A constitutively expressed fluorescent, ubiquitination-based cell cycle indicator (FUCCI) in axolotls for studying tissue regeneration

Timothy J. Duerr, Eun Kyung Jeon, Kaylee M. Wells, Antonio Villanueva, Ashley W. Seifert, Catherine D. McCusker and James R. Monaghan
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MS TITLE: A constitutively expressed fluorescence ubiquitin cell cycle indicator (FUCCI) in axolotls for studying tissue regeneration

AUTHORS: Timothy J Duerr, Eun Kyung Jeon, Kaylee M Wells, Antonio Villanueva, Ashley W Seifert, Catherine D. McCusker, and James R Monaghan

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express interest in your work and recognize its potential value, but they also have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In my view, these are challenging but valid requests given the intent of your manuscript to introduce a new too/approach to the axolotl community, and I would understand if you were to decide to submit this work elsewhere. However, If you are able to revise the manuscript along the lines suggested, which is likely to involve further experiments and analyses, I will be happy receive a revised version. It will be important to convince the reviewers that 1) the reagent can uncover novel observations that are not deduced from histology or existing strains; 2) the reagent is accurately monitoring cell cycle states; and 3) this reagent is robust, e.g. not compromised by its multiple transgene insertions.

Your revised paper will be re-reviewed by the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and

where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and 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. 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, Duerr et al., generated a new transgenic axolotl line that enables visualization of cell cycle dynamics in live animals. They take advantage of the FUCCI reporter system that shows different fluorescent colors during the progression of the cell cycle. By histologic examination they show that the reporter functions as expected in axolotl tissues. They also conduct live imaging studies during tail regeneration. The tool has the potential to give novel insights into tissue regeneration and how cell cycle dynamics is regulated on a large scale.
Overall, I find the data analyses to be properly done, and the possibility that one may investigate cell cycle regulation in live axolotl tissues during regeneration is truly exciting. However, the usefulness of the line is not convincingly showed since the authors did not apply the powerful tool to uncover at least some new biology that would have been missed with histological approaches. Below I provide comments and suggestions.

Comments for the author

Major:
1. As the line is proposed to be a powerful tool for live imaging studies of cell cycle length, the authors need to better characterize cell cycle dynamics in their movies. This should be relatively straightforward. In Movie 3, the authors recorded 60 hours of tail regeneration and showed that high-resolution cell tracking is practically available. Does the cell cycle length change across the regenerating AP axis? How are different cell cycle stages regulated across the axis? Since cell proliferation could be detected in the region that is 5 mm away from the amputation plane, do cells in average have a longer cell cycle length when they are further away from the amputation plane?
2. Figure 3. It is unclear to me how the authors can tell whether the dividing cells are epithelial cells or some other cell types? In Figures 3A-E, the authors stated these dividing cells are epithelial cells. In Figure 3F-O, they considered those dividing cells as cells constitute an early blastema (Line 158), which is often referred to as a group of mesenchymal cells of varied origin. Since the authors highlight this as the first real-time movie of blastema formation in regenerating axolotl tissue, please be very specific about how different cell types are determined from the live imaging movies. Also, it would be nice to provide illustrations for how the measurements were done in Figures 3N and O.
3. In the discussion, the authors conclude that the dying cell phenotype seen in the Movie 3 is not an accurate representation of tail blastema growth as the blastema becomes misshapen when kept in the agarose. Could it be the live cell extrusion event seen in developing zebrafish epidermis (Eisenhoffer et al., Nature 2012) and adult tailfin wound healing response (Chen et al., Dev Cell 2016)? Are those cells apoptotic or non-apoptotic?
This is a very interesting finding that could use some more insights.

Minor:
1. Line 189. …termination of the rapid proliferation program employed during "embryonic development". I think the authors mean "late stages of regeneration".
2. Line 234. Please explain what is the "R-point" of the cell cycle.
3. Line 240. Labeling error. Fig.S5G-I.
4. Line 435. Please specify the z-distance of the imaging plane. What is the objective used here?
5. Figures 5V-Y. The font size is too small to read.

6. Figure 5M. Please indicate the number of animals examined in each time point. Information on the box and whisker plot is not defined in the legend.
Please specify which statistical test is used here.
7. Figures 6L-M. Please indicate the number of animals and cells examined in each anatomical position.
8. Movies 1 to 4. All of the movies have no scale bar and time stamp. Please make sure they are added. Also, it is unclear whether the movies were recorded right after amputation injuries or a few hours later. This information should be clearly stated in each movie legend.
9. Movies 5 and 6. Please provide scale bars.
10. Line 514. The authors indicate that the custom Fiji scripts are available in the supplementary information. I found no such information in the supplementary information file. Please provide those codes as stated in the supplementary file.

Reviewer 2

Advance summary and potential significance to field

Duerr and colleagues present the transgenic FUCCI axolotl line. This represents an important and valuable contribution to the axolotl technical toolbox, enabling the study of cell-cycle progression in vivo, thereby advancing the study of regeneration, development and other biological processes in these model system. As such, this is a manuscript that presents a significant advance to the field.

