The Lifeact-EGFP Mouse is a Translationally Controlled Fluorescent Reporter of T Cell Activation

Jorge Luis Galeano Niño, Szun S. Tay, Jacqueline L. E. Tearle, Jianling Xie, Matt A. Govendir, Daryan Kempe, Jessica Mazalo, Alexander P. Drew, Feyza Colakoglu, Sarah K. Kummerfeld, Christopher G. Proud and Maté Biro
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Original submission

First decision letter

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MS TITLE: The Lifeact-EGFP Mouse is a Translationally Controlled Fluorescent Reporter of T Cell Activation

AUTHORS: Jorge Luis Galeano Nino, Szun S Tay, Jacqueline L.E. Tearle, Jianling Xie, Matt A Govendir, Daryan Kempe, Alexander P Drew, Feyza Colakoglu, Sarah Kummerfeld, Christopher G Proud, and Mate Biro
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper as a resource paper useful for the immune cell biology community.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this study, the authors describe a new application for the established Lifeact-GFP mouse as sensitive reporter for translational regulation and T-cell activation. They follow activation of primary effector T-cells both in vitro and in vivo and show that fluorescence increase of Lifeact-GFP correlates well with sustained activation (as opposed to transient early phase activation). They propose to use Lifeact-GFP as combine marker for actin dynamics and T-cell activation.
The relevance of markers for T-cell activation and T-cell morphogenesis is clear and the establishment of well-controlled markers will certainly affect many researcher focusing on infection cell biology, cancer research or immunology. The manuscript is written very well and is overall very convincing. The introduction covers all relevant background information and is very easy to follow. The material/methods section is commendable in its completeness and attention to detail. All results are presented very well and the logics of arguments can be followed throughout. In summary I congratulate the authors to a fantastic story and have only a few comments that could/should be addressed before publication.

Comments for the author

Main point:
The authors briefly mention that they generated control Lifeact-GFP expressing cells using retroviral integration. These cells did not exhibit any fluorescence increase, leading them to propose that parts of the UTR included in the original transgene are responsible for the translational regulation. This is indeed very likely and also central to the whole story. I would have expected the authors to therefore pursue this finding and determine which segment of the included UTR is actually responsible. This would have far reaching implications and applications as this sequence could potentially be used in combination with many other markers to provide simultaneous readouts for translation and intracellular organization. They should therefore stably integrate Lifeact-GFP with various UTR sequences to identify the critical motives. It would also clarify whether the translational regulation can also be observed in the absence of the BAG3 deletion, which is clearly not desirable for a general reporter mouse. Generation of the required cells should not take too much time and this point is therefore the only thing that I would actually ask to be added to a revision.

Minor points:
- One possible source for the increase in fluorescence has not been addressed: control through ROS or ROS-signaling. As increase Lifeact-GFP fluorescence is strongly correlated with T-cell activation and killing of target cells, ROS could at least modulate the observed response. Can an experiment be devised where T-cell activation can occur without accompanying ROS generation?

- The increase of eEF2 levels during T-cell activation in Fig is not convincing to me and seems not to be significant (Fig. 3J). The western should be done at least 5x to give better statistics here.

- While mTORC inhibition seems to reduce overall translation the difference between resting and activated T-cells remains (proportional increase looks the same). This would rather argue against a role for TOR-dependent translation in the observed Lifeact-GFP fluorescence increase? Can the authors clarify this points? It might be better to reduce/remove the statements on the role of mTORC in this whole process. I am not sure it is all that relevant at this stage. The identity of the relevant UTR motive might help to follow up on the mechanistic aspects here.

- The motivation for sequencing of the transgene insertion site is a bit muddled in the text. Genomic site should not affect translation but 3'/5'-UTR might – so the motivation should be whether those remain as intended by original construct (they do). The identification of the associated BAG3 deletion is a side observation that is important (and should be included) but currently has no implications for the study at hands.

- When using "arrested" in text I sometimes thought it refereed to mitotic arrest – maybe replace by "stopped moving" or "immobilized", instead.

- The fluorescence increase upon pMHC exposure (Fig. S4C) is not very clear to me and weak – remove or replace by better data?

- Include better legend for Fig. 4H: 1-2-3 not enough, put “degree of significance” below or similar

- The lag time in Fig. 1B is not very clear – include zoom in into initial section. Maybe show curve for individual cell instead to make typical kinetics more clear. I am also surprised that increase does not flatten out. This might simply reflect averaging of cells with different lag times – so I would recommend to align individual cell curves to t0 and then average curves. Then, follow time course till plateau is reached.

