Evidence for low-level translation in human erythrocytes

Sangeetha Devi Kumar, Debaleena Kar, Md Noor Akhtar, Belinda Willard, Debadrita Roy, Tanweer Hussain, Purusharth Rajyaguru, and Sandeep Eswarappa

Corresponding author(s): Sandeep Eswarappa, Indian Institute of Science

Review Timeline:

| Event                | Date       |
|----------------------|------------|
| Submission Date      | 2021-09-16 |
| Editorial Decision   | 2021-10-21 |
| Revision Received    | 2022-05-11 |
| Editorial Decision   | 2022-06-08 |
| Revision Received    | 2022-07-24 |
| Accepted             | 2022-08-08 |

Editor-in-Chief: Matthew Welch

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.)
Dear Dr. Eswarappa,

First of my apologies for the protracted review process but it turned out to be very difficult to find suitable reviewers for your manuscript at this time. However, we have now received the comments of one reviewer, and I have also read the manuscript. Whereas the expert feels that your findings could be in principle of interest to the readership of MBoC, this reviewer states that at the present stage, the data that you present in your manuscript do not yet support the conclusion that erythrocytes are active in translation. Of particular concern is the purity of your erythrocytes but other technical issues are also listed in the reviewer's report.

I concur with this assessment and therefore, we cannot offer to publish your manuscript at this point. If you however feel that you can address all the concerns of the reviewer by the addition of new experiments and additional controls, then we would be happy to consider a revised manuscript. However, given the amount of additional work, I would also understand if you prefer to submit your work elsewhere. In case you decide to submit a revised manuscript to MBoC, please make sure that you include a detailed response that list all the changes you have made to the manuscript. The revised work would then be reviewed again by the same expert and myself.

Sincerely,

Karsten Weis
Monitoring Editor
Molecular Biology of the Cell

------------------------------------------------------------------------

Dear Prof. Eswarappa,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Reviewer #1 (Remarks to the Author):

The manuscript by Kumar et al investigated the selective translation in human mature erythrocytes. They presented evidence in their views supporting that human mature erythrocyte was capable of synthesize proteins. This contrasts with the generally accepted knowledge in the field i.e. mature erythrocytes are devoid of ribosomes and mitochondria and therefore do not actively translate mRNAs. There are a number of concerns and questions regarding the evidence provided in the manuscript. Most critically, the assessment of the purity of erythrocyte preparation needs to be much more rigorous with several selection markers as details below. Additionally, whether the newly synthesized proteins are erythroid in origin remains unclear.

1. Foremost, the purity of the erythrocytes is of great concern. The authors need to be extremely careful about this since their conclusion is quite provocative and controversial. Furthermore, the rate of protein synthesis is very low about 10% of CD71+ cells (Fig. 1B). Negative selection by CD71 does not quarantine for the purity of the preparation as erythrocytes. Non-erythroid cells have lower CD71 expression and are present in the CD71- population. These cells are nucleated and are capable of protein synthesis. Furthermore, it is possible some reticulocytes may come through the negative selection. Additional selection markers should be included and erythrocytes best isolated by FACS sorting. Mitotrackers have been used to differentiate reticulocytes and RBCs. Nuclei stain should be included to eliminated nucleated cells. Lineage depletion of non-erythroid cells should also be included in the preparation of mature RBCs.

2. Fig. 1B, CD71 signal is low in CD71+ cells. What is the evidence for the isoform of GAPDH? Could it be the degraded product? In the erythroid precursors, GAPDH is 37KDa. Fig. 1D, it seems that there is lower band from both VDAC1 and MTCH2 western similarly to the lower band in GAPDH. It is important to include the CD71+ reticulocytes as a control. Mitotracker will be a superior choice to demonstrate the absence of mitochondria in RBCs.

3. Fig. 2, the low abundance of ribosomes in the CD71- preparation might originate from nucleated cells or reticulocytes as commented above in point 1.

