BLMP-1 promotes developmental cell death in *C. elegans* by timely repression of *ced-9* transcription

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DOI: 10.1242/dev.193995

Editor: Swathi Arur

Review timeline
Original submission: 17 June 2020
Editorial decision: 17 July 2020
First revision received: 16 August 2021
Accepted: 14 September 2021

Original submission

First decision letter

MS ID#: DEVELOP/2020/193995

MS TITLE: BLMP-1 promotes developmental cell death in C. elegans by timely repression of ced-9/bcl-2 transcription

AUTHORS: Hang-Shiang Jiang, Piya Ghose, Hsiao-Fen Han, Ya-Yin Tsai, Huang-Chin Lin, Wei-Chin Tseng, Jui-Ching Wu, Shai Shaham, and YI-CHUN WU

I have now received reviews of your manuscript from 3 experts. The reviewers' 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, all 3 reviewers express interest in your study, but they also have significant concerns that you would need to address before I can consider it for publication in Development. Reviewer 1 suggests that it would be better to use a mutagenized version of the blimp-1 binding site than a deletion that may disrupt other sequences or spacing. Reviewer 2 is justifiably concerned about your using high-copy transgene arrays to analyze expression patterns of various genes, the need for a negative control in Fig. 1D, and wanting to see evidence that CED-9 protein levels are down-regulated by BLMP-1. That reviewer further suggests testing if ectopic expression of BLMP-1 is sufficient to cause death of cells that don't normally die. Reviewer 3 makes numerous good points about the relationship between BLMP-1 and DRE-1.

I invite you to consider the reviewers' suggestions and submit a revised manuscript that addresses their concerns. Your revised manuscript would be re-reviewed, and acceptance would depend on your satisfactorily addressing the reviewers' concerns. Please note that Development normally permits only one round of 'major revision'.

In your revised manuscript, please clearly HIGHLIGHT all changes made in the revised version. You should avoid using 'Tracked Changes' in Word files as these are lost in PDF conversion. I also request 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 the reviewers' criticisms or suggestions, please explain why.

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 time frame of a revision. We will then provide further guidance. Please also note that we are happy to extend revision time frames as necessary.

Reviewer 1

Advance summary and potential significance to field

The tail-spike cell dies differently than the majority of programmed cell deaths in C. elegans. It follows a specialized death process called compartmentalized cell elimination which is required for tail formation. Moreover, the BH3-only protein Egl-1 only plays a minor role, and the upstream regulators that control the timing of this form of cell death have not been identified. In this paper, the authors took advantage of C. elegans forward genetics and whole genome sequencing to identify mutations that prevented tail-spike cell death. They identified 2 alleles of Blimp-1 and followed up with elegant genetic approaches to confirm the role of Blimp-1 in regulating tail spike cell death by transcriptional repression of ced-9 and acting in parallel to dre-1. Overall this paper adds insight into the diverse ways cell death can be regulated.

Comments for the author

To be addressed:
1. The authors state that ced-3 expression begins at 3.2-fold stage (page 7), but ced-3 expression is apparent at the bean stage in figure 2. Clarification is needed since the timing of the transgene expression affects conclusions. It appears that ced-3 expression is gone in stage 3, and then gets turned back on at 3.2 so this could be mentioned in this section.

2. For completion it would be best to demonstrate that blmp-1 does not affect expression of ced-3. It is possible Blimp-1 represses a repressor of ced-3 for example.

3. The precise nature of the ced-9 Δ transgenic reporter needs to be described. It would be best if a mutagenized version of the blimp-1 binding site was used, rather than a deletion which could disrupt other sequences or spacing.

Reviewer 2

Advance summary and potential significance to field

The control of programmed cell death (PCD) during development is still not fully understood. In this manuscript, the authors describe the identification of a new mechanism for the activation of programmed cell death in C. elegans embryos. Shaham and co-workers have used the tail spike cell as a model for PCD and previously found that this death depends on the C. elegans caspase CED-3 and is blocked by the Bcl-2-like protein CED-9. They now report the identification of a direct repressor of ced-9 transcription, BLMP-1, and provide evidence in support of the notion that downregulation of ced-9 expression through BLMP-1 contibutes to the activation of PCD in the tail-spike cell at a specific developmental time. The manuscript addresses an important question in developmental biology, the control of PCD, in a model in which seminal discoveries about PCD have been made.

