Decoding an organ regeneration switch by dissecting cardiac regeneration enhancers

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MS TITLE: Decoding an Organ Regeneration Switch by Dissecting Cardiac Regeneration Enhancers

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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 considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of 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 currently be unable to access the lab to undertake experimental revisions. 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 the report by Begeman and Shin et. al. their work centered on defining the minimal DNA sequence that is responsible for driving leptin b (lepb) gene expression during adult heart regeneration. The work builds form the initial identification of an enhancer region in the lepb gene that is rapidly activated after cardiac and fin injury. This led to the definition of cis-regulatory sequences that are active during regeneration. Here the authors define a critical cardiac specific enhancer (317bp fragment) that is active after injury from larval stages and in adults. Within this sequence lies multiple transcription factor binding sites that includes AP-1 NFAT, ETS, FOX and GATA sites suggesting that these factors contribute to the expression of lepb during heart regeneration. Moreover, further fine-mapping of the critical region identify a 22-bp sequence that is conserved in a variety of Danio species. Surprisingly, the authors show that reporter gene activity was aberrant prior to injury when this 22-bp sequence was deleted, i.e. in the absence of injury, GFP was expressed in the endocardium. Thus, the authors conclude that this element is a landing site for repressive factors and that upon injury, de-repression occurs offering a new paradigm in gene regulation during regeneration.

Overall the study is well executed through the generation of multiple transgenic reporter lines to carefully test each of the predicted transcription factor binding sites. The authors also show conservation of these elements with other Danio species and narrowed down to a 22bp sequence. The significant advance is that there are multiple levels of gene regulation during development and regeneration of the lepb gene. The activity of the repressor element does not occur during development but as the heart matures this is active to repress lepb expression. Important this switch is activated in response to injury and implying derepression occurs as a regenerative response.

Comments for the author

While the study is interesting and highlights the complexities of gene regulation during heart regeneration, it is lacking in direct mechanism in its current format. For example, details as to what factors bind to this 22bp fragment are not described or that direct testing candidate factors that bind to the cardiac lepb enhancer are not pursued. Addressing a mechanism as to how this enhancer is regulated would be important in a revised submission.

Major comment:

- There are outlined several transcription factor binding sites that are predicted to bind to the cardiac lepb enhancer. Some of these were ruled out with new transgenic lines where these domains were deleted and was narrowed to cLEN-act (108-238). This domain contains a predicted AP-1, FOX, NFAT and ETS sites. The authors ruled out Fox, NFAT and ETS as a fragment containing only this sequence was not active, suggesting that AP1 may be important in combination with these sites. The authors could test whether AP1 in combination with some of these sites could be sufficient. Alternatively, it may be possible to demonstrate importance of AP-1 site with mutants or chemical inhibitors of AP-1.
- Their studies highlight the FOX, NFAT and ETS sites collectively were not sufficient to drive a regenerative response. However, their deletion resulted in ectopic GFP expression in the absence of injury and when the hearts were injured, expression domain was expanded to the remote zone. What is not clear if the combination of the AP1 site with another site alone can lead to sufficient expression of GFP in injury. For example, can GFP expression be still observed if the combination of AP-1 and only the NFAT site remains?
- The identification of a 22-bp fragment within cLEN-act that when deleted leads to aberrant expression of GFP is interesting, but there are no other details given. Does this sequence contain sites for known transcriptional repressors? The authors should comment on this or are these sequences so degenerate that no firm prediction can be made.
- In figure 3, the authors quantify GFP expression in the remote zone showing ectopic expression in the absence a 47bp sequence in cLEN. It appears to this reader that expression in the border zone is also increased. Quantification of the border zone should be done. This appears to be the case in Figure 4, where border zone shows abundant GFP expression in cLEN 11-1 to 11-3, but

much weaker in 11-4 and 11-5. The authors should quantify the expression in the border zone for figure 4.

Minor Comments:

- Figure S1 should be included into Figure 1 as it shows important information for the whole study. The color-coded predicted transcription factor binding sites should be used in all the figures where the enhancer fragment is schematized. It is difficult to follow the various fragments tested without a framework as to where each of these sites are in the various transgenic lines.
- Figure S6 should be included as a main figure as it shows the importance of conservation of these sequences in other related Danio species and that lepb is activated in heart injury.
- Figure 7 depicts a model for activity of cardiac regeneration enhancers, but needs a worded description for each of the phases.

Reviewer 2

Advance summary and potential significance to field

The mechanisms by which tissues sense injury and respond to the cue at the transcriptional level remain unclear. Understanding the spatiotemporal-specific enhancer activities and binding factors involved might reveal how injury cues are transduced and how regeneration is achieved. A cardiac regeneration-specific enhancer (c-LEN) was previously identified in zebrafish. To further decipher regulatory elements regulating regeneration dependent gene expression, the authors performed extensive enhancer bashing experiments. A repressive element that inhibits enhancer activities in uninjured hearts, remote area of injured hearts and during development was identified.
Based on their data, the authors proposed that the repressive element starts to function during heart maturation and both activators and repressors coordinate to regulate the transcription during heart regeneration to prevent aberrant gene expression during heart development or in uninjured area. The data presented in this well written manuscript are of high quality. Their findings are interesting and significant and might shed light on how regenerative responses are precisely controlled at the transcriptional level.

Comments for the author

1. The authors generated an impressive panel of transgenic constructs and at least 2 lines were generated for the most constructs (supplemental Table 2). The author should indicate which lines the representative images are obtained from in the figure legends or mark them in supplemental table 2. The authors mentioned that all 4 cLENd47 lines showed ectopic expression. It would be nice if they can also comment on other constructs.

2. From the images, it is not clear if the ectopic expression of cLENd47:GFP and cLENd11-1 and 11-2 are also observed in other cell types such as cardiomyocytes or vessels. Please clarify.

3. Please clarify if the enhancer -47 bp or -22 bp repressive elements are still derepressed after the regeneration is complete or after 1 month of injury.

