ca2+-independent effects is an over-interpretation. Similarly, it is not clear that the stated mechanism for inhibition of STIM/Orai by ML-9 is, in fact, via microtubule disruption, although ML-9 does inhibition STIM1 oligomerization. However, I'm surprised that a genetic approach wasn't used. Hence, oligomerization mutants and/or SOAR mutants (such as STIM1-F394A) could be used to demonstrate the dependence on STIM1 oligomerization much more effectively than this pharmacological strategy.
3. The colocalization/immunoprecipitation studies between STIM1 and ADCY6 are unconvincing. The dynamic nature of the interaction between STIM1 and ADCY6 should be shown by FRET analysis with appropriate partners (GFP-mCherry or CFP-YFP).
4. The evidence that the PS domain of STIM1 is important for ADCY6 activation is really very thin, leaning primarily on zebrafish genes with multiple levels of genetic variation. While the authors are correct that a portion of the PS domain is different in these genes, there are other differences as well. The authors should utilize mutation/deletion analysis of the PS domain in human STIM1 and determine if it affects ADCY6 activation. Upon generation, interaction with ADCY6 should be assessed by FRET as discussed in the preceding point.
Minor comments:
1. Top of page 7: STIM and Orai proteins are known to mediate SOCE in nearly all non-excitable cells. Actually, STIM and Orai are the primary mediators of SOCE in virtually all animal cells.

2. In several places, spaces are missing between words.

1st Revision - authors' response 12 November 2017

(begins on next page)

Response to reviewers' comments

Reviewer #1

These are interesting studies reporting several new findings related to melanocytes and melanogenesis and for signaling by STIM1. The authors screens for genes and proteins that regulate melanogenesis and discovered an unexpected but separate roles for STIM1-Orai1 mediated Ca2+ influx and for STIM1 in melanogenesis. They continue to use biochemical and molecular assays together with a zebrafish model to show that Ca2+ influx by the STIM1-Orai1 complex regulates melanocytes proliferation, while STIM1 acts independent of Ca2+ to regulate the Ca2+-independent AC6 and cellular cAMP levels stimulated by aMSH. STIM1 interacts with AC6 through its S/P domain.

This is the first study to show a specific and direct role of S/P domain in STIM1 function.

Although most of the experiments are well done and for the most part the main conclusions are supported by the data, several controls are missing and how aMSH may regulate Ca2+ signaling may not be by cAMP-mediated Ca2+ release as proposed. Several experiments suggested below should strengthen the manuscript.

We really appreciate the insightful comments of the reviewer. We have performed all the experiments suggested by the reviewer and these data have made manuscript more compelling. The point wise response to the reviewer comments is as follows:

Major comments:

Comment 1. *The authors should generate STIM1(delta S/P domain) and test whether it is capable of mediating Ca^{2+} release, supporting cAMP generation, pigmentation and, most importantly, interaction with AC6. The zSTIM1b differs in several sequences from zSTIM1a and the results with these constructs although strongly suggestive, will strengthen the conclusion concerning the role of the S/P domain.*

Response: As recommended by the reviewer, we have studied STIM1 ΔS/P for its ability to mediate αMSH induced Ca^{2+} release, cAMP generation, melanogenesis and its interaction with ADCY6. We observed that:

a. The overexpression of STIM1 ΔS/P results in significant decrease in the αMSH induced ER Ca^{2+} release.
b. The ability of STIM1 ΔS/P in regulating cAMP generation and melanogenesis was examined by carrying out rescue experiments in the B16 shSTIM1 stable cells. While the STIM1 ΔS/P complementation does not restore the decrease in cAMP levels and melanogenesis, the full-length STIM1 showed complete rescue of these properties.
c. Further, co-immunoprecipitation studies performed with full length STIM1 and STIM1 ΔS/P validated an essential role of STIM1 S/P domain in interaction with ADCY6.

Collectively, this data establishes an important role of STIM1 S/P domain in regulating melanogenesis via its interaction with ADCY6. All this data is presented in the **Fig 8C-F** of the revised manuscript.

Comment 2. *Does overexpression of STIM1 and constitutively active STIM1 (like STIM1(D76A)) affect the activities above? Does expression of STIM1(D76A)+AC6 is sufficient*

to cause pigmentation? This will further reveal the role of activation of AC6 by STIM1 in melanocytes pigmentation and support the results in Figure 6.