Reviewer 2

Advance summary and potential significance to field

The authors report on an obviously incidental finding: Lifeact reporter mice, a strain widely used to visualize actin, show an increase in reporter activity in T cells upon T cell activation. This is a useful feature for researchers visually monitoring T cell activation and the authors show in a proof of principle that the phenomenon can be advantageous when imaging the cytotoxic killing of target cells.

Comments for the author

At every level this study is conducted with very high technical standards and the quantification of imaging data seems well controlled and sound. I am sure that it is true that Lifeact mice report T cell activation and that this is a useful tool for some specialized immunology applications. One issue, however, is that this tool might be interesting for some immunologists, but I cannot not really see the relevance for the readership of J Cell Sci, especially as there are no substantial new biological data shown. So in essence, this is a report that convincingly shows that Lifeact mice can be used to monitor T cell activation.
The main part of the manuscript tries to address the very obvious question, WHY Lifeact mice upregulate Lifeact GFP upon T cell activation. The authors go quite a long way in their efforts to answer this question: they probe signaling pathways, map the locus of the transgene etc.. Despite these efforts (again, technically very well conducted) the only thing they really nail down is that the regulation is post-transcriptional. This is for sure interesting and if one could understand the underlying molecular causes, this could lead to exciting findings with potentially high relevance not only for T cell activation. However - and here I see the main problem of the manuscript - this is where the work abruptly stops without offering any insight and turns towards descriptive data how the reporter function can be used.
This is why I find the manuscript somewhat unsatisfying and would recommend to either focus on one issue (the use of the reporter) or the other (the question, why it works as a reporter).

First revision

Author response to reviewers' comments

Answers to the Reviewers' Comments
We thank both reviewers for their insightful comments and suggestions. Point-by-point answers to their comments can be found below in blue, with corresponding amendments also highlighted in blue in the main text.

Reviewer 1
We would like to thank Reviewer 1 for their positive comments regarding our manuscript.

Reviewer 1 Advance Summary and Potential Significance to Field:

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We thank the reviewer for highlighting this point, which has allowed us to further clarify any involvement of the UTRs in the enhanced Lifeact-GFP signal upon T cell activation.

To evaluate the possibility that increased Lifeact-GFP fluorescence could be modulated by segments of the UTR from the original transgene, we also measured the fluorescence in resting and activated T cells derived from Lifeact-mRFPruby mice that were designed and generated with the same transgene as the Lifeact-GFP, with only replacement of the fluorescent protein (Riedl, Crevenna et al. 2008).

We confirmed that T cells from the Lifeact-GFP and -RFP mice are flanked by the same 5' and 3' UTRs by PCR of the relevant genomic DNA from each mouse, and found that the UTRs are identical. We have updated Figure S3H to reflect this result. In Figure 3B, we had found that the increase in fluorescence of activated Lifeact-RFP T cells was markedly less than that seen in activated Lifeact-GFP T cells. Therefore, the UTRs flanking Lifeact-GFP and -RFP cannot principally be responsible for the increased fluorescence in Lifeact-GFP activated T cells. We have consequently deleted the following sentence from the text: *"The Lifeact-EGFP transgene includes untranslated regions featuring a promoter, intron splice site, polyadenylation signal and 3' regulatory sequences that may have a role in translational regulation (**see Fig. 3K**)"* and instead clarified that the UTRs are common and unlikely to play a role [lines 241-252].

Minor points:

- One possible source for the increase in fluorescence has not been addressed: control through ROS or ROS-signalling. As increase Lifeact-GFP fluorescence is strongly correlated with T-cell activation and killing of target cells, ROS could at least modulate the observed response. Can

an experiment be devised where T-cell activation can occur without accompanying ROS generation?.

The reviewer correctly points out that ROS-signalling has recently become implicated in T cell signalling (reviewed in Franchina D.G., Dostert C. and Brenner D. (2018) *Trends in Immunology 39(6):489-502*). We have demonstrated that the TCR signalling pathway was responsible for the observed increase in fluorescence during T cell activation. We evaluated the most common intermediates of the TCR signalling pathway such Lck and Erk, however it remains possible that other effectors downstream of TCR signalling could be involved in this process, including ROS. While it would be interesting to extend the investigation to evaluate the effect of ROS signalling in these cells, we believe that this would be beyond the scope of the present paper, which, we hope the reviewer agrees, already contains considerable amounts of data and goes to lengths to determine that a translational mechanism underlies the increase in fluorescence.