Comments for the author

The genetic experiments are very convincing. However, I am concerned about the transgenic lines used to analyze the expression patterns of various genes especially ced-3 and blmp-1, and the conclusions made about the timing and levels of expression. As far as I can see from the methods, the authors used high-copy number arrays. It is established that those often do not reflect expression patterns, levels and control of endogenous genes. Especially when looking at

transcriptional control, the use of single-copy transgenes is to be preferred. In addition, based on the description in the methods, it is difficult to recapitulate how 'expression levels' were quantified especially in lines with extrachromosomal arrays. In my opinion, it is not possible to quantify those in a way that allows meaningful comparison between animals and lines.
Fig. 1 and 4 could be modified and show the important cells rather than entire embryos with lots of expression that is not relevant. In addition, it is unclear whether the images are from time lapse movies of one and the same embryo or different embryos.
Fig. 1D The rescue with the tail-spike cell specific promoter is not as good as with the fosmid. Could it be that there is a cell non autonomous contribution?
In addition, as a negative control, have the authors tested a promoter that is known to NOT be active in that cell?
The main conclusion of the authors is that BLMP-1-dependent downregulation of CED-9 contributes to tail-spike cell death. However, the authors do not look at levels of CED-9 protein. Have the authors considered this? They have tail-spike cell specific reporters, which should make it possible to look at CED-9 levels in this cell.
It would be interesting to test whether the overexpression of blmp-1 in cells that normally do not die can induce their PCD. This would provide additional evidence that this BLMP-1-dependent downregulation of ced-9 expression is an important trigger for PCD in cells other than the tail spike cell.
pg 10 - 'multiplicative' - why not 'synergistic'?
Conservation of BLMP-1's role in PCD. The authors propose that the role of BLMP-1 (and DRE-1) in PCD control are conserved. However, based on the papers cited it seems that mammalian BLMP-1 blocks rather than induces PCD. Could the authors clarify this? Otherwise I would remove this.

Reviewer 3

Advance summary and potential significance to field

The work is significant because it reveals a temporal developmental module linking blmp-1/BLIMP transcriptional repression of ced-9/BCL2, for the activation of cell death pathways in tissue remodeling which is likely conserved in evolution.

Comments for the author

In this work, Jiang et al demonstrate a cell intrinsic role for the transcription factor blmp-1/BLIMP in regulation of tail spike removal through the programmed cell death pathway. From genetic screens for tail spike cell persistence, they identified a number of loss of function mutations in blmp-1. They demonstrate that tail spike cell persistence is dependent on ced-9/BLC2, which is shown to be a target of blmp-1 repression at an element in its promoter. Their model suggest that upregulation of blmp-1 at the 3-fold stage of embryogenesis leads to ced-9 transcriptional repression, and the onset of cell death. This complements previous work where they had suggested that ced-9 is also regulated at the level of protein turnover, and is degraded by the dre-1/FBOX11 protein.
The work is significant because it reveals a temporal developmental module linking blmp-1/BLIMP transcriptional repression of ced-9/BCL2, for the activation of cell death pathways in tissue remodeling which is likely conserved in evolution. Generally, the work is straightforward, well done and nicely written.
However there are a number of unresolved questions regarding the model that should be addressed.

Major
1. The authors neglect to mention that previous work (including their own) has established that dre-1 and blmp-1 are not just independent regulators of larval developmental timing, but that they work in a cascade in which DRE-1 degrades BLMP-1 through ubiquitin mediated proteolysis, and that dre-1 mutation leads to persistent elevation of BLMP-1 in distal tip cells. This is actually an important point that is glossed over in regards to the embryonic mechanism (see below).

2. The authors argue that blmp-1 and dre-1 regulate ced-9 in parallel transcriptional and proteolytic pathways, respectively. This is based on the observation that dre-1 mutation can enhance blmp-1 null mutation for persistence of the tail spike. Because dre-1 has been previously shown to regulate blmp-1, it’s not clear if they are strictly parallel.

If the two processes are truly independent, shouldn't the double mutant have fully additive phenotypes? But they are not: mutating blmp-1 or dre-1 singly substantially increases tail spike persistence, while the double mutant only residually enhances this phenotype. Can the authors explain?

3. The authors also base their parallel regulation model on the observed lack of regulation of blmp-1::gfp expression by dre-1 in L1 larvae. This observation, though consistent, is preliminary. In particular, they cannot exclude the possibility that dre-1 inhibits both blmp-1 and ced-9, since they only monitored blmp-1::gfp expression in the L1 stage, probably past the point of meaningful regulation.
As their very nice data show, dre-1 and blmp-1 are expressed dynamically and inversely during embryonic development. dre-1 is expressed early on up to the three-fold stage (Fig. S2) during which blmp-1 is turned off, and ced-9 promoter is turned on (Fig. 2). Then at the three-fold stage, dre-1 expression diminishes (Fig. S2), blmp-1 increases, and ced-9 promoter shuts off (Fig.2). Cell death ensues. In the blmp-1 mutant, ced-9 expression is constitutive (Figure 4).
The inverse expression patterns of dre-1 and blmp-1 could indicate that dre-1 inhibits blmp-1 up until the 3-fold stage, at which point dre-1 goes down and blmp-1 comes on.
Given these interesting dynamics, they should examine blmp-1::gfp protein expression levels at different stages of embryogenesis in WT and dre-1 mutants/RNAi.

4. If DRE-1 only affects CED-9 protein levels, then there should be no effect on its transcription. However, if DRE-1 regulates a transcription factor (e.g. BLMP-1) that acts on the ced-9 promoter, then it should show regulation.
They should therefore monitor CED-9 protein and ced-9 promoter expression levels around the 3-fold stage in WT, dre-1, blmp-1, dre-1;blmp-1.