Response: We evaluated the efficiency of wild-type STIM1 and STIM1 D76A in variety of experimental models for the induction of αMSH induced Ca^{2+} release, cAMP generation and melanogenesis.

a. No significant differences were observed in the αMSH induced Ca^{2+} release upon overexpression of either wild type STIM1 or STIM1 D76A.
b. Both wild type STIM1 and STIM1 D76A rescue the decrease in αMSH induced cAMP generation observed in shSTIM1 stables.
c. The experiments performed in LD melanogenesis assay with shSTIM1 stable cells demonstrated that both wild type STIM1 and STIM1 D76A can restore pigmentation.
d. Further, we tested if the overexpression of STIM1 D76A alone or along with ADCY6 is enough to induce pigmentation in high-density cultured cells. We observed that just the ectopic expression of these proteins is not sufficient to cause pigmentation.
e. However, the overexpression of STIM1 D76A + ADCY6 resulted in almost three fold increase in the αMSH stimulated pigmentation.

We therefore observe that the constitutively active STIM1 (STIM1 D76A) is able to rescue the STIM1 knockdown effects. However, it cannot constitutively activate pigmentation by itself. Interestingly, simultaneous overexpression of ADCY6 and STIM1 D76A enhances αMSH induced pigmentation. We have included the data from these studies in the **Fig EV4.**

Comment 3. *In the rescue experiments, does STIM1(delta K) that does not target to the ER/PM junctions rescues cAMP generation and melanogenesis? This should reveal the specific targeting of STIM1 to the junctions in the regulation of AC6.*

Response: We have performed rescue experiments with STIM1 ΔK in the shSTIM1 stable background. The STIM1 ΔK was not able to rescue cAMP generation and melanogenesis whereas corresponding full length STIM1 control was able to completely rescue both cAMP accumulation and melanin content. We have included this data in the **Fig 8D-E** of the revised manuscript.

Comment 4. *Figure 5: It is not clear why the authors attribute the aMSH-mediated Ca^{2+} release to cAMP. aMSH stimulation generates IP_3 and this cannot be by cAMP. All the reference cited show that the increased Ca^{2+} release is due to activation of PLCbeta1 by Gβγ and this is likely the case here. The scheme is Figure 5 is not correct or supported by the experiments provided. To attribute the release to cAMP the authors needs to show that scavenging cAMP (not inhibition of production) in aMSH-stimulated cells prevents the Ca^{2+} release. Otherwise, the scheme in the Figure should be removed. Again, the important part is to show IP_3 production and Ca^{2+} release from store. All the rest is not relevant or supported by the data.*

Response: We agree with the reviewer that it's important to show IP_3 production and Ca^{2+} release upon αMSH application and have performed the experiments to demonstrate this (**Fig 5**). At this stage we would also like to remove the scheme that was initially presented in the **Fig 5A**. Additionally, as suggested by reviewer #2, we have performed experiments with PLC inhibitor U73122 and its inactive analog U73343. The data presented in revised **Fig 5** demonstrates that the PLC inhibitor completely abrogates αMSH stimulated Ca^{2+} release while its inactive analog does not affect it. Taken together, this data suggests that αMSH induced ER Ca^{2+} release is indeed downstream of PLC activation and IP_3 generation.

Comment 5. *To complete the studies with the ACs, the authors should test whether STIM1 interacts with AC4, 5 and 7 since knockdown of these ACs had an effect in one of the assays that are used all along the manuscript. If interaction is found, its role in STIM1-mediated cAMP generation and pigmentation studied and reported.*

Response: As suggested by the reviewer, we evaluated the interaction between STIM1 and ADCY4, 5 and 7. In our B16 model system, we could not observe the interaction of these ADCYs with STIM1 using commercially available antibodies (**Appendix Fig S5**). However, we would like to submit that the antibodies for these ADCYs were not very specific. While the antibodies detected the expected proteins (based on their molecular weight and antibody data sheet), multiple non-specific bands are also picked up even after substantial standardization.

Minor comments:

Figure 4: The authors should clarify if pigmentation in zebrafish is regulated hormonally by an aMSH-like mechanism and provide quantification of the results in Figure 4E.

Response: αMSH has been shown to play an important role in zebrafish pigmentation especially in the melanophore dispersion, patterning and melanization. We have included this information in the revised text and have cited relevant studies. Further, we have presented the quantification of in situ data in the **Fig 4F** of the revised manuscript.

Figure 7b,c: Inputs should be shown for all conditions, including AC6 alone and STIM1 alone.

Response: We have revised IP blots and have included ADCY6 alone and STIM1 alone input blots in the **Fig 7C, D** and **Fig 8F**.

Page 3: Ref 9 did not show direct effect of STIM1 on AC as shown here. This study only showed STIM1 effect that is independent of Orai1.

Response: We really appreciate the reviewer's comment and have modified the phrase accordingly.