Comments for the author

Duerr and colleagues present the transgenic FUCCI axolotl line. This represents an important and valuable contribution to the axolotl technical toolbox, enabling the study of cell-cycle progression in vivo, thereby advancing the study of regeneration, development and other biological processes in these model system. As such, this is a manuscript that merits publication in Development. Ahead of this, there are a few important points to be addressed.
First, the authors present validation of the FUCCI system for marking cell-cycle progression in vivo through the use of co-staining with EdU and pHH3. The high correspondence between EdU and pHH3 with mAG expression shows that the FUCCI system is able to mark cell-cycle progression in vivo in the axolotl. In addition, the authors are able to capture different cell cycle states in tissue sections, assigned by FUCCI marker expression, EdU incorporation, and nuclear morphology. As validation of the system is a critical point of this work, there are a few specific points that would merit further consideration:
- The correlation between different FUCCI states and DNA content should be presented. This can be achieved by FACS sorting mCherry+/mAG-, mCherry+/mAG+, and mCherry-/mAG+ populations combined with DNA staining and conventional cell cycle content profiling for the different cell populations. This is a critical aspect of validating FUCCI systems, which will inform on how faithfully the FUCCI system captures cell cycle progression in the axolotl.
- At present, the authors have characterised EdU+ cells/FUCCI population in 14dpa spinal cords yet the analysis of pHH3 is done in 10dpa limb blastemas. For consistency of the analysis, both EdU and pHH3 should be analysed in the same tissue/stage (eg parallel sections/same animal batch)
- I find it puzzling that there is little-to-no cells with dual mAG/mCherry staining whereas these are significantly higher in almost every other context in which the FUCCI system is used. It would be good if the authors expand on their discussion/interpretation of this observation
Second, The authors proceed to follow live FUCCI animals, and follow cell cycle progression during tail regeneration (Fig 3). No direct transitions between mAG-mCherry and vice versa are observed in the present study. This is attributed to the shortened G1 length during development. However, it would be useful to observe direct transitions between mCherry to mAG.
- This could be addressed by examining older animals, for example, in the epidermis, which is known to be a high turnover tissue. Ideally, one could start by focusing on G1 cells, as there is an increased probability of detecting a transition to mAG (given the overall cell cycle distribution profile and the high percentage of cells in S-G2-M)
- Alternatively, the authors can attempt to sort mCherry+ cells and track them in vitro to prove that direct transition can be observed.

- Regarding the tracking system (which is a useful tool), I am not clear if one can be certain of 'tracking cell migration' given that the images are taken every 30 minutes and no rainbow system for genetic tracing of individual cells is used (one cannot discard proliferation/death as one cannot be certain of individual cell fate)– this should be considered/discussed.

Next, The authors also present multi-modal imaging to permit cell-type characterization in FUCCI animals, with serial staining and visualization for endogenous FUCCI proteins, RNA, protein and click-chemistry (Fig 4). This method is extremely useful for the field, and beyond the context of FUCCI animals.

The authors then characterize the cell-cycle progression during limb regeneration in FUCCI animals. They demonstrate that during limb regeneration (Fig 5), mAG+ cycling cells are induced proximal to the amputation plane, and observe the induction of a non-cycling population in the distal portion of the blastema mesenchyme during the latter stages of regeneration.

- While the authors provide detailed images of the blastema (Fig), it would be useful to present images of equivalent resolution for the intact limb.
- The authors refer to 'determine the cell type of the mAG and mCherry cells' yet they do not show any cell type specific staining. The latter could be provided or the fact that cell type assessment is based purely on morphology should be made clear.

Lastly, the authors study the induction of proliferation during spinal cord regeneration (Fig 6), and interestingly describe the induction of proliferation up to 5000 µm distal to the amputation plane. This is a very important observation and, given that the field will pay considerable attention to it, it would be desirable to validate the FUCCI/EdU based observations with additional markers of cell-cycle progression which are established for the axolotl system (e.g. PCNA, Ki67 or (as previously used by the authors) pHH3).

Additional comments:

- Please indicate what number of mAG+ cells are PHH3+ (in mitosis, text ref to Fig. 2).
- It would be helpful to the reader to add the n, animal size and age to the figure legends.
- Fig 5 G-I and Fig 5 J-K are annotated as 'Fig S X-X…'
- Perturbations that lead to cell cycle arrest (eg compounds targeting DNA topoisomerase and alike leading to S/G2 arrest, or even irradiation to see how the proportions of FUCCI indicators change) could be very informative on the usefulness and validity of the FUCCI system.

Reviewer 3

Advance summary and potential significance to field

Live monitoring of cell cycle progression is crucial to study spatiotemporal dynamics of cell proliferation and behavior during animal development and regeneration. In the manuscript, Duerr et al generated CAG-FUCCI axolotl and revealed cell proliferation during axolotl development and regeneration of various tissues and/or organs. A common problem for FUCCI system is that inappropriate expression level of the fluorescent proteins may affect the interpretation of proliferation, which is also a major problem in the manuscript.

Comments for the author

Major issues:

1. The authors performed several experiments to demonstrate that mAG+ cells are real proliferating cells, for example, EdU staining, pHH3 staining, and live cell imaging. However, I am not fully convinced by the results given that: 1) the authors measured the correlation between EdU and mAG signals of spinal cord sections (without representative images in Fig. 2 and Fig. S1), but the EdU and mAG signals were poorly colocalized in Fig. S1; 2) the majority of mAG+ cells did not divide in the live imaging of Movie 1.
2. The images in Fig. 2D-M are misleading. As far as I know, mAG-zGem should be localized within the nuclei when nuclear envelops are intact. However, the mAG signals exist in both nuclei and cytosol in Fig. 2H'-I' at early S stages. Similarly, the mCherry-zCdt1 signal cannot be observed once they disappear until next G1 stage but the mCherry signals are evident in Fig. 2K''-M'' from late S to M stages. Are the images misinterpreted or phenomena unique to axolotl?

3. The authors mentioned that the FUCCI animals has multiple transgenic insertions. They should perform southern blot to characterize the insertion number and select animals with single insertion for further experiments. Otherwise, it may be difficult to reproduce the results.