4. It is interesting that cLENd11-4 and cLENd11-5 showed very week GFP expression in the border zone expression. Is this due to the deletion of potential activator elements? Please clarify or speculate.

5. Phylogenetic comparison revealed moderate sequence conservation within the Cyprinidae family and that the cLEN-act enhancer is highly conserved with two other Danio species. A highly conserved AP-1 site and GTCA sequences were identified.
Do the constructs cLEN-FNE and cLEN-act suggest that the AP-1 site (between 108-118) is indeed essential for the enhancer activity? The authors can clarify and speculate.

6. What is the functional consequence of deleting the repressive element on heart development or regeneration? The authors might try to delete the 22 bp element in the genome with CRISPR/Cas9. Since this experiment might take a long time to perform, the authors can just

examine the potential phenotypes in heart development if they have generated the lines. It is certainly possible there are redundant repressive elements and the deletion will not result in any phenotypes in heart development and regeneration. If this is the case, the authors can also comment on this.

Reviewer 3

Advance summary and potential significance to field

The authors study how an enhancer which they have previously identified to be specifically activated during zebrafish heart and fin regeneration, is regulated. In particular, they identify smaller sequence elements that are sufficient to drive activation during heart regeneration, and, interestingly identify an element that represses expression in noninjured hearts. The findings are of great interest and provide substantial novel mechanistic insight into a highly intriguing biological problem. The data are generally of high quality. However, at several points I think the data do not yet fully support the conclusions drawn. Yet, when the authors can address the following issues, I can recommend publication in Development.

Comments for the author

Major:

1) Throughout: Positional effects can massively influence expression of transgenes, in particular in adults. Thus, deriving well-expressing transgenic lines using random integrations of transgenes into the genome can be a challenge even for well characterized “ubiquitous” promoters. Thus, I find it generally quite puzzling in studies like this and similar ones, that the authors are able to derive clear, unambiguous conclusions from the analyses of a few transgenic lines. Maybe specific enhancers are less prone to positional effects and silencing than ubiquitous promoters? While I can hardly ask the authors of this study to figure this out, I would like to see more transparency in relation to this issue. They mention at several places in the manuscript that several independent lines (sublines / integrations) have been analyzed, but this is not enough information to judge how reproducible their findings really are between independent sublines / integrations. They should provide a table giving data for each individual subline / integration analyzed for each of the constructs used in the paper that summarizes which of the described expression patterns were actually seen in which line. In addition, for a few select particularly important results, quantifications of different sublines should be shown, eg. for cLENDelta47.

2) Fig. 1C: induction in 5dpf embryos does not seem to be specific to the heart. Which tissues / cells is the reporter expressed in? Did the authors perform further controls to make sure it’s not the Mtz treatment on it’s own that causes induction (treat single reporter transgenics with Mtz). Is induction outside the heart in response to CM ablation also seen in juvenile and adult stages?

2b) somewhat related to issue 2: an interesting question that has also not been addressed by the previous publication of Junsu Kang on the LEN is to which extent activation is regeneration-specific or occurs also in response to other types of injuries which would conventionally not be considered to result in regeneration. E.g. epidermal wounds? Larval fin folds or adult fins (incisions in the interray tissue) would be convenient models to test this.

3) for non-quantified experiments, like in Fig. 1C-E, Fig. 2, the number of samples (embryos, hearts) that showed a phenotype / appearance similar to the one shown in the representative image and the total number of analyzed samples should be given (eg. 12/15 embryos).

4) Fig. 1H: While the expression pattern is suggestive, these images do not support the statement that expression is mainly in endocardial cells. In fact, at the wound border, expression appears to be not just confined to endothelial cells (e.g. Fig. 3B), as far as this can be judged without co-staining. Co-staining with endothelial markers (e.g. in double transgenics) and higher magnification would be needed to unambiguously identify the expressing cells. Same is true for the deletion constructs in Fig. 2 & 3 &

4. Thus, authors should first unambiguously demonstrate endothelial expression of the full transgene, which will make the deletion construct data more interpretable as well.

5) Related to issue 3, the statement in line 177 “a significant portion of endocardial cells” needs to be substantiated by a) co-staining and b) quantification of expressing cells.

6) Fig. 3B: authors imply that cLENDelta47 still induces at the border. How do they know that? S4 images are suggestive, but intensity measurements of the border zone vs. uninjured and remote areas would be required to verify that activity is indeed higher at the border.

7) Fig.4. area covered by EGFP should be also measured for the border zone for these deletion lines to support the statement that all lines support induction there. In addition, images suggest that expression at the border is also much weaker in some deletion lines than others. Thus, expression in the remote zone should also be shown relative to the one in the border zone.
In case the author’s conclusion is correct that some deletions only result in derepression of expression in the remote zone (and in uninjured hearts) and do not effect overall expression levels, this effect should still be seen when normalized for expression in the border zone.

8) Fig. 6. While it is interesting that ectopic expression of the deletion line in noninjured hearts can only be seen after 5 dpf, I am not sure whether I can agree with the authors that this shows that repression emerges at or after that time point. If repression were not active earlier shouldn’t the complete LEN element be expressed at early stages? It’s rather the ectopic expression that gets activated later, which I think is not quite the same thing...

Minor:

Language: Line 160 “as reported...”

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
In the report by Begeman and Shin et. al. their work centered on defining the minimal DNA sequence that is responsible for driving leptin b (lepb) gene expression during adult heart regeneration. The work builds form the initial identification of an enhancer region in the lepb gene that is rapidly activated after cardiac and fin injury. This led to the definition of cis-regulatory sequences that are active during regeneration. Here the authors define a critical cardiac specific enhancer (317bp fragment) that is active after injury from larval stages and in adults. Within this sequence lies multiple transcription factor binding sites that includes AP-1, NFAT, ETS, FOX and GATA sites suggesting that these factors contribute to the expression of lepb during heart regeneration. Moreover, further fine-mapping of the critical region identify a 22-bp sequence that is conserved in a variety of Danio species. Surprisingly, the authors show that reporter gene activity was aberrant prior to injury when this 22-bp sequence was deleted, i.e. in the absence of injury, GFP was expressed in the endocardium. Thus, the authors conclude that this element is a landing site for repressive factors and that upon injury, de-repression occurs offering a new paradigm in gene regulation during regeneration.