5. Relatedly, the epistasis model in Figure 7 does not fully capture the regulatory dynamics they depict in their embryo pictures. This might be useful even if not all the answers are clear.

6. The Discussion should be strengthened with what is known about dre-1 and blmp-1 regulating other processes and other factors (e.g. ces-1), and could speculate that they too might be involved. In addition, it would appropriate to mention that apoptotic protease ced-3 plays a non-canonical role in regulating developmental timing and lin28.
Perhaps bring out the idea that several components involved in larval timing also function in embryonic cascades?

Minor
7. Abstract Change "temporally managed" to controlled or regulated.
8. Fig 1 b Please show the nature of mutations somewhere in Figure or legend.
9. Fig 2 blmp-1 comes on at 3-fold, transiently shuts off the tail spike reporter. Why does this occur?
10. There are two blmp-1 isoforms. Which one is used in the cDNA constructs? I guess it is the smaller B isoform, based on the yk clone used to make it.
The authors might consider using the genomic construct in the experiments suggested in 4 to ensure all isoforms are included.
11. Fig. 3 Indicate how many biological replicates were done.
12. Fig 3b Full-length ced-9 transgene does not fully rescue the ced mutant phenotype. Why not?
Further, the authors claim:
"The full-length transgene, therefore, efficiently complements the ced-9 mutation, and transgenics resemble wild-type animals."
Please modify.
13. Fig. 5 How many times was experiment done?
14. Fig 5A Why does blmp-1 RNAi significantly reduce expression of ced-9 promoter deletion construct ?
Could it be that it has both negative and positive effects on expression of ced-9 promoter? Are there other elements in the promoter?

First revision

Author response to reviewers' comments

Point-by-point responses to reviewer comments

Reviewer 1:

1. **The authors state that ced-3 expression begins at 3.2-fold stage (page 7), but ced-3 expression is apparent at the bean stage in figure 2. Clarification is needed since the timing of the transgene expression affects conclusions. It appears that ced-3 expression is gone in stage 3, and then gets turned back on at 3.2 so this could be mentioned in this section.**

   Our response:
   We thank the reviewer for this comment. According to the suggestion of Reviewer 2, we have redone the experiment by changing the high-copy *ced-3* promoter::GFP transgenes to single-copy *ced-3* promoter::GFP transgenes and using the tail-spike cell marker P_{aff-1}::mKate2PH, instead of $P_{cbr-ced-3}$::mRFP, to label the tail-spike cell. The single-copy *ced-3* promoter::GFP transgene shows that *ced-3* expression begins at the 3.2-fold stage and not earlier.

   We added the data to Supplementary Figure S1A-D and inserted the following sentences on page 9, 2nd paragraph, line 1: "As previously reported, *ced-3* transcription initiates at the 3.2-fold stage when the tail-spike cell is about to die (Maurer et al., 2007). We confirmed this result using a single-copy transgene (Supplementary Figure S1)."

2. **For completion it would be best to demonstrate that blmp-1 does not affect expression of ced-3. It is possible Blimp-1 represses a repressor of ced-3 for example.**

   Our response:
   We thank the reviewer for the suggestion. We have done the experiment and found that *blmp-1* does not affect *ced-3* expression.

   We added the data to Supplementary Figure S1E-I and inserted the following sentences on page 9: "As previously reported, *ced-3* transcription initiates at the 3.2-fold stage when the tail-spike cell is about to die (Maurer et al., 2007). We confirmed this result using a single-copy transgene (Supplementary Figure S1), and examined the effect of *blmp-1* RNAi on this reporter. We found that *blmp-1* is not required for *ced-3* transcriptional activation (Supplementary Figure S1)."

3. **The precise nature of the ced-9Δ transgenic reporter needs to be described. It would be best if a mutagenized version of the blimp-1 binding site was used, rather than a deletion which could disrupt other sequences or spacing.**

   Our response:
   We thank the reviewer for the comment. We generated and analyzed both transcriptional and translational P_{ced-9m}*::gfp* and P_{ced-9m}*::ced-9::gfp* transgenes carrying a mutagenized version of the BLMP-1 binding site in the promotor region and added the data to Figure 4I-P and Figure 3B Lanes 11 and 12, respectively. We also added sentences on pages 7-8 to describe the results and in the MATERIALS AND METHODS section on page 15 to mention the precise nature of the $P_{ced-9\Delta}$ and P_{ced-9m} transgenic reporters.

   On page 7, 2nd paragraph, line 11, the inserted sentences read: "This band is not evident when DNA of a different sequence is used (mutant probe, changing TTTCAATTT to AGGGTTAGG). Importantly, a *ced-9* promoter::GFP reporter transgene harboring the mutant sequence (P_{ced-9m}::GFP) is no longer down-regulated and shows a similar expression level when compared to P_{ced-9}::GFP in the *blmp-1(s71)* mutant (Figure 4I-L and P)."