-The increase of eEF2 levels during T-cell activation in Fig is not convincing to me and seems not to be significant (Fig. 3J). The western should be done at least 5x to give better statistics here.

The reviewer is right in pointing out that the increase of eEF2 in activated T cells is not statistically significant compared with the resting counterparts. As the reviewer suggested, the western blot was repeated to obtain a total of 5 independent experiments. After pooling the data (n=5) we did not find any statistical difference in the amount of eEF2 between activated and resting T cells. Accordingly, we no longer state that eEF2 is increased in activated T cells and have removed the corresponding figures (previous Fig. 3J and Fig. S3H) and the following text [deletion at line 220]: "*We noted that activation of T cells increases the overall levels of the elongation factor eEF2, which would tend to increase elongation rates, and that this effect was blocked by INK128 (Fig. 3J). The synthesis of eEF2 is known to be strongly activated by mTOR signalling as it is encoded by an mRNA containing a terminal oligopyrimidine (TOP) motif (Meyuhas and Kahan, 2015). However, the phosphorylated form of eEF2 (Fig. S3H), which is inactive in translation elongation (Proud, 2019), was not detected despite an abundance of total eEF2 protein, suggesting that control of other translation effectors is involved in regulating initiation and/or elongation rates in this context.*"

-While mTORC inhibition seems to reduce overall translation the difference between resting and activated T-cells remains (proportional increase looks the same). This would rather argue against a role for TOR-dependent translation in the observed Lifeact-GFP fluorescence increase? Can the authors clarify this points? It might be better to reduce/remove the statements on the role of mTORC in this whole process. I am not sure it is all that relevant at this stage. The identity of the relevant UTR motive might help to follow up on the mechanistic aspects here.

We thank the reviewer for pointing out this important consideration. Inhibition of mTOR resulted in reduced fluorescence intensity of Lifeact-GFP in resting T cells. However, following activation, the proportional increase in GFP intensity is very similar between T cells treated with mTOR inhibitor or vehicle. This increase could thus also be mediated by other kinases that are involved in the translational regulation of Lifeact-GFP. We have followed the reviewer's suggestion and reduced any statements regarding the involvement of mTOR [lines 209-220], notably by deletion of the following text, and removed previous panels F and G from Figure 3: "*We next tested if inhibiting mammalian target of rapamycin (mTOR) kinase, a protein kinase and master regulator of protein synthesis, affected Lifeact-EGFP protein expression by application of INK128, an ATP-competitive inhibitor of mTOR. GFP intensity (Fig. 3F) and Lifeact-EGFP protein (Fig. 3G) were significantly reduced by INK128 treatment in both activated and resting effector T cells.*"

-The motivation for sequencing of the transgene insertion site is a bit muddled in the text. Genomic site should not affect translation but 3'/5'-UTR might – so the motivation should be whether those remain as intended by original construct (they do). The identification of the associated BAG3 deletion is a side observation that is important (and should be included) but currently has no implications for the study at hands.

We agree with the reviewer. The original motivation for sequencing the mouse was to determine whether the Lifeact-GFP transgene was integrated in regions of active transcription during T cell activation. Following the reviewer's recommendation, we have removed the statement that the insertion site of Lifeact-GFP transgene could be responsible for its translational regulation ("*… and that the insertion site of the Lifeact-EGFP transgene therefore may play a role in its translational regulation*" [line 284]), however conserve the text regarding the BAG3 deletion in the main text.

-When using "arrested" in text I sometimes thought it refereed to mitotic arrest – maybe replace by "stopped moving" or "immobilized", instead.

Thank you for this recommendation, we have updated the text as suggested by clarifying that we are referring to a migratory arrest.

-The fluorescence increase upon pMHC exposure (Fig. S4C) is not very clear to me and weak – remove or replace by better data?

The reviewer is quite right in pointing out that the enhanced GFP fluorescence in activated T cells is weaker than in other experiments. This is mainly due to photo-bleaching effects of the cells immobilised in the field of view during imaging. Following addition of the cognate peptide, the activated T cells rapidly stop migrating (Fig. S4B) and thus, combined with live imaging at high spatiotemporal resolution (as described in the methods), the time of laser light exposure was longer per cell. As a consequence, the increased GFP fluorescence appears less significant at population level. We had applied photo-bleaching correction for individual cells (Fig. S4E, see Methods) for the analysis of data in Figure 4F-H. To avoid this apparent ambiguity, we have followed the reviewer's suggestion and removed the raw data before photo-bleaching correction (previous S4C).