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-Their studies highlight the FOX, NFAT and ETS sites collectively were not sufficient to drive a regenerative response. However, their deletion resulted in ectopic GFP expression in the absence of injury and when the hearts were injured, expression domain was expanded to the remote zone. What is not clear if the combination of the AP1 site with another site alone can lead to sufficient expression of GFP in injury. For example, can GFP expression be still observed if the combination of AP-1 and only the NFAT site remains?

We thank the reviewer for suggesting interesting experiments. We agree with the comments of the reviewer on the importance of transcription factor binding sites, including AP-1 and others. To address this comment, we have performed two additional experiments.

1) AP-1 binding site mutations
We generated two new transgenic constructs:
a) AP-1 mutated *cLEN* (*cLEN*$^{AP\text{-}1m}$): Two AP-1 binding sites in *cLEN* were mutated.
b) Synthetic enhancer (AFNEGx3): We generated a synthetic enhancer consisting of three tandem copies of AP-1-FOX-NFAT-ETS-GATA binding sites.

These transgenic constructs were injected, and EGFP expression was examined in injured hearts. To injure the hearts, we ablated cardiomyocytes (CMs) using *cmlc2:mCherry-NTR* as described in the method and Fig. 1C and D. Although a significant number of larvae injected with the *cLEN:EGFP* (positive control) induce EGFP expression in the hearts upon injury, the majority of larvae injected with *P2:EGFP* (negative control, minimal promoter), *cLEN*$^{AP\text{-}1m}$*P2:EGFP* and *AFNEGx3-P2:EGFP* do not have injury-responsive expression in hearts. These new results are now included in Fig. 2C-E and described in the text on Page 9 as follows:

"To test the involvement of the AP-1 binding sites in injury-responsive activation, two AP- 1 motifs in *cLEN* were mutated to generate *cLEN*$^{AP\text{-}1m}$, and their activity was examined in the F0 mosaic injured hearts. Several constructs were generated, including *P2:EGFP* (minimal promoter, negative control), *cLEN:EGFP* (positive control), or *cLEN*$^{AP\text{-}1m}$*:EGFP* (**Fig. 2C**). These constructs were injected into one-cell stage of the *cmlc2:mCherry-NTR* embryos, and EGFP expression was examined before and after cardiac ablation (**Fig. 2D**). None of these constructs were able to direct EGFP expression in the uninjured hearts. Upon cardiac injury, a significant number of larvae carrying the intact *cLEN* directed EGFP induction (**Fig. 2E**). However, the majority of larvae with *P2* or *cLEN*$^{ap\text{-}1m}$ were not able to direct injury-responsive expression (**Fig. 2E**), indicating that AP-1 binding sites are required for injury-responsive activity.

We next attempted to build a synthetic version of a cardiac regeneration enhancer by combining TF binding sites present in *cLEN-act*. Three copies of AP-1-FOX-NFAT- ETS-GATA sites were coupled with the *P2* minimal promoter and EGFP sequences to produce *AFNEGx3*, and its activity was examined in the F0 mosaic injured hearts. EGFP was undetectable in 3 dpf uninjured hearts. Noticeable EGFP induction was detected in some mosaic injured hearts carrying *AFNEGx3* (**Fig. S6D**); however, the majority of larvae did not demonstrate injury-responsive EGFP induction (**Fig. 2E**). These data suggest

that further dissection of *cLEN* is required to create a functional synthetic regeneration enhancer. In addition, enhancer assays with stable lines will be required to conclude the activity of *AFNEGx3*."

2) mRNA injection

We generated mRNA of AP-1 complex and *nfatc1* and examined whether mRNA injection of these transcription factors in early *cLENP2:EGFP* embryos can drive EGFP expression. As AP-1 complex functions as a dimer of the *jun* and *fos* subunits, we selected *junba* and *fosl1a* to represent *fos* and *jun*, respectively. A recent study demonstrated that these two factors are potential AP-1 components contributing to the cardiac injury-responsive gene expression in zebrafish (Beisaw et al., 2020). Although *mCherry* or *nfatc1* mRNA injection into *cLENP2:EGFP* heterozygote embryos does not direct EGFP expression, AP-1 alone or AP-1+*nfatc1* can direct EGFP expression in the early embryos. Since AP-1 overexpression in the early embryos cause developmental defects, we were not able to test EGFP induction in uninjured heart in the later larval stages. These new results are now included in Fig. S6 and described in the text on Page 10 as follows:

"A recent study demonstrated that co-injection of mRNAs of the AP-1 complex components, *junba* and *fosl1a*, into one-cell-stage embryos is sufficient to activate the expression of AP-1 target genes (Beisaw et al., 2020). To determine whether AP-1 TFs can activate *cLEN*, *mCherry*, AP-1 (*junba*/*fosl1a*), *nfatc1*, or *nfatc1*+AP-1 mRNAs were injected into the one-cell stage of the *cLEN:EGFP* heterozygote embryos, and EGFP expression was assayed at 1 day (**Fig. S6A**). Although *mCherry* or *nfatc1* mRNA injection was unable to induce EGFP, we observed that a significant number of embryos injected with AP-1 or *nfatc1*+AP-1 demonstrated EGFP expression (**Fig. S6C**), suggesting that AP-1 contributes to the activation of *cLEN*. Overall, our data suggest that AP-1 is an important injury-activating factor for *cLEN*."