4. Fig. 3A, SDS-PAGE shall be performed with higher % of gel to include globin monomer around 15KDa as shown in Fig. 3B. Presently, Fig 3A suffered from the missing information of globin protein synthesis, the major protein produced in erythroid cells. Why the inhibition by harringtonine is only 50%?

5. Fig. 3C, bright field image shall be included. Does all cells label with puromycin? It will also be important to include the western blot analysis of puromycin incorporation into nascent polypeptides.

6. Fig. 3D, again as point out in point 3, globin monomer is missing in the gel. It is also unclear why globin chains shall appear as 30 KDa in SDS-PAGE, should be 15KDa as a monomeric form. There is no doubt that carbonic anhydrases and globin are the dominant bands in Coomassie stained gels of the erythrocyte preparation. However, whether the newly synthesized proteins are carbonic anhydrases and globin remain to be investigated, i.e. immunoprecipitation of the S35-methionine labeled proteins.

7. Table 1, the enrichment of Fos and JunB mRNAs, which encodes nuclear transcription factor in the polysomes, further flag the possibility of presence of nucleated cells in the preparation. More surprising and problematic was that CA1 and CA2 mRNA is not detectable in the polysomes (table 1).

8. Fig. 4E, globin chain in SDS-PAGE shall be of 15 KDa, which is not included in the western results. Do not understand the presence of 25KDa dimer as authors claimed. None the less, the monomer shall be the predominant form in SDS-PAGE as the authors have shown in Fig. 3B.

9. Other general comments in the Introduction:
line 63-64. Ribosomes are also eliminated during erythroid maturation
line 72-73, Why not? In addition, protein synthesis is the most energy intensive process in the cell. It would be difficult to do so without mitochondria in the mature RBCs.
Line 75-76 Why shall RBCs continue to supply hemoglobin? Citation?
Responses to Reviewer’s comments  
(Reviewer’s comments are in **bold** and our responses are in *italics*)

Reviewer #1 (Remarks to the Author):
The manuscript by Kumar et al investigated the selective translation in human mature erythrocytes. They presented evidence in their views supporting that human mature erythrocyte was capable of synthesize proteins. This contrasts with the generally accepted knowledge in the field i.e. mature erythrocytes are devoid of ribosomes and mitochondria and therefore do not actively translate mRNAs. There are a number of concerns and questions regarding the evidence provided in the manuscript. Most critically, the assessment of the purity of erythrocyte preparation needs to be much more rigorous with several selection markers as details below. Additionally, whether the newly synthesized proteins are erythroid in origin remains unclear.

We thank the Reviewer for the constructive comments and valuable suggestions. We have performed all the experiments/analyses suggested. We believe that our manuscript has improved significantly because of them. The changes incorporated in the revised manuscript are explained in detail below.

1. Foremost, the purity of the erythrocytes is of great concern. The authors need to be extremely careful about this since their conclusion is quite provocative and controversial. Furthermore, the rate of protein synthesis is very low about 10% of CD71+ cells (Fig. 1B). Negative selection by CD71 does not quarantine for the purity of the preparation as erythrocytes. Non-erythroid cells have lower CD71 expression and are present in the CD71- population. These cells are nucleated and are capable of protein synthesis. Furthermore, it is possible some reticulocytes may come through the negative selection.

Response: We completely agree that the purity of mature erythrocyte preparations is of paramount importance for our study. We have performed the following experiments suggested by the Reviewer, and mass-spectrometry to establish the purity of our preparations:

1. We have performed MitoTracker Red staining to rule out the presence of mitochondria in mature erythrocyte preparations.
2. We have performed nucleus staining to rule out the presence of nucleated cells in mature erythrocyte preparations.
3. We have performed mass spectrometry analysis of lysates from mature erythrocyte preparations to rule out mitochondrial contamination as all other cells (including reticulocytes) contain mitochondria.