Minor issues:
1. The authors should disclose detailed information for the EdU pulse experiments, especially for the duration of treatment and interval before tissue collection.
2. The authors should disclose the stages of axolotl used in all experiments.
3. The authors should include the timepoints for each frame in the supplementary movies.
4. Line 118: Fig. 3B should be Fig. 2B.
5. Line 173: Fig. J-K.
6. Line 193: this limitation is not caused by the CAG promoter not the fluorescent proteins.
7. Movie 2 is difficult to play.

First revision

Author response to reviewers' comments

We thank the reviewers for the comments and suggestions. We have performed further experiments based upon these suggestions that we feel have strengthened the manuscript. We have alsomade significant changes throughout the text. Below you will find responses to each reviewer comment, the change that was made in the manuscript, and the line number where the change was made.

Reviewer 1 Advance Summary and Potential Significance to Field...

In this study, Duerr et al., generated a new transgenic axolotl line that enables visualization of cell cycle dynamics in live animals. They take advantage of the FUCCI reporter system that shows different fluorescent colors during the progression of the cell cycle. By histologic examination, they show that the reporter functions as expected in axolotl tissues. They also conduct live imaging studies during tail regeneration. The tool has thepotential to give novel insights into tissue regeneration and how cell cycle dynamics is regulated on a large scale.
Overall, I find the data analyses to be properly done, and the possibilitythat one may investigate cell cycle regulation in live axolotl tissues during regeneration is truly exciting. However, the usefulness of the line is not convincingly showed since the authors did not apply the powerful tool to uncover at least some new biology that would have been missed with histological approaches. Below I provide comments and suggestions.

Reviewer 1 Comments for the Author...

Major:
1. As the line is proposed to be a powerful tool for live imaging studies ofcell cycle length, the authors need to better characterize cell cycle dynamics in their movies. This should be relatively straightforward. In Movie 3, the authors recorded 60 hours of tail regeneration and showed that high-resolution cell tracking is practically available. Does the cell cycle length change across the regenerating AP axis? How are different cell cyclestages regulated across the axis? Since cell proliferation could be detected in the region that is 5 mm away from the amputation plane, do cells in average have a longer cell cycle length when they are further away from theamputation plane?

A technical limitation to addressing these questions is the long cell cycle length of axolotl cells (some estimated to be >100 hours (Rost et al. 2016)), and the fact that many dividing cells in the regenerating tail seem to sink deeper into the tissue following division. To better understand the cell cycle length, we would need to use two photon microscopy. However, our goal for this manuscript was to introduce the animal line and to demonstrate its capabilities for *in-vivo* imaging. For this reason, we chose not to pursethe question of cell cycle length in this study.

2. Figure 3. It is unclear to me how the authors can tell whether the dividing cells are epithelial cells or some other cell types? In Figures 3A-E, the authors stated these dividing cells are epithelial cells. In Figure 3F-O, they considered those dividing cells as cells constitute an early blastema (Line 158), which is often referred to as a group of mesenchymal cells of varied origin. Since the authors highlight this as the first real-time movie of blastema formation in regenerating axolotl tissue, please bevery specific about how different cell types are determined from the live imaging movies. Also, it would be nice to provide illustrations for how the measurements were done in Figures 3N and O.

In Figures 3A-E, we image the superficial surface of a stage 32 FUCCI larvae. It is for this reason we know these cells are of epithelial nature. We have clarified this on lines 169-171 It reads "To determine the feasibility of real-time, in-vivo imaging of FUCCI tissue, we imaged cycling epithelial cells on the surface of an anesthetized stage 32 larva mounted in 0.3% agarose (Fig. 3A-E, Movie 1)." With regard tofigure 3F-O however, we cannot determine if the dividing cells are mesenchymal or epithelial in nature. We have clarified this on lines 184-186. It now reads 'By 8 hours post amputation (hpa), the tail stump was completely covered by a thin layer of both mAG+ cells and mCherry+ cells (Fig. 3G)".

3. In the discussion, the authors conclude that the dying cell phenotypeseen in the Movie 3 is not an accurate representation of tail blastema growth as the blastema becomes misshapen when kept in the agarose. Could itbe the live cell extrusion event seen in developing zebrafish epidermis (Eisenhoffer et al., Nature 2012) and adult tailfin wound healing response (Chen et al., Dev Cell 2016)? Are those cells apoptotic or non-apoptotic?
This is a very interesting finding that could use some more insights.

Our results cannot exclude the possibility of both these phenomena. We have added the sentences "During blastema growth, we observed a large number of dying cells and cells sluffing from the blastema.After removing the larvae from the agarose, we also noticed that the blastema was misshapen. We suspect that this is not a true representation of tail blastema growth and is more likely a result of the blastema growing in the agarose. However, we cannot exclude the possibility of live cell extrusion (Chen et al., 2016; Eisenhoffer et al., 2012) or apoptosis" in the discussion on lines 425-431.

Minor:
1. Line 189. …termination of the rapid proliferation program employed during"embryonic development". I think the authors mean "late stages of regeneration".

We have clarified this line. It is now found on lines 216-218 and reads "These results indicate an increasein the total number of cells in G1 phase, which may represent an accumulation of cells in resting state after rapid proliferation during early embryonic development."

2. Line 234. Please explain what is the "R-point" of the cell cycle.

This statement was removed for brevity. The edited sentence is located on lines 261-263 and now reads"Most muscle cells observed were mAG+/mCherry+, which is consistent with a similar G1/S arrest in FUCCI mouse cardiomyocytes (Alvarez et al., 2019)."