On page 7, 3rd paragraph, line 2, the inserted sentences read: “To assess the physiological significance of BLMP-1 DNA binding, we generated three sets of *ced-9(n2812); ced-3(n2427)* transgenic animals, carrying either a functional *ced-9* promoter::*ced-9*::GFP transgene, a *ced-9Δ* promoter::*ced-9*::GFP transgene lacking the BLMP-1 binding site TTTCAATTT, or a *ced-9m* transgene in which the *ced-9* promoter contains a mutant BLMP-1 binding site (changing TTTCAATTT to AGGGTTAGG).”

On page 8, 1st paragraph, line 6, the inserted sentences read: “By contrast, the binding-site-deleted or binding-sequence-altered transgenes cause tail-spike cell survival, even without *blmp-1* RNAi. Survival is similar in extent to that observed in animals carrying the wild-type transgene and treated with *blmp-1* RNAi (Figure 3B).”

On page 15, the inserted sentences read: “The deletion or mutation of BLMP-1 binding site TTTCAATTT (922-930 bp upstream of the ATG of *ced-9*) was constructed by site-directed mutagenesis. Briefly, the plasmid $P_{ced\text{-}9::gfp}$ and $P_{ced\text{-}9::ced\text{-}9::gfp}$ mentioned above were amplified by primers,
5’- ACGCACCGCCCTGTTTCTTTTGATAAGAAAATCAGCATTG-3’ and
5’-CAATGCTGATTTTCTTATCAAAAGAAACAGGGCGGTGCGT-3’ for deleted BLMP-1 binding site and
5’-ACGCACCGCCCTGTTTCTTTAGGGTTAGGTGATAAGAAAATCAGCAT TG-3’ and
5’-CAATGCTGATTTTCTTATCACCTAACCCTAAAGAAACAGGGCGGTGC GT-3’ for mutated BLMP-1 binding site, and the resulting PCR products were treated with *Dpn*I and then transformed to competent cells. Plasmids were verified by sequencing.”

Response to Reviewer 2:

1. **The genetic experiments are very convincing. However, I am concerned about the transgenic lines used to analyze the expression patterns of various genes, especially ced-3 and blmp-1, and the conclusions made about the timing and levels of expression. As far as I can see from the methods, the authors used high-copy number arrays. It is established that those often do not reflect expression patterns, levels and control of endogenous genes. Especially when looking at transcriptional control, the use of single-copy transgenes is to be preferred. In addition, based on the description in the methods, it is difficult to recapitulate how 'expression levels' were quantified especially in lines with extrachromosomal arrays. In my opinion, it is not possible to quantify those in a way that allows meaningful comparison between animals and lines.**

   Our response:
   We thank the reviewer for this comment. We have now generated single-copy transgenes for all transcriptional reporters, including *blmp-1*, *ced-3, ced-9* and *dre-1*. We have also analyzed a single-copy BLMP-1::GFP line generated by CRISPR/cas9 to verify the result of our transcriptional reporter *blmp-1* promotor::gfp. To specifically answer the reviewer’s question, we have added the *ced-3* and *blmp-1* data to Supplementary Figure S1A-D and Figure 2A-D, respectively, and inserted sentences describing the results on pages 9 and 5-6.

   On page 9, 2nd paragraph, line 1, the inserted sentences read: “As previously reported, *ced-3* transcription initiates at the 3.2-fold stage when the tail-spike cell is about to die (Maurer et al., 2007). We confirmed this result using a single-copy transgene (Supplementary Figure S1), and examined the effect of *blmp-1* RNAi on this reporter. We found that *blmp-1* is not required for *ced-3* transcriptional activation (Supplementary Figure S1)”

   On page 5, last paragraph, line 3, the inserted sentences read: “To determine when *blmp-1* is expressed relative to cell death onset, we generated animals carrying a single copy *blmp-1* promoter::GFP transgene using PhiC31 integrase-mediated insertion (Yang et al., 2020), and crossed these with animals expressing the *aff-1* promoter::myristoyl-KatePH (mKatePH) tail-spike cell reporter. We found that *blmp-1* transcription is detected in the tail-spike cell as early as the mKatePH reporter (1.5-fold stage; Figure 2A). *blmp-1* transcription continues until the tail-spike cell dies with a characteristic rounded refractile morphology at the 3.7-

fold stage (Figure 2B-D)."

On page 6, 1st paragraph, the inserted paragraph reads: "To determine whether BLMP-1 protein accumulation follows its transcriptional expression pattern, we examined animals carrying the *cshIs41[BLMP-1::GFP]* single-copy translational reporter, in which GFP is fused to the BLMP-1 C terminus (Stec et al., 2021). We found that, like the transcriptional reporter, endogenous BLMP-1::GFP is detected in the tail-spike cell from the 1.5-fold stage until the cell dies (Figure 2E-H)."