It is unlikely that the translation signal we have observed is coming from the contaminated cells because of the following reasons:

1. In our RiboPuromycylation assay, majority of cells in a field show signal (Fig 3C), which is not possible if the signal was due to contaminating cells.
2. Reticulocytes make up just 1% of erythrocytes in healthy individuals. Thus, the translation signal we have observed, which is 10% of that in reticulocytes (Fig 3B), is much higher than the signal that is expected due to reticulocyte contamination.

3. We observe > 50% reduction in β-globin level in mature erythrocytes treated with translation inhibitors (Fig 4E). Since β-globin is specific to erythroid cells, this observation rules out the possibility of contamination with non-erythroid cells (which don’t express β-globin). Furthermore, > 50% reduction is much higher than the reduction expected if the signal was due to reticulocyte contamination, which constitute just 1% of erythrocytes.

4. Our results are consistent with studies that have reported the presence of both mRNAs and miRNAs in mature erythrocytes (Chen et al., 2008, PMID: 18523662; Doss et al., 2015, PMID: 26573221).

the rate of protein synthesis is very low about 10% of CD71+ cells (Fig. 1B).

Response: We agree that the level of translation is lower than that is observed in other nucleated cells. However, its contribution will be physiologically significant given the long life and large number of mature erythrocytes, compared to reticulocytes. Low level of translation could be due to the following reasons:

(i) Mature erythrocytes require less number of proteins compared to other nucleated cells as they lack many physiological processes (e.g., cell division) and all membrane-bound organelles

(ii) Energy (ATP) requirement for translation is high. In the absence of mitochondria, mature erythrocytes can afford only low-level translation.

Additional selection markers should be included and erythrocytes best isolated by FACS sorting.

Response: In the revised manuscript, we have investigated translation in FACS sorted mature erythrocytes. We sorted CD71-negative cells using FACS and performed metabolic labelling using [35S]methionine. Like MACS-sorted mature erythrocytes, we observed a signal around 15 kDa in FACS-sorted mature erythrocytes, which reduced in intensity when the cells were treated with harringtonine (Fig S1D).

Mitotrackers have been used to differentiate reticulocytes and RBCs.

Response: We have used MitoTracker Red to differentiate CD71^{+ve} (reticulocytes) and CD71^{−ve} erythrocytes. We observed MitoTracker signal in CD71^{+ve} cells (pattern similar to previous reports, Moura et al 2018, PMID: 30076174) and K562 cells, but not in CD71^{−ve} erythrocytes (Fig 1E).

Nuclei stain should be included to eliminated nucleated cells.
Response: We have used CyQUANT NF, a DNA-binding dye, to stain the nucleus. We did not observe any nuclear staining in our mature erythrocytes preparation. K562 cells were used as positive control (Fig S1A).

Lineage depletion of non-erythroid cells should also be included in the preparation of mature RBCs.

Response: In the revised manuscript, we have performed lineage cell depletion (using a kit from Miltenyi Biotec) coupled with MACS separation to get CD71$$^-$$ mature erythrocytes as suggested here. These cells also showed clear evidence of translation when treated with $[^{35}\text{S}]$methionine (Fig S1E). One drawback of this technique is that it can remove a portion of erythroid cells (they express CD235a/glycophorin A, whose antibody is a component of lineage cell depletion kits) including mature erythrocytes.

2. Fig. 1B, CD71 signal is low in CD71+ cells.

Response: We have replaced Fig 1B with a new blot where the CD71 signal is stronger.

What is the evidence for the isoform of GAPDH?

Response: An isoform of human GAPDH with molecular weight ~ 32 kDa is reported (NCBI ID: NP_001243728.1; Uniprot ID: P04406-2; PMID: 34966852). This is a product of alternatively spliced GAPDH mRNA.

Could it be the degraded product? In the erythroid precursors, GAPDH is 37KDa.

Response: Yes, degradation is a possibility.

Fig. 1D, it seems that there is lower band from both VDAC1 and MTCH2 western similarly to the lower band in GAPDH.

Response: Mass spectrometry analysis performed on mature erythrocyte lysate did not detect VDAC1 or MTCH2. Lower bands could be non-specific.