3. Line 240. Labeling error. Fig.S5G-I.

4. Line 435. Please specify the z-distance of the imaging plane. What is the objective used here?

This information is now provided on lines 524-528. It now reads "For live imaging of tail regeneration, weimaged two adjacent tiles to accommodate for growth during imaging at 10X magnification. Additionally, to accommodate for cells moving in and out of the focal plane, we imaged four planes in the z axis spanning 119.4 µm and merged these planes together in a maximum intensity projection."

5. Figures 5V-Y. The font size is too small to read.

We have made the font larger and moved the plots to Figure S8

6. Figure 5M. Please indicate the number of animals examined in each timepoint. Information on the box and whisker plot is not defined in the legend. Please specify which statistical test is used here.

A legend has been added to this chart for clarification. Animal number and the statistical test were addedto the figure caption.

7. Figures 6L-M. Please indicate the number of animals and cells examined ineach anatomical position.

We have included this information in Table S1.

8. Movies 1 to 4. All of the movies have no scale bar and time stamp. Pleasemake sure they are added. Also, it is unclear whether the movies were recorded right after amputation injuries or a few hours later. This information should be clearly stated in each movie legend.

Scale bars and time stamp were added to the figures. Additional information pertaining to time sinceamputation was added to the captions.

9. Movies 5 and 6. Please provide scale bars.

Scale bars were included at the end of the movies.

10. Line 514. The authors indicate that the custom Fiji scripts are available in the supplementary information. I found no such information in the supplementary information file. Please provide those codes as stated inthe supplementary file.

We have now included the scripts.

Reviewer 2 Advance Summary and Potential Significance to Field...

Duerr and colleagues present the transgenic FUCCI axolotl line. This represents an important and valuable contribution to the axolotl technical toolbox, enabling the study of cell-cycle progression in vivo, thereby advancing the study of regeneration, development and other biological processes in these modelsystem. As such, this is a manuscript that presents a significant advance to the field.

Reviewer 2 Comments for the Author...

Duerr and colleagues present the transgenic FUCCI axolotl line. This represents an important and valuable contribution to the axolotl technical toolbox, enabling the study of cell-cycle progression in vivo, thereby advancing the study of regeneration, development and other biological processes in these modelsystem. As such, this is a manuscript that merits publication in Development. Ahead of this, there are a few important points to be addressed.

First, the authors present validation of the FUCCI system for marking cell-cycle progression in vivo through the use of co-staining with EdU and pHH3. The high correspondence between EdU and pHH3 with mAG expression shows that the FUCCI system is able to mark cell-cycle progression in vivo in the axolotl. In addition, the authors are able to capture different cell cycle states in tissue sections, assigned by FUCCI marker expression, EdU incorporation, and nuclear morphology. As validation of the system isa critical point of this work, there are a few specific points that would merit further consideration:

-The correlation between different FUCCI states and DNA content should be presented. This can be achieved by FACS sorting mCherry+/mAG-, mCherry+/mAG+, and mCherry-/mAG+ populations combined with DNA staining and conventional cell cycle content profiling for the different cell populations.This is a critical aspect of validating FUCCI systems, which will inform on how faithfully the FUCCI systemcaptures cell cycle progression in the axolotl.

We have performed this experiment and included it in Figure S3. Experimental details are discussed onlines 147-166.

-At present, the authors have characterized EdU+ cells/FUCCI population in 14dpa spinal cords, yet theanalysis of pHH3 is done in 10dpa limb blastemas. For consistency of the analysis, both EdU and pHH3should be analysed in the same tissue/stage (eg parallel sections/same animal batch)

We chose to use the limb blastema as a means to quantify pHH3+ cells because there are many more dividing cells in this environment than that of regenerating spinal cords. Because of this we are able to quantify more cells in fewer sections, allowing us to demonstrate the correlation between mAG+ cells andpHH3+ cells more effectively. We feel that the evidence for this conclusion is sufficient in the limb blastema.

-I find it puzzling that there is little-to-no cells with dual mAG/mCherry staining whereas these are significantly higher in almost every other context in which the FUCCI system is used. It would be good ifthe authors expand on their discussion/interpretation of this observation

It is likely few $mAG^+/mCherry^+$ cells were observed since few homeostatic cells were observed in the study as the vast majority of cells quantified were observed in a regenerating context. See figure 5H-N to observe many homeostatic, $mAG^+/mCherry^+$ cells (as represented by the white coloration) in the musculature of the limb. We have included the sentences “Interestingly, we observed few mAG+/mCherry+ cells. This observation is corroborated in similar FUCCI axolotl lines (Costa et al., 2021),and is likely because the cells are in a regenerative environment where they are receiving mitogens instructing them enter the cell cycle instead of resting at the G1/S boundary (Takahashi et al., 2021)” inthe results section on lines 111-115 to clarify this observation.

Second, the authors proceed to follow live FUCCI animals, and follow cell cycle progression during tailregeneration (Fig 3). No direct transitions between mAG-mCherry and vice versa are observed in the present study. This is attributed to the shortened G1 length during development. However, it would beuseful to observe direct transitions between mCherry to mAG.

-This could be addressed by examining older animals, for example, in the epidermis, which is known tobe a high turnover tissue. Ideally, one could start by focusing on G1 cells, as there is an increased probability of detecting a transition to mAG (given the overall cell cycle distribution profile and the high percentage of cells in S-G2-M)
Alternatively, the authors can attempt to sort mCherry+ cells and track them in vitro to prove that directtransition can be observed.