2. **Fig. 1 and 4 could be modified and show the important cells rather than entire embryos with lots of expression that is not relevant. In addition, it is unclear whether the images are from time lapse movies of one and the same embryo or different embryos.**

Our response:
We thank the reviewer for this comment. We have modified the figures as suggested by the reviewer and now show the tail-spike cell, rather than entire embryo, for all tail-spike cell figures, including Figures 2, 4, 6 and S1. We have also inserted a sentence in the MATERIALS AND METHODS section on page 14 to indicate that the images shown are from different embryos.

On page 14, the inserted sentence reads: "Representative images of different stages are from different embryos. Images were deconvolved to remove out-of-focus light."

3. **Fig. 1D The rescue with the tail-spike cell specific promoter is not as good as with the fosmid. Could it be that there is a cell non autonomous contribution? In addition, as a negative control, have the authors tested a promoter that is known to NOT be active in that cell?**

Our response:
We thank the reviewer for this comment. While there is variation among different transgenic lines, generally, the rescuing activities are similar for the fosmid and the tail-spike cell specific promoter::*blmp-1* cDNA (7-25% and 10-30%, respectively). The difference in the Y-axis in the original Figure 1C (0-100%) and D (0-80%) may have been misleading. We have therefore modified the Y-axis of Figure 1D to the same scale (0-100%) as that of Figure 1C. In addition, we have generated constructs expressing *blmp-1* cDNA in hyp10, located in the tail region near the tail-spike cell, to address the reviewer's concern. However, we could not obtain lines using various injection concentrations. We are not sure if the transgene might be toxic. Nevertheless, our combined molecular, genetic and biochemical results together indicate that BLMP-1 binds to the *ced-9* promotor and represses its expression to regulate tail-spike cell death. This strongly supports that *blmp-1* acts cell-autonomously in the tail-spike cell to control its demise.

On page 5, 1st paragraph, line 9, the inserted sentences read: "Importantly, expression of *blmp-1* cDNA specifically in the tail-spike cell can restore tail-spike cell death to the same extent as the fosmid (Figure 1D). Taken together, these studies suggest that *blmp-1* is required cell-autonomously for tail-spike cell death. Overexpression of *blmp-1* cDNA in the phasmid sheath cells, located in the tail region near the tail-spike cell, does not cause phasmid sheath cell death (Figure 1E). Thus, *blmp-1* is unlikely to be a direct component of the tail-spike cell killing apparatus, and is more likely to function as a regulator."

4. **The main conclusion of the authors is that BLMP-1-dependent downregulation of CED-9 contributes to tail-spike cell death. However, the authors do not look at levels of CED-9 protein. Have the authors considered this? They have tail-spike cell specific reporters, which should make it possible to look at CED-9 levels in this cell.**

Our response:
We thank the reviewer for this suggestion. We have tried very hard to observe both single-copy and high-copy P_{ced-9}*::ced-9::gfp* transgenes using various microscopy tools to enhance

our detection sensitivity. Unfortunately, we cannot observe CED-9::GFP protein in the tail-spike cell. This is consistent with our observation that the transcriptional reporter of *ced-9* is barely observable in the tail-spike cell.

5. **It would be interesting to test whether the overexpression of blmp-1 in cells that normally do not die can induce their PCD. This would provide additional evidence that this BLMP-1-dependent downregulation of ced-9 expression is an important trigger for PCD in cells other than the tail spike cell.**

   Our response:
   We thank the reviewer for this comment. We expressed *blmp-1* in the phasmid sheath cells, which normally do not die, and found that *blmp-1* does not cause ectopic phasmid sheath cell death. Perhaps this is due to the absence of the rest of the PCD machinery. We added the data to Figure 1E and inserted a sentence on page 5.

   On page 5, 1st paragraph, line 12, the inserted sentence reads: "Overexpression of *blmp-1* cDNA in the phasmid sheath cells, located in the tail region near the tail-spike cell, does not cause phasmid sheath cell death (Figure 1E)."

6. **pg 10 - 'multiplicative' - why not 'synergistic'?**

   Our response:
   We thank the reviewer for the comment. We have changed "multiplicative" to "synergistic" on Page 8, 2nd paragraph, line 10.

7. **Conservation of BLMP-1's role in PCD. The authors propose that the role of BLMP-1 (and DRE-1) in PCD control are conserved. However, based on the papers cited, it seems that mammalian BLMP-1 blocks rather than induces PCD. Could the authors clarify this? Otherwise I would remove this.**

   Our response:
   We have removed the references.