It is important to include the CD71+ reticulocytes as a control.

Response: As per this suggestion, Western blot using lysates from CD71+ cells is shown in Fig.S1B.

Mitotracker will be a superior choice to demonstrate the absence of mitochondria in RBCs.

Response: As per this suggestion, we have performed MitoTracker Red staining and we did not detect any MitoTracker signal in our mature erythrocytes preparation.
K562 cells and CD71^{+ve} erythrocytes showed MitoTracker signal as expected (Fig 1E).

3. Fig. 2, the low abundance of ribosomes in the CD71- preparation might originate from nucleated cells or reticulocytes as commented above in point 1.

Response: Mass spectrometry analysis, Western blot analysis, MitoTracker Red staining and nuclear staining experiments rule out the contamination with other cells in our mature erythrocyte preparation. This is explained in detail above.

4. Fig. 3A, SDS-PAGE shall be performed with higher % of gel to include globin monomer around 15KDa as shown in Fig. 3B. Presently, Fig 3A suffered from the missing information of globin protein synthesis, the major protein produced in erythroid cells.

Response: We thank the Reviewer for this excellent suggestion. The Reviewer’s statement is correct. We do observe a prominent band around 15 kDa. We have replaced Fig. 3A with a new autoradiogram showing that band.

Why the inhibition by harringtonine is only 50%?

Response: We do not have a specific answer for this. Some possible reasons could be reduced uptake of harringtonine by mature erythrocytes, or inactivation/degradation of harringtonine in the cellular environment of mature erythrocytes.

5. Fig. 3C, bright field image shall be included. Does all cells label with puromycin?

Response: We have included the bright field images in the revised manuscript. Most of the cells show puromycin signal.

It will also be important to include the western blot analysis of puromycin incorporation into nascent polypeptides.

Response: As per this suggestion, we have included this data in Fig S1F.

6. Fig. 3D, again as point out in point 3, globin monomer is missing in the gel.

Response: We have removed this data from the revised manuscript as the analysis was performed on 26-30 kDa band. However, as the Reviewer rightly pointed out, the primary band is around 15 kDa. We have now performed mass spectrometry analysis of the whole lysate from mature erythrocytes, and there also globins appear in the top of the list (Table S1).

It is also unclear as why globin chains shall appear as 30 KDa in SDS-PAGE, should be 15KDa as a monomeric form.
Response: Globin dimers have been observed in SDS-PAGE previously. For example,
A. Fig 1 in Hosomi et al, 2016, PMID: 30263494
B. Fig 7 in Hamdane et al 2007, PMID: 17194704
C. Fig 1 in Hamdan et al 2012, PMID: 23057022

The reference image provided in the web page of the commercial antibody (Santa Cruz, SC-130320) we have used in this study also shows a band around 30 kDa (https://www.scbt.com/p/hemoglobin-beta-antibody-18)

Globin proteins are abundant in the erythrocyte lysate (Table S1). Some portion of the dimers possibly escape reducing agents’ action and move as dimers during electrophoresis.

There is no doubt that carbonic anhydrases and globin are the dominant bands in Coomassie stained gels of the erythrocyte preparation. However, whether the newly synthesized proteins are carbonic anhydrases and globin remain to be investigated, i.e. immunoprecipitation of the S35-methionine labeled proteins.

Response: This point has been addressed in the following ways:
1. Using polysome fractionation, we have isolated polysome-associated mRNAs and performed RNA-seq. This analysis reveals that globin mRNAs are enriched, but not the CA mRNAs, in the translating pool (Table 1)
2. Quantitative real-time PCR also shows the enrichment of globin mRNAs in translating pool compared to non-translating pool. However, the CA1 transcript was undetectable and Ct value of CA2 (carbonic anhydrase) was comparable in both translating (29.52) and non-translating (30.27) pools. This indicates that the globin mRNAs are preferentially translated, but not CA mRNAs (Fig 4A).
3. To further confirm the translation of globin mRNAs, we precipitated the globins in [$^{35}$S]methionine-treated cells and we could observe [$^{35}$S]methionine-signal in the precipitated globin proteins (Fig 4B).