Page 13, line 6: references should be given for the effect of 2ABP on STIM1 oligomerization.

Response: We have included reference on 2APB's effect on STIM1 oligomerization.

Reviewer #2

The manuscript entitled "STIM1 activation of Adenylyl cyclase 6 connects Ca2+ and cAMP signaling through a positive feedback loop during melanogenesis" is an intriguing study describing multi-level crosstalk mechanisms between SOCE and cAMP for control of melanin synthesis. Hence, the authors provide circumstantial evidence that cAMP mediates PLC activation by aMSH, leading to ER Ca2+ depletion. This then causes STIM1 oligomerization and direct activation of adenylate cyclase 6. The later point is the primary source of novelty for this study; although STIM1 was shown to induce cAMP production 8 years ago, the identity of the AC mediating this effect has not been published. Further, subsequent studies by other groups have focused on the role of Orai1 in cAMP production. These papers combined with the lack of prior identification of the AC targeted by STIM1 despite continued efforts to do so by the laboratory that made this finding provide both impact and controversy to these observations. With that in mind, I think that these findings could be high impact, but that the work needs to be more convincing and complete than in its current form. This and others concerns described below:

We highly appreciate the constructive comments of the reviewer. We have completed all the suggested experiments and these studies have further strengthened the manuscript. The point wise response to the reviewer comments is as follows:

Major comments:

Comment 1. *In figure 5f, the authors show that forskolin stimulates ER Ca2+ release. The stated purpose of this experiment was to show that Gs mediates PLC activation through AC, however, this experiment is insufficient to demonstrate this. An inhibitor approach should have been used to show that AC mediates PLC activation by MSH. Assuming that this experiment will, in fact, show that AC mediates PLC activation, further effort is needed to delineate the relationship between AC and Ca2+ signaling, since AC is required both for ER Ca2+ depletion and subsequent AC activation. Indeed, considering that a screen of all ACs was performed in this study, it is surprising that the contribution of AC to MSH-induced ER Ca2+ depletion was not assessed. This would be a strong and seemingly achievable addition to the study. The implications of this apparent feedback loop between AC and ER Ca2+ depletion also requires discussion that was not provided.*

Response: We have performed several αMSH induced ER Ca^{2+} release experiments with ADCYs inhibitor SQ22536; PLC inhibitor U73122; its inactive analog U73343 and siRNAs targeting ADCYs. In these studies, we observed:

a. Complete abrogation of ER Ca^{2+} release upon treatment with U73122 while its inactive analog did not affected Ca^{2+} release.
b. Further, the general ADCYs inhibitor SQ22536 significantly inhibited the αMSH induced ER Ca2+ release. The inhibitor data is presented in the **Fig 5E** of the revised manuscript.

c. As suggested by the reviewer, we next performed siRNA screening of ADCYs for evaluating their role in αMSH induced ER Ca^{2+} release. Our imaging assays show that the knockdown of ADCY5 and ADCY6 results in significant decrease in the αMSH stimulated ER Ca^{2+} release (**Fig 7B**).

Additionally, we have included the implications of the feedback loop in the revised discussion (second last paragraph of the discussion).

Comment 2. *In figure S6, the authors use 2-APB to disrupt STIM1 oligomerization. 2-APB has complex concentration-dependent effects on STIM-Orai function. As such, the author's interpretation of this data as demonstrating that STIM1 oligomerization has Ca2+-independent effects is an over-interpretation. Similarly, it is not clear that the stated mechanism for inhibition of STIM/Orai by ML-9 is, in fact, via microtubule disruption, although ML-9 does inhibition STIM1 oligomerization. However, I'm surprised that a genetic approach wasn't used. Hence, oligomerization mutants and/or SOAR mutants (such as STIM1-F394A) could be used to demonstrate the dependence on STIM1 oligomerization much more effectively than this pharmacological strategy.*

Response: We really appreciate the reviewer's recommendation of using SOAR mutant for establishing Orai1 independent role of STIM1 oligomerization in pigmentation. We employed SOAR mutant (STIM1 F394H) and evaluated its ability to rescue cAMP generation and pigmentation observed upon STIM1 silencing. As reported in the **Fig 6E and H**, this SOAR mutant is able to restore both cAMP generation and pigmentation. Further, we have rephrased the statement on the ML-9 and have included relevant references.