Response to Reviewer 3:

1. **The authors neglect to mention that previous work (including their own) has established that dre-1 and blmp-1 are not just independent regulators of larval developmental timing, but that they work in a cascade in which DRE-1 degrades BLMP-1 through ubiquitin mediated proteolysis, and that dre-1 mutation leads to persistent elevation of BLMP-1 in distal tip cells. This is actually an important point that is glossed over in regards to the embryonic mechanism (see below).**

   Our response:
   We thank the reviewer for this comment. DRE-1-mediated degradation of BLMP-1 is important for hypodermal development and distal tip cell migration.
   However, it may not be important for the regulation of tail-spike cell death because if it was, we would expect to observe the opposite phenotypes for *blmp-1* and *dre-1* mutants. Instead, *blmp-1* and *dre-1* mutants have a similar phenotype: tail-spike cell survival. To address these points, we added the following on page 8, 2nd paragraph, line 3: "DRE-1 has been shown to directly bind BLMP-1 and to mediate BLMP-1 degradation during larval development (Horn et al., 2014; Huang et al., 2014), suggesting opposite functions for DRE-1 and BLMP-1. However, we suspected that this may not be the case for the tail-spike cell, as *blmp-1* and *dre-1* mutants both harbor surviving tail-spike cells."

2. **The authors argue that blmp-1 and dre-1 regulate ced-9 in parallel transcriptional and proteolytic pathways, respectively. This is based on the observation that dre-1 mutation can enhance blmp-1 null mutation for persistence of the tail spike. Because dre-1 has been previously shown to regulate blmp-1, it's not clear if they are strictly parallel. If the two processes are truly independent, shouldn't the double mutant have fully additive**

phenotypes? But they are not: mutating blmp-1 or dre-1 singly substantially increases tail spike persistence, while the double mutant only residually enhances this phenotype. Can the authors explain?

Our response:
We thank the reviewer for this comment, however we disagree with the conclusion. As shown in Figure 6A, combining a *blmp-1* mutation with either of two *dre-1* alleles results in enhanced tail-spike cell persistence compared to either single mutant alone. If the genes act independently in parallel we predict that persisting cells in the double mutant are a result of either one or the other of the mutations, or both. Therefore, we expect the fraction of persisting cells to be 1-WT(*dre-1*)xWT(*blmp-1*), where WT(*dre-1*) is the number of dying tail-spike cells in *dre-1* single mutants and WT(*blmp-1*) is the number of dying tail-spike cells in *blmp-1* single mutants. For the combination of *blmp-1(s71)* and *dre-1(ns39)* these number are 0.4 and 0.18, respectively. Therefore, we predict persistence of 1-(0.4)(0.18)= 0.93, which is precisely what we see experimentally. For the *dre-1(dh279)* allele, we don't see a defect in this single mutant. Given that heterozygous animals for a strong *dre-1* mutation are wild type, we can posit that a 50% activity reduction in the *dre-1(dh279)* mutant would have no phenotype as well. We therefore predict that the *blmp-1(dh279)*; *dre-1(ns39)* double mutant will have at most 1-(0.4)(0.5)=0.8 persisting cells, which is exactly what we see.

3. **The authors also base their parallel regulation model on the observed lack of regulation of blmp-1::gfp expression by dre-1 in L1 larvae. This observation, though consistent, is preliminary. In particular, they cannot exclude the possibility that dre-1 inhibits both blmp-1 and ced-9, since they only monitored blmp-1::gfp expression in the L1 stage, probably past the point of meaningful regulation. As their very nice data show, dre-1 and blmp-1 are expressed dynamically and inversely during embryonic development. dre-1 is expressed early on up to the three-fold stage (Fig. S2) during which blmp-1 is turned off, and ced-9 promoter is turned on (Fig. 2). Then at the three-fold stage, dre-1 expression diminishes (Fig. S2), blmp-1 increases, and ced-9 promoter shuts off (Fig.2). Cell death ensues. In the blmp-1 mutant, ced-9 expression is constitutive (Figure 4). The inverse expression patterns of dre-1 and blmp-1 could indicate that dre-1 inhibits blmp-1 up until the 3-fold stage, at which point dre-1 goes down and blmp-1 comes on. Given these interesting dynamics, they should examine blmp-1::gfp protein expression levels at different stages of embryogenesis in WT and dre-1 mutants/RNAi.**

Our response:
We thank the reviewer for this comment. As suggested by reviewer 2, we redid our experiment on the *blmp-1* expression pattern using a single-copy *blmp-1* promotor::*gfp* transcriptional reporter, rather than high-copy transgenes, and using the *aff-1* promoter::myristoyl-KatePH (mKatePH) as the tail-spike cell reporter, rather than $P_{cbr\text{-}ced\text{-}3}$::mRFP. The new data show that *blmp-1* starts to express in the tail-spike cell shortly after its birth at the1.5-fold stage. Like the transcriptional reporter, the endogenous BLMP-1::GFP protein expressed by the *cshIs41[BLMP-1::GFP]* single-copy translational reporter generated by CRISPR/cas9 is also detected in the tail-spike cell from the 1.5-fold until the cell dies. We added the results to Figure 2 and described the results in the 2nd paragraph on page 5 and the 1st paragraph on page 6. The reason that we did not observe early expression of *blmp-1* in the original manuscript might be due to instability of the high-copy arrays or a difference in the tail-spike cell reporter used. We have also generated and analyzed single-copy transcriptional *gfp* reporter for *ced-3, dre-1* and *ced-9* using the better tail-spike cell reporter *aff-1* promoter::myristoyl-KatePH and replaced the old data with the respective new ones.