-Include better legend for Fig. 4H: 1-2-3 not enough, put "degree of significance" below or similar

We thank the reviewer for pointing this out. In Fig. 4H we have added "Degree of significance" to clarify the axis labels.

-The lag time in Fig. 1B is not very clear – include zoom in into initial section. Maybe show curve for individual cell instead to make typical kinetics more clear. I am also surprised that increase does not flatten out. This might simply reflect averaging of cells with different lag times – so I would recommend to align individual cell curves to t0 and then average curves. Then, follow time course till plateau is reached.

The reviewer suggested that the lag time is not clear in Fig. 1B. However, Fig. 1B only shows the increase in GFP fluorescence over time in T cells co-embedded with cognate tumour cells. The lag time was calculated in normalised data from Fig. S1A that shows the divergence of the mean GFP intensity of T cells co-embedded with cognate or non-cognate tumour cells. In Fig. 1C we show the lag times from three independent experiments (n=3). The increase in GFP intensity over time for each individual cell is plotted in Fig. S4D and Fig. 4H and we could not detect any plateau, due to limitations in the amount of time we are able to image the samples. As the reviewer suggests, we now provide a zoomed-in section of the divergence point in Fig. S1A to visualise clearer the difference between the two GFP fluorescence intensities (Cognate vs. non-Cognate).

Reviewer 2

We would like to thank Reviewer 2 for their positive comments regarding our manuscript.

Reviewer 2 Advance Summary and Potential Significance to Field:

The authors report on an obviously incidental finding: Lifeact reporter mice, a strain widely used to visualize actin, show an increase in reporter activity in T cells upon T cell activation. This is a useful feature for researchers visually monitoring T cell activation and the authors

show in a proof of principle that the phenomenon can be advantageous when imaging the cytotoxic killing of target cells.

Reviewer 2 Comments for the Author:

At every level this study is conducted with very high technical standards and the quantification of imaging data seems well controlled and sound. I am sure that it is true that Lifeact mice report T cell activation and that this is a useful tool for some specialized immunology applications. One issue, however, is that this tool might be interesting for some immunologists, but I cannot not really see the relevance for the readership of J Cell Sci, especially as there are no substantial new biological data shown. So in essence, this is a report that convincingly shows that Lifeact mice can be used to monitor T cell activation. The main part of the manuscript tries to address the very obvious question, WHY Lifeact mice upregulate Lifeact GFP upon T cell activation. The authors go quite a long way in their efforts to answer this question: they probe signaling pathways, map the locus of the transgene etc.. Despite these efforts (again, technically very well conducted) the only thing they really nail down is that the regulation is post-transcriptional. This is for sure interesting and if one could understand the underlying molecular causes, this could lead to exciting findings with potentially high relevance not only for T cell activation. However - and here I see the main problem of the manuscript - this is where the work abruptly stops without offering any insight and turns towards descriptive data how the reporter function can be used. This is why I find the manuscript somewhat unsatisfying and would recommend to either focus on one issue (the use of the reporter) or the other (the question, why it works as a reporter).

The authors thank Reviewer 2 for their insight and recommendations in terms of the focus of the paper.

In regard to concerns with readership relevance, we would like to clarify that this paper is intended for the “Cell Biology of the Immune System” special edition of J Cell Sci, which therefore presents a suitable opportunity to share our findings of an interesting tool for immunology-focussed readers of J Cell Sci.

In response to the reviewer’s comments we have decided to focus more on the use of the Lifeact-GFP mouse as a reporter for T cell activation and have removed the majority of the main text discussing mTorc signalling. We believe however that the evidence for translational regulation during T cell activation presented in this work may be of great interest to a broader research audience. We find it important to report how we have narrowed down the mechanism to translational regulation, and consequently have left that discussion in the text.

Second decision letter

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AUTHORS: Jorge Luis Galeano Nino, Szun S Tay, Jacqueline L.E. Tearle, Jianling Xie, Matt A Govendir, Daryan Kempe, Jessica Mazalo, Alexander P Drew, Feyza Colakoglu, Sarah Kummerfeld, Christopher G Proud, and Mate Biro
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

In this study, the authors describe a new application for the established Lifeact-GFP mouse as sensitive reporter for translational regulation and T-cell activation. They follow activation of primary effector T-cells both in vitro and in vivo and show that fluorescence increase of Lifeact-GFP correlates well with sustained activation (as opposed to transient early phase activation). They propose to use Lifeact-GFP as combine marker for actin dynamics and T-cell activation.

Comments for the author

I am fully satisfied by the correction and modifications in the revision and again congratulate the authors for a very nice story!