- The identification of a 22-bp fragment within cLEN-act that when deleted leads to aberrant expression of GFP is interesting, but there are no other details given. Does this sequence contain sites for known transcriptional repressors? The authors should comment on this or are these sequences so degenerate that no firm prediction can be made.

We updated our manuscript with the explanation of why we mutated GTCA to CATT.

On Page 15, we added:

"Our motif analysis of *cLEN-22* using the JASPAR database predicted binding sites for two repressors: *growth factor independent 1b* (*gfi1b*) and *PR/SET domain 1a* (*prdm1a)*. *gfi1b* is the key repressor controlling hematopoiesis (Dahl et al., 2007; Li et al., 2010; Saleque et al., 2007). *prdm1* acts as a repressor in the intestine and immune cells (Harper et al., 2011; Hohenauer and Moore, 2012; Kallies et al., 2006; Muncan et al., 2011). To test whether their binding sites are responsible for the repression, the JASPAR database was used to design a mutation that disrupts the Prdm1a and Gfi1b binding sites and is not predicted to create new binding sites or disturb spacing between other candidate sites. Based on this analysis, the GTCA sequence was mutated to CATT. To test whether the GTCA sequence comprises the critical repressive motif, new transgenic fish with the GTCA to CATT mutation were generated, named *cLENm*^{gtca}."

-In figure 3, the authors quantify GFP expression in the remote zone showing ectopic expression in the absence a 47bp sequence in cLEN. It appears to this reader that expression in the border zone is also increased. Quantification of the border zone should be done. This appears to be the case in Figure 4, where border zone shows abundant GFP expression in cLENΔ11-1 to 11-3, but much weaker in 11-4 and 11-5. The authors should quantify the expression in the border zone for figure 4.

We have added the quantification data of the border zone in Fig. 3C and 4D and explanation on Pages 11 and 13:

Page 11

"In 3 dpa hearts, both *cLEN* and *cLENΔ47* directed EGFP expression in the border zone (5.66, 10.95, 12.99 and 15.62 μm^2/100 μm^2 in *cLEN* line 3 and 7 and *cLENΔ47* line 3 and 6, respectively), demonstrating their activation abilities in response to injury (**Fig. 3B, D**)."

Page 13

"In 3 dpa regenerating adult hearts, all five constructs were capable of directing injury-responsive gene expression in the border zone (16.77, 15.86, 20.98, 5.12 and 6.96 $\mu m^2/100\ \mu m^2$ for *cLENΔ11-1* to *-5*, respectively; **Fig. 4B, D**), indicating that injury-responsive activity is intact regardless of these 11 bp deletions. *cLENΔ11-4* and *-5* had lower area of EGFP expressing cells, compared to that in other *cLENΔ11* lines, suggesting that deletion of the potential activation motifs, such as ETS, influences injury-responsive activity in the border zone."

Minor Comments:
-Figure S1 should be included into Figure 1 as it shows important information for the whole study. The color-coded predicted transcription factor binding sites should be used in all the figures where the enhancer fragment is schematized. It is difficult to follow the various fragments tested without a framework as to where each of these sites are in the various transgenic lines.

Thank you for the suggestion. We modified Fig. 1 by adding a new schematic indicating predicted TF binding sites. However, whole *cLEN* sequence information is not closely related to the main point of Fig. 1 that *LEN* is a regeneration enhancer. Thus, we did not change Fig. S4 (corresponding Fig. S1 in the previous manuscript). We also added the color-coded predicted TF binding sites in Figure 2A.

-Figure S6 should be included as a main figure as it shows the importance of conservation of these sequences in other related Danio species and that lepb is activated in heart injury.

Figure S6 is included in Figure 5.

-Figure 7 depicts a model for activity of cardiac regeneration enhancers, but needs a worded description for each of the phases.

We have updated the figure legend:

"**Figure 7. Proposed model to regulate the activity of cardiac tissue regeneration enhancer elements (TREEs) in uninjured and regenerating hearts.** In uninjured embryonic hearts, cardiac TREEs are inactive and are activated upon injury. Repression of cardiac TREEs is not functional in the heart in early development. During maturation, cardiac TREEs are actively repressed to prevent aberrant activation in uninjured tissues. Upon injury, the dual function of distinct *cis*-regulatory elements restricts cardiac TREE activation to the wound area."

Reviewer 2 Advance Summary and Potential Significance to Field:
The mechanisms by which tissues sense injury and respond to the cue at the transcriptional level remain unclear. Understanding the spatiotemporal-specific enhancer activities and binding factors involved might reveal how injury cues are transduced and how regeneration is achieved. A cardiac regeneration-specific enhancer (c-LEN) was previously identified in zebrafish. To further decipher regulatory elements regulating regeneration dependent gene expression, the authors performed extensive enhancer bashing experiments. A repressive element that inhibits enhancer activities in uninjured hearts, remote area of injured hearts, and during development was identified. Based on their data, the authors proposed that the repressive element starts to function during heart maturation and both activators and repressors coordinate to regulate the transcription during heart regeneration to prevent aberrant gene expression during heart development or in uninjured area. The data presented in this well written manuscript are of high quality. Their findings are interesting and significant and might shed light on how regenerative responses are precisely controlled at the transcriptional level.

Reviewer 2 Comments for the Author:
1. The authors generated an impressive panel of transgenic constructs and at least 2 lines were generated for the most constructs (supplemental Table 2). The author should indicate which lines the representative images are obtained from in the figure legends or mark them in supplemental table 2. The authors mentioned that all 4 cLENd47 lines showed ectopic expression. It would be nice if they can also comment on other constructs.

We thank the reviewer for this suggestion. We generated Supplemental Table 3 to include the total

number of animals used in this study.

2. From the images, it is not clear if the ectopic expression of cLENd47:GFP and cLENd11-1 and 11-2 are also observed in other cell types such as cardiomyocytes or vessels. Please clarify.