7. Table 1, the enrichment of Fos and JunB mRNAs, which encodes nuclear transcription factor in the polysomes, further flag the possibility of presence of nucleated cells in the preparation.

Response: Several nuclear proteins have important functions in the cytoplasm. Even Fos has a cytoplasmic function in the synthesis of phospholipids (Caputto et al., Biochim Biophys Acta. 2014 (9):1241-6). Furthermore, there were no cells with nuclei in our mature erythrocytes preparations (Fig S1A).

More surprising and problematic was that CA1 and CA2 mRNA is not detectable in the polysomes (table 1).
Response: Yes, this is an unexpected result. Our mass spectrometry analysis revealed that CA1 and CA2 are among the abundant proteins in mature erythrocytes along with globins (Table S1). However, their mRNAs were not detectable in polysomes. This suggests that these cells have other mechanisms to maintain CA1 and CA2 protein levels. For example, they may have very long half-lives.

8. Fig. 4E, globin chain in SDS-PAGE shall be of 15 KDa, which is not included in the western results.
Response: Thanks for this suggestion. We do observe prominent band at 15 kDa, which reduces in intensity when cells are treated with translation inhibitors (Fig 4E).

Do not understand the presence of 25KDa dimmer as authors claimed.
Response: ~ 25 kDa band is consistent with the globin dimer, which has been observed in previous reports as well (See response to Point 6). We do observe this band in the Western blot performed using anti-beta globin antibody (Fig 4E). Corresponding band can be seen in Coomassie-stained gels also (Fig 3A and 3B). Mass-spectrometry of the ~ 25 kDa band (results shown in the previous version) revealed many peptides specific to globins (>80% coverage and > 0.1 NSAF value (normalized spectral abundance factor)).

None the less, the monomer shall be the predominant form in SDS-PAGE as the authors have shown in Fig. 3B.
Response: We agree with this comment, and this is observed in the experiments shown in Fig 3A and B, Fig 4E and Fig S1D-F.

9. Other general comments in the Introduction:

line 63-64. Ribosomes are also eliminated during erythroid maturation
Response: We have included this statement in the revised manuscript (line 65).

line 72-73, Why not?
Response: Mature erythrocytes stay functional for about 115 days. To survive and stay functional for 115 days, these cells would need constant supply of globin proteins for hemoglobin. They would need enzymes of glycolysis and pentose phosphate pathway, and other housekeeping proteins. This is not possible without translation. These points are explained with references in the Introduction (Page 75-79).

In addition, protein synthesis is the most energy intensive process in the cell. It would be difficult to do so without mitochondria in the mature RBCs.
Response: Yes, translation is energy intensive process. In the absence of mitochondria, mature erythrocytes will have to rely on Glycolysis. This could explain the low-level selective translation we observe in our study.

Line 75-76 Why shall RBCs continue to supply haemoglobin? Citation?
Response: This is because the mature erythrocytes have functional proteasome machinery suggesting protein turnover in them (Kakhniashvili et al., 2004, PMID: 14963112; Neelam et al., 2011, PMID: 21508250). This information with references is given in the manuscript, line 79-81.
Dear Dr. Eswarappa,

Thank you for submitting a revised manuscript, which was now seen by the original reviewer. This expert feels that your revisions address most prior concerns and that you now provide convincing data supporting a low level of protein synthesis in human erythrocytes. However, there remain four concerns that you will need to addressed before we can accept of the manuscript for publication.

I am therefore returning the manuscript to you again for another round of revisions.