Observing a color change is a goal for our lab but is technically very challenging. Live imaging adult animals presents a myriad of issues which have not yet been worked out in the system. It is infeasible tokeep an animal moist and anesthetized for the length of time required to witness a color change given that the axolotl cell cycle has been reported to take over 100 hours (Rost et al. 2016, Costa et al. 2021).Furthermore, culturing axolotl cells in vitro also tends to have many technical challenges. Our validation with EdU and pHH3 in Figure 2 clearly demonstrates that the fluorescence emitted from FUCCI cells matches our expectations for cell cycle state. We have added the sentences “Importantly, we observe mAG+/mCherry+/EdU+ cells (Fig. 2H-H’’’), indicating the presence of cells in very early S phase. Thesecells have not yet fully degraded mCherry but have started producing mAG while incorporating EdU in early S phase. This demonstrates that cells in our FUCCI system faithfully change color upon transition from G1 phase into S phase” on lines 139-143 to clearly indicate that $FUCCI^+$ cells are changing color.

-Regarding the tracking system (which is a useful tool), I am not clear if one can be certain of ‘tracking cellmigration’ given that the images are taken every 30 minutes and no rainbow system for genetic tracing of individual cells is used (one cannot discard proliferation/death as one cannot be certain of individual cell fate)- this should be considered/discussed.

We have added the sentence “Using available programs for cell tracking, we also demonstrate the capability for tracking FUCCI cells during the regenerative process. While images were taken every 30 minutes, we cannot exclude the possibility of uncertainty in the fate of some cells given this time intervalwithout identification of specific cells” on lines 431-435 in the discussion to address

this possibility.

Next, the authors also present multi-modal imaging to permit cell-type characterization in FUCCI animals,with serial staining and visualization for endogenous FUCCI proteins, RNA, protein and click-chemistry (Fig 4). This method is extremely useful for the field, and beyond the context of FUCCI animals.

The authors then characterize the cell-cycle progression during limb regeneration in FUCCI animals. Theydemonstrate that during limb regeneration (Fig 5), mAG+ cycling cells are induced proximal to the amputation plane, and observe the induction of a non-cycling population in the distal portion of the blastema mesenchyme during the latter stages of regeneration.

-While the authors provide detailed images of the blastema (Fig), it would be useful to present images ofequivalent resolution for the intact limb.

We have now included an image of an uninjured limb in Figure 5.

-The authors refer to 'determine the cell type of the mAG and mCherry cells' yet they do not show any celltype specific staining. The latter could be provided or the fact that cell type assessment is based purely on morphology should be made clear.

These observations were made purely on morphology. We have clarified this on lines 259-261. It now reads "Based on cell morphology, we observed that the majority of uninjured tissue including fibroblasts,epithelial cells, and chondrocytes were mCherry$^+$."

Lastly, the authors study the induction of proliferation during spinal cord regeneration (Fig 6), and interestingly describe the induction of proliferation up to 5000 µm distal to the amputation plane. This is a very important observation and, given that the field will pay considerable attention to it, it would be desirable to validate the FUCCI/EdU based observations with additional markers of cell-cycle progressionwhich are established for the axolotl system (e.g. PCNA, Ki67 or (as previously used by the authors) pHH3).

We have validated our FUCCI probes with pHH3 and showed that a small fraction of mAG$^+$ cells are pHH3$^+$. Given the small number of cells in each spinal cord, the probability of locating mAG$^+$/pHH3$^+$ is small and may not be informative. Regarding additional markers of cell cycle progression, our lab has notbeen able to obtain reliable staining with Ki67 in axolotls. Additionally, PCNA stains the majority of cells and is not often used in axolotls. It also tends to stain many cells in regenerating and non-regenerating tissues observed by us and others (Albors et al., 2015).

Additional comments:
-Please indicate what number of mAG+ cells are PHH3+ (in mitosis, text ref to Fig. 2).

This information is now found in Fig. S2, and in text on lines 128-130. It reads "Further, we found that 3.91% of mAG+ cells were pHH3+ while 96.09% were pHH3- (Fig. S2C), suggesting that the vast majorityof mAG+ limb ¬¬blastema cells are in S/G2."

-It would be helpful to the reader to add the n, animal size and age to the figure legends.

Number of replicates, age, and animal sizes are now included in figure legends.

-Fig 5 G-I and Fig 5 J-K are annotated as 'Fig S X-X…'

-Perturbations that lead to cell cycle arrest (eg compounds targeting DNA topoisomerase and alike leading to S/G2 arrest, or even irradiation to see how the proportions of FUCCI indicators change) couldbe very informative on the usefulness and validity of the FUCCI system.

Denervating the regenerating limb leads to a reduction of cell proliferation. We have included an experiment exploring the impacts of denervation on the cell cycle in FUCCI animals. Data can be found inFigure 6 and the new results section titled "Blastema cells arrest in G1 phase in the absence of an intact nerve supply" on lines 297-322.

Reviewer 3 Advance Summary and Potential Significance to Field...

Live monitoring of cell cycle progression is crucial to study spatiotemporal dynamics of cell proliferationand behavior during animal development and regeneration. In the manuscript, Duerr et al generated CAG-FUCCI axolotl and revealed cell proliferation during axolotl development and regeneration of varioustissues and/or organs. A common problem for FUCCI system is that inappropriate expression level of the fluorescent proteins may affect the interpretation of proliferation, which is also a major problem in the manuscript.

Reviewer 3 Comments for the Author...

Major issues:
1. The authors performed several experiments to demonstrate that mAG+ cells are real proliferating cells,for example, EdU staining, pHH3 staining, and live cell imaging. However, I am not fully convinced by theresults given that: 1) the authors measured the correlation between EdU and mAG signals of spinal cord sections (without representative images in Fig. 2 and Fig. S1), but the EdU and mAG signals were poorlycolocalized in Fig. S1; 2) the majority of mAG+ cells did not divide in the live imaging of Movie 1.