We have analyzed more images of injured hearts in multiple transgenic fish, including *P2:EGFP*, *cLEN:EGFP* and *cLENΔ47:EGFP*. Cardiomyocytes (CMs) and endocardial cells were stained with MHC and raldh2 antibodies, respectively. This analysis demonstrated that the *P2* minimal promoter used for transgenic assays drives weak expression in some CMs upon injury. However, *P2* does not direct the expression in any cell types in the uninjured heart. Additionally, *P2* does not drive endocardial expression in the injured heart. Similarly, *cLEN:EGFP* and *cLENΔ47:EGFP* direct EGFP expression in some CMs. In contrast to *P2*, *cLEN:EGFP* and *cLENΔ47:EGFP* drive strong endocardial expression in the border zone. In the remote zone of *cLEN:EGFP* and *cLENΔ47:EGFP* injured heart, EGFP is not detectable in CMs. Our analysis demonstrated that *cLEN* causes injury-induced ectopic expression in CMs at the border zone but the main activity is observed in endocardial cells in border and remote zones upon injury. These new results are included in Fig. S3.

3. Please clarify if the enhancer -47 bp or -22 bp repressive elements are still derepressed after the regeneration is complete or after 1 month of injury.

We have performed additional experiments to address this comment. In Fig. 1H, we demonstrated that *cLEN* activity is significantly reduced to the level similar to that detected in uninjured heart. We examined EGFP expression in 42 dpa heart of *cLENΔ47:EGFP*, *cLENΔ11-1:EGFP* and *cLENΔ11-2:EGFP*. Interestingly, EGFP expression in 42 dpa heart of these deletion lines does not return to the level detected in uninjured hearts. Quantification analysis in *cLENΔ47:EGFP* indicates significant induction at 42 dpa, indicating that the repressive element remains derepressed at 42 dpa heart. These new results are added in Fig. S8D, E and described in the text on Pages 12 and 13 as follows:

Page 12
"*cLEN* activity returned to the level similar to that in the uninjured hearts at 42 dpa (**Figs. 1H, I and S8D**); however, significant EGFP expression was detected in the *cLENΔ47* hearts at 42 dpa (**Fig. S8D, E**), suggesting that the repressive elements are functional and restrict TREE activity after the completion of regeneration."

Page 13
"Similar to *cLENΔ47*, *cLENΔ11-1* and *-2* exhibited significant GFP expression at 42 dpa (**Fig. S8D).**"

4. It is interesting that cLENd11-4 and cLENd11-5 showed very week GFP expression in the border zone expression. Is this due to the deletion of potential activator elements? Please clarify or speculate.

We have added the quantification data of the border zone in Fig. 4D and explanation on Pages 13:

"In 3 dpa regenerating adult hearts, all five constructs were capable of directing injury-responsive gene expression in the border zone (16.77, 15.86, 20.98, 5.12 and 6.96 μm^2/100 μm^2 for *cLENΔ11-1* to *-5*, respectively; **Fig. 4B, D**), indicating that injury-responsive activity is intact regardless of these 11 bp deletions. *cLENΔ11-4* and *-5* had lower area of EGFP expressing cells, compared to that in other *cLENΔ11* lines, suggesting that deletion of the potential activation motifs, such as ETS, influences injury-responsive activity in the border zone."

5. Phylogenetic comparison revealed moderate sequence conservation within the Cyprinidae family and that the cLEN-act enhancer is highly conserved with two other Danio species. A highly conserved AP-1 site and GTCA sequences were identified.

Do the constructs cLEN-FNE and cLEN-act suggest that the AP-1 site (between 108-118) is indeed essential for the enhancer activity? The authors can clarify and speculate.

We agree with the reviewer that transcription factor binding sites, including AP-1, are important. To address this comment, we have performed two additional experiments.

1) AP-1 binding site mutations
We generated two new transgenic constructs:
c) AP-1 mutated *cLEN* (*cLEN*$^{AP\text{-}1m}$): Two AP-1 binding sites in *cLEN* were mutated.
d) Synthetic enhancer (AFNEGx3): We generated a synthetic enhancer consisting of three tandem copies of AP-1-FOX-NFAT-ETS-GATA binding sites.

These transgenic constructs were injected, and EGFP expression was examined in injured hearts. To injure the hearts, we ablated cardiomyocytes (CMs) using *cmlc2:mCherry-NTR* as described in the method and Fig. 1C and D. Although a significant number of larvae injected with the *cLEN:EGFP* (positive control) induce EGFP expression in the hearts upon injury, the majority of larvae injected with *P2:EGFP* (negative control, minimal promoter), *cLEN*$^{AP\text{-}1m}$*P2:EGFP* and *AFNEGx3-P2:EGFP* do not have injury-responsive expression in hearts. These new results are now included in Fig. 2C-E and described in the text on Page 9 as follows:

"To test the involvement of the AP-1 binding sites in injury-responsive activation, two AP- 1 motifs in *cLEN* were mutated to generate *cLEN*$^{AP\text{-}1m}$, and their activity was examined in the F0 mosaic injured hearts. Several constructs were generated, including *P2:EGFP* (minimal promoter, negative control), *cLEN:EGFP* (positive control), or *cLEN*$^{AP\text{-}1m}$*:EGFP* (**Fig. 2C**). These constructs were injected into one-cell stage of the *cmlc2:mCherry-NTR* embryos, and EGFP expression was examined before and after cardiac ablation (**Fig. 2D**). None of these constructs were able to direct EGFP expression in the uninjured hearts. Upon cardiac injury, a significant number of larvae carrying the intact *cLEN* directed EGFP induction (**Fig. 2E**). However, the majority of larvae with *P2* or *cLEN*$^{ap\text{-}1m}$ were not able to direct injury-responsive expression (**Fig. 2E**), indicating that AP-1 binding sites are required for injury-responsive activity.