Comment 3. *The colocalization/immunoprecipitation studies between STIM1 and ADCY6 are unconvincing. The dynamic nature of the interaction between STIM1 and ADCY6 should be shown by FRET analysis with appropriate partners (GFP-mCherry or CFP-YFP).*

Response: In accordance with the reviewer's suggestion, we have performed FRET studies with ADCY6-CFP and STIM1-YFP. The data presented in the **Fig 7E** demonstrate that STIM1 and ADCY6 interact upon Ca^{2+} store depletion in the melanocytes. Additionally, as suggested by the reviewer #1, we have included input control blots for the co-immunoprecipitation data that further provides confidence to the data.

Comment 4. *The evidence that the PS domain of STIM1 is important for ADCY6 activation is really very thin, leaning primarily on zebrafish genes with multiple levels of genetic variation. While the authors are correct that a portion of the PS domain is different in these genes, there are other differences as well. The authors should utilize mutation/deletion analysis of the PS domain in human STIM1 and determine if it affects ADCY6 activation. Upon generation, interaction with ADCY6 should be assessed by FRET as discussed in the preceding point.*

Response: This comment of reviewer #2 is also specified by reviewer #1 (comment 1); therefore we performed several independent experiments with STIM1 ΔS/P for evaluating its role in ADCY6 activation. We observe that STIM1 S/P domain plays an important role in

a. cAMP generation
b. Melanogenesis
c. ADCY6 interaction

Further, as advised by the reviewer, we performed FRET studies with ADCY6-CFP and STIM1 ΔS/P-YFP. These experiments corroborated with the other biochemical and cellular data. Taken

together, all these data (**Fig 8D-G**) demonstrate that STIM1 S/P domain plays a critical role in ADCY6 activation.

Minor comments:

1. Top of page 7: STIM and Orai proteins are known to mediate SOCE in nearly all non-excitable cells. Actually, STIM and Orai are the primary mediators of SOCE in virtually all animal cells.

Response: We have rephrased this sentence in the revised manuscript.

2. In several places, spaces are missing between words.

Response: We apologize for the spacing typos. They have been corrected in the revised manuscript.

2nd Editorial Decision 22 November 2017

Thank you for submitting a revised version of your manuscript. It has now been seen by the two original referees, who find that their main concerns have now been addressed. There are just a few minor mainly editorial issues to be dealt with formal acceptance here. Congratulations on a nice study!

1. Please address the remaining comments of reviewer #2 regarding the discussion of results and data presentation.

Referee #1:

The authors thoroughly addressed all my concerns and I find the manuscript suitable for publication in The EMBO Journal in its current form.

Referee #2:

The manuscript is much improved upon revision, which has served to increase its impact. Still, some concerns remain.

The fact that STIM1-deltaK and STIM1-deltaSP have the exact same lack of effect on both cAMP production and melanin content is something of a concern. I do not accept the authors' claim that STIM1-deltaK does not target to ER-PM junctions. It will not do so in the absence of Orai1, but will target ER-PM junctions when Orai1 (and perhaps other targets) are present (Xiao et al, Nat Cell Biol, 2011). Similar points are actually made in some of the papers that the author has cited (Calloway, 2011). As such, the implications of this finding needs further discussion.

It is notable that both siADCY and SQ22536 only partially inhibited ER Ca2+ release. Representative data would be helpful - perhaps in the supplement. Irrespective, while interesting, the authors should discuss alternative mechanisms of PLC activation (is Gq activated by MSH?).

STIM1 expression is presented diagonally in Figure 7D. Surely this can be corrected.

2nd Revision - authors' response 29 November 2017

(begins on next page)

Response to Reviewers Comments:

Referee #1:

The authors thoroughly addressed all my concerns and I find the manuscript suitable for publication in The EMBO Journal in its current form.

We are pleased to know that the reviewer found our revised manuscript suitable for publication in the EMBO Journal.

Referee #2:

The manuscript is much improved upon revision, which has served to increase it's impact. Still, some concerns remain.

We really appreciate the positive feedback of the reviewer. We have address the remaining comments of the reviewer and a point wise response to the comments is provided below:

1. The fact that STIM1-deltaK and STIM1-deltaSP have the exact same lack of effect on both cAMP production and melanin content is something of a concern. I do not accept the authors' claim that STIM1-deltaK does not target to ER-PM junctions. It will not do so in the absence of Orai1, but will target ER-PM junctions when Orai1 (and perhaps other targets) are present (Xiao et al, Nat Cell Biol, 2011). Similar points are actually made in some of the papers that the author has cited (Calloway, 2011). As such, the implications of this finding needs further discussion.

Response: *We appreciate the reviewers comment on targeting of STIM1 ΔK mutant to ER-PM junctions. It is important to note that earlier studies have clearly demonstrated that the polybasic clusters in several PM targeting proteins play a critical role in their recruitment to PM (Heo et al, Science, 2006). Similarly, it has*

been corroborated that STIM1 ΔK mutant is not able to translocate to ER-PM junctions (Liou et al, PNAS, 2007).