We have now included representative images of EdU pulsed spinal cords and pHH3 stained blastemas inFigure S2. To assist in observing mAG/EdU colocalization in Fig. S1, we have increased the brightness ofthe mAG channel. However, we still see some mAG^-/EdU^+ cells. We predict that this is as a result of DNAdamage, as EdU is known to incorporate into cells undergoing DNA repair (Verbruggen et al., 2014) This may not explain the strong EdU signal in mAG^- cells in the gut. One potential explanation for this is rapid division and migration of enterocytes along the intestinal crypt. Supporting this is the fact that the EdU signal is weakest at the tip of the villi, suggesting that the EdU signal was rapidly diluted during the 3 hourEdU pulse. Few studies have been conducted on the intestinal stem cell niche of the axolotl, and it is our hope this finding may aid in future studies. We have included the sentences "An interesting finding in our study is the abundance of mAG-/EdU+ enterocytes in the axolotl gut (Figure S1F-F''''). One explanation for this could be the rapid clonal expansion and migration of enterocytes from the intestinal crypt to the tipof the villi. This is supported by a decrease in EdU intensity at the tip of the villi, suggesting EdU dilution after division despite a relatively short 3 hour pulse of EdU. More studies on the cell cycle length in the intestinal stem cell niche in the axolotl are required to elucidate this phenomenon." in the discussion on lines 413-419 to expand on this.

In some cases the length of the axolotl cell cycle has been reported to be 117 ± 12 hours (Rost et al. 2016), and the length of Movie 1 is 16 hours. It is thus reasonable to expect the level of cell divisionsobserved in Movie 1.

2. The images in Fig. 2D-M are misleading. As far as I know, mAG-zGem should be localized within the nuclei when nuclear envelops are intact. However, the mAG signals exist in both nuclei and cytosol in Fig.2H'-I' at early S stages. Similarly, the mCherry-zCdt1 signal cannot be observed once they disappear untilnext G1 stage but the mCherry signals are evident in Fig. 2K''-M'' from late S to M stages. Are the imagesmisinterpreted or phenomena unique to axolotl?

The vast majority of an axolotl cell is its nuclei due to its large genome size, and the cytosolic space isoften indistinguishable from the nuclei (see FISH in figure 4 to observe cytosolic mRNA surrounding massive nuclei). Some levels of fluorescence are to be expected in the cytosol, as the mAG/mCherry fluorescent proteins are first translated in the cytoplasm. However, most of the mAG/mCherry signal is located in the nucleus. Regarding the mCherry signal evident in late S to M phase, several plausible explanations exist: autofluorescence, early cytosolic translation of mCherry, or both. We believe that the former explanation is most likely, as the mCherry histograms for panels D''- M'' in figure 2 are matched forinterpretation across each cell. Axolotl cells are highly autofluorescent, which we believe is why some mCherry signal is observed in panels K''-M'' of figure 2.

3. The authors mentioned that the FUCCI animals has multiple transgenic insertions. They should perform southern blot to characterize the insertion number and select animals with single insertion forfurther experiments. Otherwise, it may be difficult to reproduce the results.

This is a future goal for our lab, and any potential issue will be diluted with subsequent offspring generations. However, the axolotl takes 8-14 months to reach sexual maturity, making immediate dilutions impractical to obtain. Furthermore, in recognition of this potential issue we were careful in ourstudies to not compare cell fluorescence from animal to animal, mitigating any potential difficulties in interpretation and replication.

Minor issues:
1. The authors should disclose detailed information for the EdU pulse experiments, especially for theduration of treatment and interval before tissue collection.

This information can be found in the main text on lines 107-109, animal procedures section in the methods on lines 461-463, and the figure legend of figure 2 on lines 816-818.

2. The authors should disclose the stages of axolotl used in all experiments.

Number of replicates, age, and animal sizes are now included in figure legends.

3. The authors should include the timepoints for each frame in the supplementary movies.

Timestamps and scale bars were added to the supplementary movies.

4. Line 118: Fig. 3B should be Fig. 2B.

5. Line 173: Fig. J-K.

6. Line 193: this limitation is not caused by the CAG promoter not the fluorescent proteins.

We have modified this sentence for clarification. It now reads “The use of two fluorescent proteins in FUCCI sensors limits the acquisition of cell type information, precluding robust cell characterization usingimaging modalities including immunohistochemistry (IHC) and fluorescence in-situ hybridization (FISH) inconjunction with FUCCI probes” on lines 221-224.

7. Movie 2 is difficult to play.

We have tried to re-save the movie to make it easier to play.

Second decision letter

MS ID#: DEVELOP/2021/199637

MS TITLE: A constitutively expressed fluorescence ubiquitin cell cycle indicator (FUCCI) in axolotls for studying tissue regeneration

AUTHORS: Timothy J Duerr, Eun Kyung Jeon, Kaylee M Wells, Antonio Villanueva, Ashley W Seifert, Catherine D. McCusker, and James R Monaghan

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

You will see that Reviewer's #1 and #2 are enthusiastic and recommend publication of the manuscript, whereas Reviewer #3 feels that certain points must be addressed. Both Reviewer #1 and #3 recommend this manuscript be considered as a Techniques and Resources piece, as they feel the new reagent is central to the work versus new concepts/mechanisms. I have thought about this and generally agree that this manuscript fits better as a Techniques and Resources article. While it is unusual to ask for a second round of revisions and review, I feel another revision is needed to

improve your manuscript and its potential impact given the change in format and additional information that is requested.