We next attempted to build a synthetic version of a cardiac regeneration enhancer by combining TF binding sites present in *cLEN-act*. Three copies of AP-1-FOX-NFAT- ETS-GATA sites were coupled with the *P2* minimal promoter and EGFP sequences to produce *AFNEGx3*, and its activity was examined in the F0 mosaic injured hearts. EGFP was undetectable in 3 dpf uninjured hearts. Noticeable EGFP induction was detected in some mosaic injured hearts carrying *AFNEGx3* (**Fig. S6D**); however, the majority of larvae did not demonstrate injury-responsive EGFP induction (**Fig. 2E**). These data suggest that further dissection of *cLEN* is required to create a functional synthetic regeneration enhancer. In addition, enhancer assays with stable lines will be required to conclude the activity of *AFNEGx3*."

2) mRNA injection
We generated mRNA of AP-1 complex and *nfatc1* and examined whether mRNA injection of these transcription factors in early *cLENP2:EGFP* embryos can drive EGFP expression. As AP-1 complex functions as a dimer of the *jun* and *fos* subunits, we selected *junba* and *fosl1a* to represent *fos* and *jun*, respectively. A recent study demonstrated that these two factors are potential AP-1 components contributing to the cardiac injury-responsive gene expression in zebrafish (Beisaw et al., 2020). Although *mCherry* or *nfatc1* mRNA injection into *cLENP2:EGFP* heterozygote embryos does not direct EGFP expression, AP-1 alone or AP-1+*nfatc1* can direct EGFP expression in the early embryos. Since AP-1 overexpression in the early embryos cause developmental defects, we were not able to test EGFP induction in uninjured heart in the later larval stages. These new results are now included in Fig. S6 and described in the text on Page 10 as follows:

"A recent study demonstrated that co-injection of mRNAs of the AP-1 complex components, *junba* and *fosl1a*, into one-cell-stage embryos is sufficient to activate the expression of AP-1 target genes (Beisaw et al., 2020). To determine whether AP-1 TFs can activate *cLEN*, *mCherry*, AP-1 (*junba*/*fosl1a*), *nfatc1*, or *nfatc1*+AP-1 mRNAs were injected into the one-cell stage of the *cLEN:EGFP* heterozygote embryos, and EGFP expression was assayed at 1 day (**Fig. S6A**). Although *mCherry* or *nfatc1* mRNA injection was unable to induce EGFP, we observed that a significant number of embryos injected with AP-1 or *nfatc1*+AP-1 demonstrated EGFP expression (**Fig. S6C**), suggesting that AP-1 contributes to the activation of *cLEN*. Overall, our data suggest that AP-1 is an important injury-activating factor for *cLEN*."

6. What is the functional consequence of deleting the repressive element on heart development or regeneration? The authors might try to delete the 22 bp element in the genome with CRISPR/Cas9. Since this experiment might take a long time to perform, the authors can just examine the potential

phenotypes in heart development if they have generated the lines. It is certainly possible there are redundant repressive elements and the deletion will not result in any phenotypes in heart development and regeneration. If this is the case, the authors can also comment on this.

As the reviewer pointed out, this experiment takes a long time to perform. We have not started to generate a 22-bp deletion line using CRISPR/Cas9; thus it is not feasible to conduct this experiment in a timely manner. Although we were unable to address this comment, the potential outcome of impeding repression of injury-responsive genes in tissue regeneration are discussed in the Discussion section on Page 19 as follows:

"The impact of repression in the uninjured tissues is a fundamental problem. Previous studies demonstrated that repression plays certain roles in supporting terminal differentiation and safeguarding proper tissue homeostasis (Ma et al., 2015; Sun et al., 2016). Schwann cells and liver constitute regenerative tissues in mammals. Loss of PRC2 in Schwann cells results in upregulation of injury-responsive genes, implying that repression is required for inhibition of aberrant gene expression in the absence of injury. Importantly, Schwann cells lacking PRC2 function display myelin abnormalities, including morphological changes and progressive hypermyelination (Ma et al., 2015), emphasizing the impact of repression on the function of uninjured tissues. In the mammalian liver, *Arid1a*, a key component of the ATP-dependent chromatin-remodeling complex, plays repressive roles, which contribute to differentiation of the liver after birth (Sun et al., 2016). Although the mutation of *lepb*, a target gene of *LEN*, is unlikely to influence regeneration ability in zebrafish (Kang et al., 2016), it will be interesting to determine whether disruption of the endogenous repressive motif in *cLEN* yields ectopic *lepb* expression in uninjured hearts and subsequently influences endocardial function.
"

Reviewer 3 Advance Summary and Potential Significance to Field:
The authors study how an enhancer which they have previously identified to be specifically activated during zebrafish heart and fin regeneration, is regulated. In particular, they identify smaller sequence elements that are sufficient to drive activation during heart regeneration, and, interestingly, identify an element that represses expression in noninjured hearts. The findings are of great interest and provide substantial novel mechanistic insight into a highly intriguing biological problem. The data are generally of high quality. However, at several points I think the data do not yet fully support the conclusions drawn. Yet, when the authors can address the following issues, I can recommend publication in Development.

Reviewer 3 Comments for the Author:

Major:

1) Throughout: Positional effects can massively influence expression of transgenes, in particular in adults. Thus, deriving well-expressing transgenic lines using random integrations of transgenes into the genome can be a challenge even for well characterized "ubiquitous" promoters. Thus, I find it generally quite puzzling in studies like this and similar ones, that the authors are able to derive clear, unambiguous conclusions from the analyses of a few transgenic lines. Maybe specific enhancers are less prone to positional effects and silencing than ubiquitous promoters? While I can hardly ask the authors of this study to figure this out, I would like to see more transparency in relation to this issue. They mention at several places in the manuscript that several independent lines (sublines / integrations) have been analyzed, but this is not enough information to judge how reproducible their findings really are between independent sublines / integrations. They should provide a table giving data for each individual subline / integration analyzed for each of the constructs used in the paper, that summarizes which of the described expression patterns were actually seen in which line. In addition, for a few select particularly important results, quantifications of different sublines should be shown, eg. forcLENDelta47.