To address the reviewer's question directly, we examined the effect of *dre-1* RNAi on BLMP-1 protein level by treating the *cshIs41[BLMP-1::GFP]* single-copy translational reporter with *dre-1* RNAi. We found that *dre-1* RNAi did not affect BLMP-1::GFP expression pattern at different stages, supporting the notion that DRE-1 does not degrade BLMP-1 in the tail-spike cell.

We added the data to Figure 2E-M and inserted the following sentence on page 9: "Supporting this conclusion, RNAi against *dre-1* does not affect BLMP-1::GFP levels (Figure 2I-M)"

4. **If DRE-1 only affects CED-9 protein levels, then there should be no effect on its transcription. However, if DRE-1 regulates a transcription factor (e.g. BLMP-1) that acts on the ced-9 promoter, then it should show regulation. They should therefore monitor CED-9 protein and ced-9 promoter expression levels around the 3-fold stage in WT, dre-1, blmp-1, dre-1;blmp-1.**

Our response:
As suggested by the reviewer, we generated both high-copy and single-copy translational CED-9::GFP transgenes ($P_{ced\text{-}9}$*::ced-9::gfp*) and tried very hard to examine expression using various microscopy tools to enhance detection sensitivity. However, we cannot observe the CED-9::GFP signal in the tail-spike cell. It is possible that the level of CED-9 protein is too low to observe. Although the transcriptional reporter of *ced-9* is barely observable in the tail-spike cell in the wild-type, its signal is weakly enhanced in the *blmp-1* mutant at the 3.2-fold stage. We have quantified the expression of *ced-9* transcription at the 3.2-fold stage in wild-type, *dre-1*, *blmp-1*, and *blmp-1; dre-1* animals. Results show that *blmp-1*, but not *dre-1*, affects *ced-9* transcription.

We added the data to Figure 4 and inserted a sentence on page 9, 1st paragraph, line 3, as follows: "Furthermore, knockdown of *dre-1* does not increase *ced-9* transcription in the wild-type or *blmp-1(s71)* mutant (Figure 4N, O and P)."

5. **Relatedly, the epistasis model in Figure 7 does not fully capture the regulatory dynamics they depict in their embryo pictures. This might be useful even if not all the answers are clear.**

Our response:
Given our new data, we have removed Figure 7.

6. **The Discussion should be strengthened with what is known about dre-1 and blmp-1 regulating other processes and other factors (e.g. ces-1), and could speculate that they too might be involved. In addition, it would appropriate to mention that apoptotic protease ced-3 plays a non-canonical role in regulating developmental timing and lin28. Perhaps bring out the idea that several components involved in larval timing also function in embryonic cascades?**

Our response:
We thank the reviewer for this comment. We added two paragraphs on pages 10-11 to discuss these points and at the end bring out the idea that components involved in larval developmental timing also temporally regulate tail spike cell death.

On page 10 and 11 the paragraphs read: "Blimp-family members have been shown to control the timing of developmental processes in *C. elegans* (Horn et al., 2014; Huang et al., 2014), *Drosophila* (Agawa et al., 2007; Ng et al., 2006), zebrafish (Lee and Roy, 2006) and mice (Harper et al., 2011). In *C. elegans*, BLMP-1 and DRE-1 control the timing of several developmental events, including larval distal-tip cell migration and seam cell development (Horn et al., 2014; Huang et al., 2014). However, other timing genes, including *lin-29* and *daf-12*, are not required for tail-spike cell death (Table 1), suggesting that if BLMP-1 and DRE-1 function as a timing regulator in the tail-spike cell the mechanism may be different. Consistent with this notion, it has been shown that DRE-1 mediates BLMP-1 proteolysis to temporally control distal tip cell migration and seam cell development (Horn et al., 2014; Huang et al., 2014), whereas DRE-1 does not appear to affect BLMP-1 protein levels in tail-spike cell death (Figure 2I-M).

FBXO11, the human homolog of DRE-1, has been reported to recognize and promote ubiquitin-mediated degradation of multiple Snail family members of zinc-finger transcription factors in mammalian cells (Jin et al., 2015). Interestingly, *C. elegans* Snail-like gene *ces-1*, which represses *egl-1* transcription in the NSM sister cells and therefore prevents their death, genetically interacts with *dre-1* in seam cell development (Jin et al., 2015; Metzstein and

Horvitz, 1999; Thellmann et al., 2003). Loss of *ces-1* suppresses the precocious phenotype of seam cell development in the *dre-1* mutant, raising a possibility that CES-1 might function as a DRE-1 target during seam cell development. However, it is yet unclear whether CES-1 may be involved in regulation of tail-spike cell death, and if so, whether CES-1 might function as a DRE-1 target in the timing control of tail-spike cell death. Intriguingly, CED-3 plays a non-canonical role in regulating developmental timing in seam cell development by acting together with the Arg/N-end rule pathway (Weaver et al., 2017; Weaver et al., 2014). Specifically, CED-3 forms a complex with Arg/N-end rule E3 ligase UBR-1 and Arginyltransferase ATE-1 to efficiently cleave LIN-28, which is subsequently degraded through the Arg/N-end rule pathway, and prevents abnormal temporal seam cell divisions (Weaver et al., 2017). Therefore, several components involved in larval timing control appear to also function in tail-spike cell death."