I expect to send the revised manuscript to Reviewer #3 only. My impression is that you can address comments 1, 2, 4 with explanation in a response letter and as text changes in the manuscript. I do not feel it is necessary to perform tissue culture experiments for comment 5. I ask that you pay particular attention in your response to comment 3, which is likely to involve new analyses. I suspect you will understand how important it is to understand the nature of insertions and how they might relate to the reported visual observations. Other investigators wishing to use the multi-insertion line may receive animals with a subset of insertions, potentially with different FUCCI expression domains and dynamics. While reporting the tool as a single insertion line is standard and ideal, in this case it will be key to offer at least as much insight as possible into the number of insertions among animals as requested.

Please attend to all of the reviewers' comments and 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.

Reviewer 1

Advance summary and potential significance to field

The manuscript presents a new transgenic tool to study cell cycle dynamics in axolotls.

Comments for the author

The authors have addressed most of the points in a manner that significantly improves the manuscript. Just a minor comment: since the significance of the study lies in the validation and examination of the FUCCI reporter in the transgenic line, and proof-of-principle live imaging experiments during limb and tail regeneration, the manuscript might be a better fit for the “Techniques and Resources” section. The transgenic tool is promising and would be a useful resource to the field. Yet, unlike typical Research articles published in the journal, the study falls short of providing new mechanistic insight into our understanding of the regenerative process.

Reviewer 2

Advance summary and potential significance to field

Duerr and colleagues have satisfactorily addressed my comments, both experimental and communicational. I have no further points to raise and thus endorse this manuscript for publication in Development.

Comments for the author

Duerr and colleagues have satisfactorily addressed my comments, both experimental and communicational. I have no further points to raise and thus endorse this manuscript for publication in Development.

Reviewer 3

Advance summary and potential significance to field

The authors made some modification to previous version.

Comments for the author

1. I am not convinced that the revision is well prepared, actually, it looks like immature for both the original manuscript and this revision. As pointed by me and the other two reviewers, there were many typo and mislabeling in the original ms. The new revision also contains many typo and mislabeling, for example, "Fig. 6H-N" in line 259 should be "Fig. 5H-N". Most importantly, the main figures are totally disordered.
2. The authors argue that "The vast majority of an axolotl cell is its nuclei due to its large genome size, and the cytosolic space is often indistinguishable from the nuclei" to explain why "the mAG signals exist in both nuclei and cytosol in Fig. 2H'-I' at early S stages." However, the boundary of the nuclei is clearly represented by the DAPI signal. I checked the movies closely in the Sakaue-Sawano paper (doi: 10.1016/j.cell.2007.12.033) and Costa paper (doi: 10.7554/eLife.55665), showing that the predominant nuclear localization of mAG signals at S/G2 phase. There explanation is not persuasive.
3. This manuscript is more like a resource paper, but the transgenic line is not well characterized. Even though the author cannot screen single insertion line within two to three months, they can perform southern blot to characterize the identify the copy number for the animals with different fluorescent levels. By the way, it has been five months since their first submission. At least, they should be able to analyze the embryos as they did in several experiments. Please improve the data quality of this manuscript first.
4. The authors performed cell cycle analysis of the denervated and innervated limb regenerates in the revision, but the results were not statistically analyzed with biological repeats, which damps their conclusion.
5. I can play Movie 2 this time, but a single dividing nucleus is insufficient. I recommend the authors to culture and live image the dissociated cells or tissue trunks to visualize and analyze the cell cycle progression of animals with different fluorescent levels. This can help to improve accuracy of their data and conclusion.

Second revision

Author response to reviewers' comments

Reviewer 3 Advance Summary and Potential Significance to Field:

The authors made some modification to previous version.

Reviewer 3 Comments for the Author:
1. I am not convinced that the revision is well prepared, actually, it looks like immature for boththe original manuscript and this revision. As pointed by me and the other two reviewers, there were many typo and mislabeling in the original ms. The new revision also contains many typo and mislabeling, for example, "Fig. 6H-N" in line 259 should be "Fig. 5H-N". Most importantly, the main figures are totally disordered.

We have meticulously gone through the manuscript again and identified any typos or mislabeling and corrected them. We have rearranged several figures to make sure the figures follow the order as presented in the manuscript. We have also presented the figures to both specialists and non-specialists to check if they were clear.

2. The authors argue that "The vast majority of an axolotl cell is its nuclei due to its large genome size, and the cytosolic space is often indistinguishable from the nuclei" to explain why "the mAG signals exist in both nuclei and cytosol in Fig. 2H'-I' at early S stages." However, the boundary of the nuclei is clearly represented by the DAPI signal. I checked the movies closely in the Sakaue-Sawano paper (doi: 10.1016/j.cell.2007.12.033) and Costa paper (doi: 10.7554/eLife.55665), showing that the predominant nuclear localization of mAG signals at S/G2 phase. There explanation is not persuasive.

As a reminder to Reviewer 3, here are their comments from the first round of reviews*:*

“The images in Fig. 2D-M are misleading. As far as I know, mAG-zGem should be localized within the nuclei when nuclear envelops are intact. However, the mAG signals exist in both nuclei and cytosol in Fig. 2H’-I’ at early S stages. Similarly, the mCherry-zCdt1 signal cannot beobserved once they disappear until next G1 stage but the mCherry signals are evident in Fig. 2K’’-M’’ from late S to M stages. Are the images misinterpreted or phenomena unique to axolotl?”

We agree with Reviewer 3's comment that Video 2 in the Costa et al paper shows predominant nuclear expression of the Gemenin degron in S phase, but it is definitely not equally strong throughout the S-phase. For example, in second 2 on Movie 2 in the Costa paper, there is weak nuclear staining during the transition from the G1 to S phase. The movie then increases nuclear intensity when progressing into the S-phase, which is just what our Figure 2 H and I show. By showing both channels simultaneously, it makes the contrast look more striking. For example, H"" looks to have more nuclear localization than H'.