We completely agree with this comment. We have added the Supplementary Table 3 to clarify our results. We also quantified additional *cLEN* and *cLENΔ47* lines, which are included in Fig. 3C- E and explanation on Pages 11 and 12 as follows:

"While EGFP was undetectable in uninjured hearts of *cLEN* lines (0.001 and 0.016 $\mu m^2/100\ \mu m^2$ in

line 3 and 7, respectively), EGFP was expressed in *cLENΔ47* uninjured hearts (0.912 and 1.04 μm^2/100 μm^2 in line 3 and 6, respectively)."

"In 3 dpa hearts, both *cLEN* and *cLENΔ47* directed EGFP expression in the border zone (5.66, 10.95, 12.99 and 15.62 μm^2/100 μm^2 in *cLEN* line 3 and 7 and *cLENΔ47* line 3 and 6, respectively), demonstrating their activation abilities in response to injury (**Fig. 3B, D**)."

"While the EGFP$^+$ area was very limited in the remote zone of *cLEN* (0.35 and 0.55 μm^2/100 μm^2 in line 3 and 7, respectively), regenerating *cLENΔ47* hearts demonstrated EGFP expression in a significant endocardial area of the remote zone (4.94 and 5.43 μm^2/100 μm^2 in line 3 and 6, respectively) (**Figs. 3E, S3A, B and S8A-C**)."

2) Fig. 1C: induction in 5dpf embryos does not seem to be specific to the heart. Which tissues / cells is the reporter expressed in? Did the authors perform further controls to make sure it's not the Mtz treatment on it's own that causes induction (treat single reporter transgenics with Mtz). Is induction outside the heart in response to CM ablation also seen in juvenile and adult stages?

We thank the reviewer for suggesting several control experiments. We confirmed that *cLEN:EGFP* is not induced by Mtz treatment. We also tested *cLEN:EGFP* expression outside the heart upon cardiac injury in larvae and adults. Cardiac ablation in larvae directed EGFP induction in epidermis. In contrast, cardiac ablation does not induce epidermal EGFP induction in the adult stages. These results are added in Fig. S2 and described on Page 6 as:

"Cardiac ablation in larvae results in a pericardial edema phenotype, potentially inducing a systemic injury response due to insufficient circulation and subsequently activating *cLEN* in the epidermis (**Fig. S2A**). By contrast, cardiac ablation in adults did not direct ectopic expression in epidermis (**Fig. S2A**). Epidermal induction upon local injury was not observed in amputated adult caudal fins, suggesting that ectopic *cLEN* activation in epidermis is repressed in the adults (**Fig. S1C**). Mtz treatment did not induce EGFP in the absence of *cmlc2:mCherry-NTR*, confirming the specificity of cardiac injury-responsive activation of *cLEN* (**Fig. S2B**)."

2b) somewhat related to issue 2: an interesting question that has also not been addressed by the previous publication of Junsu Kang on the LEN is to which extent activation is regeneration- specific or occurs also in response to other types of injuries which would conventionally not be considered to result in regeneration. E.g. epidermal wounds? Larval fin folds or adult fins (incisions in the interray tissue) would be convenient models to test this.

We thank the reviewer for interesting question. As described in the previous paper (Nature, 2016), the *P2* minimal promoter directs EGFP expression at the wound area upon larval fin fold amputation. However, *P2* is unable to direct EGFP induction upon fin amputation in adults. This is the method used to screen transgenic fish because most of the transgenic lines do not have obvious EGFP expression in the larval and adult stages. Thus, we were unable to use larval fin folds to address this comment. Instead, we determined whether full-length *LEN* is responsible for injury-induced expression using epidermis wound in the adult fin caused by incisions of the inter- ray tissue. Incision injury directs EGFP induction of *LEN*, but EGFP expression level is noticeably less robust, compared to that detected after whole-fin amputation. These data are added in Fig. S1 and described on Page 5 as follows:

"We previously demonstrated that *lepb* regeneration enhancer (*LEN*) is strongly activated during fin and heart regeneration (Kang et al., 2016). To determine the extent of regeneration- specific activation of *LEN*, *LEN* activation was compared in two different injury models: a whole- fin amputation model, which causes massive loss of multiple tissues, such as bones, fibroblasts, and epidermis, and a fin incision injury model that does not cause tissue loss. A small incision within an interray region induced EGFP signal in *LEN:EGFP*, but the signal intensity was noticeably less robust than that observed after whole-fin amputation (**Fig. S1B**). These results indicate that *LEN* is activated by injury and is TREE."

3) for non-quantified experiments, like in Fig. 1C-E, Fig. 2, the number of samples (embryos, hearts) that showed a phenotype / appearance similar to the one shown in the representative image and the total number of analyzed samples should be given (eg. 12/15 embryos).

We have added the Supplementary Table 3 to clarify the total number of analyzed samples.

4) Fig. 1H: While the expression pattern is suggestive, these images do not support the statement that expression is mainly in endocardial cells. In fact, at the wound border, expression appears to be not just confined to endothelial cells (e.g. Fig. 3B), as far as this can be judged without co- staining. Co-staining with endothelial markers (e.g. in double transgenics) and higher magnification would be needed to unambiguously identify the expressing cells. Same is true for the deletion constructs in Fig. 2 & 3 & 4. Thus, authors should first unambiguously demonstrate endothelial expression of the full transgene, which will make the deletion construct data more interpretable as well.