Sincerely,
Karsten Weis
Monitoring Editor
Molecular Biology of the Cell

------------------------------------------------------------------------

Dear Prof. Eswarappa,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor’s decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor’s and reviewers’ comments have been addressed. (The file type for this letter must be “rebuttal letter”; do not include your response to the Monitoring Editor and reviewers in a "cover letter." ) Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,
Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

------------------------------------------------------------------------

Reviewer #1 (Remarks to the Author):
1. Title: Suggested title: Evidence for low level translation in human erythrocytes
Selective translation is misleading as authors discussed in the manuscript, the translation of globin mRNAs can simply because of the abundance of the globin mRNAs (line 260). The role of the 5'UTR for globin mRNA translation has been demonstrated in the reticulocytes and is not unique to erythrocytes.
Erythrocyte by definition is mature RBCs. Thus, it is not necessary to include mature.
2. Remove selective translation throughout the manuscript
3. Results of Fig. 3B showed that erythrocytes have about 10X lower translation than reticulocytes. This point and the low level of ribosomes shall be made clearly to the readers.
4. Figure 4E: 10days of cycloheximide or harringtonine treatment was quite a long time. How were the erythrocytes maintained during these 10days and what were the morphology and integrity of the erythrocytes at the end of 10days of treatments? It would be desirable to include these results in the manuscript. Also, a time course of cycloheximide or harringtonine treatment with earlier time points will be important to minimize the cytotoxic effects of these inhibitors in erythrocytes and to sustain authors' claim on the degradation and the replenishment of the globins in erythrocytes.
Responses to Reviewer’s comments  
(Reviewer’s comments are in bold and our responses are in italics)

Reviewer #1 (Remarks to the Author):

1. Title: Suggested title: Evidence for low level translation in human erythrocytes. Selective translation is misleading as authors discussed in the manuscript, the translation of globin mRNAs can simply because of the abundance of the globin mRNAs (line 260). The role of the 5'UTR for globin mRNA translation has been demonstrated in the reticulocytes and is not unique to erythrocytes. Erythrocyte by definition is mature RBCs. Thus, it is not necessary to include mature.
   Response: We agree with this comment, and we have changed the title as per this suggestion.

2. Remove selective translation throughout the manuscript
   Response: As per this suggestion, we have removed ‘selective translation’ throughout the manuscript.

3. Results of Fig. 3B showed that erythrocytes have about 10X lower translation than reticulocytes. This point and the low level of ribosomes shall be made clearly to the readers.
   Response: This point is included in the Abstract and the Results (Lines 144 and 284) of the manuscript.

4. Figure 4E: 10days of cycloheximide or harringtonine treatment was quite a long time. How were the erythrocytes maintained during these 10days and what were the morphology and integrity of the erythrocytes at the end of 10days of treatments? It would be desirable to include these results in the manuscript.
   Response: Mature erythrocytes were treated with 100 μg/ml of cycloheximide (CHX) or 3 μg/ml of harringtonine (HGT). Media containing translation inhibitors was replaced every alternate day, until the end of the experiment (mentioned in the Legend to Figure 4E and F). Cells did not show any observable changes during the period we tested in this study (10 days). Microscope images showing the morphology and the % survival values are shown in the revised manuscript (Fig S2C and D).
   Also, a time course of cycloheximide or harringtonine treatment with earlier time points will be important to minimize the cytotoxic effects of these inhibitors in erythrocytes and to sustain authors' claim on the degradation and the replenishment of the globins in erythrocytes.
Response: As per this suggestion, we have shown the $\beta$-globin expression at 0, 5 and 10 days after treatment. Reduction in expression can be observed as early as 5 days after the treatment.
Dear Dr. Eswarappa,

Thank you for revising your manuscript once again. I have now evaluated your changes and feel that you have adequately addressed the remaining concerns of the reviewer. I am therefore pleased to accept your manuscript for publication in MBoC. Congratulation to you and your co-workers.

Sincerely,
Karsten Weis

------------------------------------------------------------------------

Dear Prof. Eswarappa:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

Would you like to see an image related to your accepted manuscript on the cover of MBoC? Please contact the MBoC Editorial Office at mboc@ascb.org to learn how to submit an image.

Authors of Articles and Brief Communications are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

We are pleased that you chose to publish your work in MBoC.

Sincerely,
Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

------------------------------------------------------------------------