We also feel that cells electroporated with the FUCCI plasmid (as in the Costa paper) should have considerably higher expression and therefore signal to noise compared to our in vivo images, which have limited genomic inserts and in vivo imaging.

The individual cells in Figures 2H-I were transitioning from mCherry^{+} to mAG^{+}. For this reason, we expected low levels of both mCherry and mAG, as mCherry should be degraded simultaneously as mAG is being translated. This is just what we observed. The low intensity of mAG in early S phase likely led to low signal to noise between nuclear and cytoplasmic staining.Once the cells enter the S phase, it is very clear that the signal is exclusively nuclear in Figure 2.
This can also be observed in figures 4, 7, Supp Fig 2, Supp Fig 5, and Supp Fig 8. In particular Figures 4 and Supp Fig 8 show clear nuclear staining of both mCherry and mAG in the majority of cells in the tissue sections. Therefore, we feel that the reporter provided signal asexpected.

3. This manuscript is more like a resource paper, but the transgenic line is not well characterized. Even though the author cannot screen single insertion line within two to three months, they can perform southern blot to characterize the identify the copy number for the animals with different fluorescent levels. By the way, it has been five months since their firstsubmission. At least, they should be able to analyze the embryos as they did in several experiments. Please improve the data quality of this manuscript first.

Although we would prefer to wait for the F2 generation for all characterization, this can be up to three years in the axolotl and the community has shown great interest in obtaining this line before then. There have been 27 axolotl lines generated to date, and all but two are readily available to the entire community. The line described here is already almost ready for shipping from the Ambystoma Genetic Stock Center and has shown high interest from the community. Our strategy of using F1's for analysis is to increase accessibility to the community for these important transgenic lines.

To attempt to address this question of insertion number, we discussed this point with three of the leading experts in axolotl genomics and transgenics, Dr. Prayag Murawala, Dr. Randal Voss, and Dr. Jeramiah Smith. They were all in agreement that neither southern blots or inversePCR has been done in any of the lines. We agree in the importance of understanding the genomic integration, but we did not see this as necessary when crafting the original manuscript considering many of these unmapped lines have been published in the world's top journals.

All three colleagues independently said that southern blotting with a 32gb genome is much easier said than done, which we agreed. Therefore, we attempted to perform inverse PCR to identify the location of the insert within the axolotl genome (and thus the number of inserts) with little success. All of our control amplifications of the transgene and other genomic loci worked well, but we were unable to amplify the flanking regions of the insert. However, we agree more characterization of the transgenic line is important. To this end we have crossed F1 FUCCI animals to other F1 FUCCI siblings or to a d/d animal. The results of these crosses can be found in supplementary table 1. Our quantification confirms the presence of multiple insertions within the F1 offspring. We have added the following sentences on lines 94-100 “To confirm this, we crossed F1 FUCCI animals to an F1 FUCCI sibling or a d/d white animal. In each cross,we observed a non-

mendelian distribution of offspring (Table S1), confirming the presence of multiple integrations in the F1 generation. F2 larave exhibit similar mAG and mCherry expression patterns as in the F1 generation through embryonic development (data not shown). All subsequent experiments in the forthcoming sections were conducted on FUCCI animals fromthe F1 generation."

4. The authors performed cell cycle analysis of the denervated and innervated limb regenerates in the revision, but the results were not statistically analyzed with biological repeats, which damps their conclusion.

We agree that biological repeats would strengthen our conclusion. However, the intention of our manuscript was to showcase the versatility of the FUCCI system in the axolotl and the impact of decreased cell cycling after denervation has been a known phenomenon for decades. For this

reason, our goal was to demonstrate the utility of the line and not further this point. A full investigation of the impact of denervation on specific cell cycle kinetics is an interesting question though, which will require the use of this FUCCI line on top of in vitro and biochemical analyses.

5. I can play Movie 2 this time, but a single dividing nucleus is insufficient. I recommend the authors to culture and live image the dissociated cells or tissue trunks to visualize and analyze the cell cycle progression of animals with different fluorescent levels. This can help to improve accuracy of their data and conclusion

Movie 2 is simply a zoom in to a diving cell. Movie 1 and 3 show hundreds of dividing cells over many hours of recording. We do not feel removing cells to image in culture strengthens the clear observation of cell divisions in the movies provided. Movie 2 is included to provide a zoomed in image of a dividing cell.

As we had written to reviewer two:

Our validation with EdU and pHH3 in Figure 2 demonstrates that the fluorescence emitted from FUCCI cells matches our expectations for cell cycle state. We have added the sentences "Importantly, we observe mAG+/mCherry+/EdU+ cells (Fig. 2H-H''''), indicating the presence of cells in very early S phase. These cells have not yet fully degraded mCherry but have started producing mAG while incorporating EdU in early S phase. This demonstrates that cells in our FUCCI system faithfully change color upon transition from G1 phase into S phase" on lines 139-144 to indicate that FUCCI^{+} cells are changing color.

Third decision letter

MS ID#: DEVELOP/2021/199637

MS TITLE: A constitutively expressed fluorescent, ubiquitination-based cell cycle indicator (FUCCI) in axolotls for studying tissue regeneration

AUTHORS: Timothy J Duerr, Eun Kyung Jeon, Kaylee M Wells, Antonio Villanueva, Ashley W Seifert, Catherine D. McCusker, and James R Monaghan
ARTICLE TYPE: Techniques and Resources Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 3

Advance summary and potential significance to field

This study generated a novel FUCCI-based axolotl line and characterized cell proliferation during axolotl development and regeneration, which will be a valuable resource for the field.

Comments for the author

The revised manuscript has been improved. Although the authors did not fully address my concern partially due to technical difficulties, the manuscript is still worth publishing as a resource report.