We thank the reviewer for the suggestion. We stained cardiac sections of *P2:EGFP*, *cLEN:EGFP*, *cLENΔ47:EGFP*, *cLENΔ11-1:EGFP* and *cLENΔ11-2:EGFP* using an EGFP antibody along with MHC antibody to detect CMs or Raldh2 antibody to detect endocardial cells. This analysis demonstrated that the *P2* minimal promoter used for transgenic assays drives weak expression in some CMs upon injury. However, *P2* does not direct the expression in any cell types in uninjured hearts. *P2* also does not drive endocardial expression in injured hearts. Similarly, *cLEN:EGFP* and *cLENΔ47:EGFP* direct EGFP expression in some CMs. In contrast to *P2*, *cLEN:EGFP* and *cLENΔ47:EGFP* drive strong endocardial expression in the border zone. In the remote zone of *cLEN:EGFP* and *cLENΔ47:EGFP* injured hearts, EGFP is not detectable in CMs. Our analysis demonstrated that *cLEN* causes injury-induced ectopic expression in CMs at the border zone but the main activity is observed in endocardial cells in border and remote zones upon injury. These new results are included in Fig. S3.

5) Related to issue 3, the statement in line 177 “a significant portion of endocardial cells” needs to be substantiated by a) co-staining and b) quantification of expressing cells.

As described above, we confirmed that EGFP is co-localized with Raldh2$^+$ endocardial cells.

6) Fig. 3B: authors imply that cLENDelta47 still induces at the border. How do they know that? S4 images are suggestive, but intensity measurements of the border zone vs. uninjured and remote areas would be required to verify that activity is indeed higher at the border.

We addressed this comment by quantifying EGFP intensity in Raldh2$^+$ endocardial cells between the border and remote zones in the same heart. These new data are included in Fig. S3.

7) Fig.4. area covered by EGFP should be also measured for the border zone for these deletion lines to support the statement that all lines support induction there. In addition, images suggest that expression at the border is also much weaker in some deletion lines than others. Thus, expression in the remote zone should also be shown relative to the one in the border zone. In case the author’s conclusion is correct that some deletions only result in derepression of expression in the remote zone (and in uninjured hearts) and do not effect overall expression levels, this effect should still be seen when normalized for expression in the border zone.

We have added the quantification data in the border zone in Fig. 3C and 4D and explanation on Page 11 and 13:

Page 11
“In 3 dpa hearts, both *cLEN* and *cLENΔ47* directed EGFP expression in the border zone (5.66, 10.95, 12.99 and 15.62 μm^2/100 μm^2 in *cLEN* line 3 and 7 and *cLENΔ47* line 3 and 6, respectively), demonstrating their activation abilities in response to injury (**Fig. 3B, D**).”

Page 13
“In 3 dpa regenerating adult hearts, all five constructs were capable of directing injury-responsive gene expression in the border zone (16.77, 15.86, 20.98, 5.12 and 6.96 μm^2/100 μm^2 for *cLENΔ11-1* to *-5*, respectively; **Fig. 4B, D**), indicating that injury-responsive activity is intact regardless of these 11 bp deletions. *cLENΔ11-4* and *-5* had lower area of EGFP expressing cells, compared to that in other *cLENΔ11* lines, suggesting that deletion of the potential activation motifs, such as ETS, influences injury-responsive activity in the border zone.”

8) Fig. 6. While it is interesting that ectopic expression of the deletion line in noninjured hearts

can only be seen after 5 dpf, I am not sure whether I can agree with the authors that this shows that repression emerges at or after that time point. If repression were not active earlier, shouldn't the complete LEN element be expressed at early stages? It's rather the ectopic expression that gets activated later, which I think is not quite the same thing...

We thank the reviewer for pointing out this consideration. Our data demonstrated that injury signal is required for *cLEN* activation in early larval stage, such as 5 dpf (Fig. S10.). While larvae develop, the heart matures to provide more blood to the body. Increase in the cardiac burden may generate an injury-like signal, which can nonspecifically activate injury-responsive enhancers in the uninjured heart. Our data suggest that zebrafish utilize repression to prevent this non-specific enhancer activation in uninjured tissues. Although this is an interesting hypothesis, it is beyond the scope of our current manuscript demonstrating that dual regulation via distinct types of *cis*- regulatory elements ensures regeneration-restricted activation of cardiac regeneration enhancers. We plan to address this interesting finding in the future studies.

Minor:
Language: Line 160 "as reported..."

This has updated as follows:
"Developmental enhancers are known to regulated by multiple regulatory elements (Spitz and Furlong, 2012); hence, these results indicate that cardiac regeneration enhancers are also regulated by multiple *cis*-regulatory elements."

Second decision letter

MS ID#: DEVELOP/2020/194019

MS TITLE: Decoding an Organ Regeneration Switch by Dissecting Cardiac Regeneration Enhancers

AUTHORS: Ian Begeman, Kwangdeok Shin, Daniel Osorio-Mendez, Andrew Kurth, Nutishia Lee, Trevor Chamberlain, Francisco Pelegri, and Junsu Kang
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

See previous review comments.

Comments for the author

In this revision the authors have addressed the comments I made.
Specifically, the authors have added new experiments to show the importance of the AP-1 site within the LEN.
This includes the generation of new AP-1 reporter constructs and testing these in the larval hearts. In addition ectopic expression of AP-1 was included in these experiments. Furthermore the authors have include new data that quantified expression of GFP in the remote zone of the regenerating heart, which was absent in the first submission,

Overall this study highlights transcriptional activity in the regenerating zebrafish heart and provides a new mechanism of derepression as a means to control gene expression in response to injury.

Reviewer 2

Advance summary and potential significance to field

The enhancer and repressor elements analyses further elucidate how heart development and regeneration are regulated at the transcriptional level. Based on their data, the authors proposed that both activators and repressors coordinate to regulate transcription during heart regeneration to prevent aberrant gene expression during heart development or in uninjured areas. The potential binding factors of these elements might lead to the upstream regulators.

Comments for the author

The authors have addressed my previous concerns with new results and detailed explanations. I don't have further questions and comments.

Reviewer 3

Advance summary and potential significance to field

not relevant for revision.

Comments for the author

The authors have addressed all issues I had raised in a satisfactory manner. I recommend publication in Development.