Although in some instances STIM1 ΔK mutant was shown to target ER-PM junctions, it does so only upon overexpression of Orai1 in these cells (Park et al, Cell, 2009). Actually, one of the studies specified by the reviewer also suggests that ectopic Orai1 expression is required for STIM1 ΔK mutant puncta formation at the ER-PM junctions (Xiao et al, Nat Chem Biol, 2011; Park et al, Cell, 2009). However, in our studies we have not performed STIM1 ΔK mutant experiments along with Orai1 overexpression. Therefore, we believe that under these experimental conditions STIM1 ΔK mutant will not target ER-PM junctions.

2. It is notable that both siADCY and SQ22536 only partially inhibited ER Ca2+ release. Representative data would be helpful - perhaps in the supplement.
Irrespective, while interesting, the authors should discuss alternative mechanisms of PLC activation (is Gq activated by MSH?).

Response: *Reviewer has specifically pointed to the partial inhibition of Ca^{2+} release with siADCY and SQ22536. This incomplete abrogation could be explained by the extent of ADCYs silencing by the siRNAs used in these studies. Indeed, our data suggests that the siADCYs were able to decrease the expression of ADCYs by 50-60% (**Appendix Figure 5**) and we observe a corresponding decrease in ER Ca^{2+} release experiments (**Figure 7B**).*

*Further, previous studies have used up to 500μM SQ22536 for complete inhibition of ADCYs while in order to rule out any non-specific effects, we have used 100μM SQ22536 in the Ca^{2+} release assays. This could be a possible reason behind partial inhibition of ER Ca^{2+} release (**Figure 5E**). As suggested by the reviewer, we have presented the representative data in the **Appendix Figure 4.***

Interestingly, αMSH was recently reported to activate an orphan G-coupled receptor GPR139. Upon overexpression in CHO cells, this orphan receptor was demonstrated to behave like a Gq receptor (Nøhr et al. Neurochemistry International, 2017). It is important to note that αMSH was reported to be 100 fold less potent agonist of GPR139 in comparison to MC4R (Gs receptor) (Nøhr et al. Neurochemistry International, 2017). Further, the expression of GPR139 is limited to brain and

central nervous system. However, at this stage we cannot completely rule out the possibility of existence of GRP139 or any similar αMSH activated Gq receptor in melanocytes.
We have deliberated upon these possibilities in the discussion of revised manuscript.

3. STIM1 expression is presented diagonally in Figure 7D. Surely this can be corrected.

Response: *As suggested, we have improved the data presented in the* ***Figure 7D*** *of the revised manuscript.*

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Rajesh S Gokhale and Rajender K Motiani
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-97597R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 $\chi 2$ 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.

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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

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?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	In accordance with the field norms, more than 200 zebrafish embryos were screened for phenotypic changes.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	Zebrafish embryos were randomly distributed for morpholino injections.
For animal studies, include a statement about randomization even if no randomization was used.	Zebrafish embryos from several breeding pairs were pooled and then randomly distributed in three groups for morpholino injections.
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.	Results were analyzed by two independent investigators. Further, the exact number of embryos with phenotypic changes were recorded and they are reported in the manuscript.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done but sceening was performed by two independent investigators.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Kolmogorov-Smirnov test
Is there an estimate of variation within each group of data?	Yes
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), 1DegreeBio (see link list at top right).	All the antibodies used in the study were from Abcam (Cambridge, UK) except STIM1 (GOK) antibody used for IP studies with STIM1 (delta 5/P). This GOK antibody was procured from BD Biosciences. The antibody details are included in the supplementary information.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Primary Human Melanocytes were sourced from Lonza (Switzerland) and B16 cell line was procured from ATCC (USA). B16 cells were authenticated by STR and we routinely perform mycoplasma contamination tests using MycoAlert kit (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.	Assam wild type zebrafish embryos were used in the study. These embryos were obtained by in-house breeding of the adult zebrafish pairs. For further details on the housing and experimental setup please refer supplementary information.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Zebrafish experiments were performed in strict accordance with the recommendations and guidelines laid down by the CSIR-Institute of Genomics and Integrative Biology, India. The Institutional Animal Ethics Committee (IAEC) of the CSIR-Institute of Genomics and Integrative Biology approved the protocol (Proposal No 45a).

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.	Ok

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	The microarray data is submitted to Gene Expression Omnibus and its accession number is GSE107450.
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).	NA
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).	NA
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 Biomodels (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.	NA

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.	No