7. **Abstract**
Change "temporally managed" to controlled or regulated

Our response:
We changed "managed" to "regulated" on Page 2.

8. **Fig 1b Please show the nature of mutations somewhere in Figure or legend.**

Our response:
The nature of the newly identified alleles *ns823* and *ns830* is already described in context on page 4-5. We inserted sentences that describe the remaining mutations in the Figure legend as follows: "The positions of the *blmp-1* mutant alleles, including the region corresponding to the *tm548* deletion, are marked. *s71*, *tk41*, and *tp5* have, respectively, non-sense mutations in codon 281, 381, or 434, and are predicted to encode truncated BLMP-1 proteins without zinc fingers."

9. **Fig 2 blmp-1 comes on at 3-fold, transiently shuts off the tail spike reporter. Why does this occur?**

Our response:

We thank the reviewer for this comment. As for question 3, we redid the experiment to examine the expression pattern of *blmp-1* by using the single-copy *blmp-1* promoter::GFP transgene and the *cshIs41[BLMP-1::GFP]* single-copy translational reporter generated by CRISPR/cas9. We found that GFP in both transcriptional and translational fusion lines is detected in the tail-spike cell from the 1.5-fold until the cell dies. As reviewer 2 pointed out, single-copy transcriptional reporter are more stable and hence more reliable than high-copy reporters. We added the 2nd paragraph on page 5 and 1st paragraph on page 6 to describe the new results, as follows: "Tail-spike cell death is initiated at the 3.2-fold stage of embryogenesis, ~550 minutes post-fertilization (Ghose et al., 2018; Sulston et al., 1983). To determine when *blmp-1* is expressed relative to cell death onset, we generated animals carrying a single copy *blmp-1* promoter::GFP transgene using PhiC31 integrase-mediated insertion (Yang et al., 2020), and crossed these with animals expressing the *aff-1* promoter::myristoyl-KatePH (mKatePH) tail-spike cell reporter. We found that *blmp-1* transcription is detected in the tail-spike cell as early as the mKatePH reporter (1.5-fold stage; Figure 2A). *blmp-1* transcription continues until the tail-spike cell dies with a characteristic rounded refractile morphology at the 3.7-fold stage (Figure 2B-D).

To determine whether BLMP-1 protein accumulation follows its transcriptional expression pattern, we examined animals carrying the *cshIs41[BLMP-1::GFP]* single-copy translational reporter, in which GFP is fused to the BLMP-1 C terminus (Stec et al., 2021). We found that, like the transcriptional reporter, endogenous BLMP-1::GFP is detected in the tail-spike cell from the 1.5-fold stage until the cell dies (Figure 2E-H)."

10. **There are two blmp-1 isoforms. Which one is used in the cDNA constructs? I guess it is the smaller B isoform, based on the yk clone used to make it. The authors might consider**

using the genomic construct in the experiments suggested in 4 to ensure all isoforms are included.

Our response:
We used isoform A because it is the longer isoform. Genomic DNA was not chosen for the cell autonomous rescue experiment as it may have regulatory elements allowing for expression in other nearby cells that would make it challenging to interpret the results.

11. **Fig. 3 Indicate how many biological replicates were done.**

Our response:
We inserted a sentence in the figure legend as follows: "At least three biological replicates were analyzed for each genotype."

12. **Fig 3b Full-length ced-9 transgene does not fully rescue the ced mutant phenotype. Why not? Further, the authors claim: "The full-length transgene, therefore, efficiently complements the ced-9 mutation, and transgenics resemble wild-type animals." Please modify.**

Our response:
We thank the reviewer for this comment. Lack of full rescue may be caused by transgene array instability or insufficient expression levels of the *ced-9* transgene due to missing some regulatory regions. We have removed the sentence "The full-length transgene, therefore, efficiently complements the *ced-9* mutation, and transgenics resemble wild-type animals" from the text.

13. **Fig. 5 How many times was experiment done?**

Our response:
We inserted a sentence to the figure legend as follows: "The experiment was repeated three times"

14. **Fig 5A Why does blmp-1 RNAi significantly reduce expression of ced-9 promoter deletion construct ? Could it be that it has both negative and positive effects on expression of ced-9 promoter? Are there other elements in the promoter?**

Our response:
As reviewer 1 pointed out, it would be best if a mutagenized version of the *blimp-1* binding site was used, rather than a deletion which could disrupt other sequences or spacing in the experiment. We therefore generated single-copy P_{ced-9}::GFP and P_{ced-9m}::GFP transgenes and examined their GFP intensity at the 3.2-fold stage. We found that the *blmp-1(s71)* mutation increases expression of P_{ced-9}::GFP to an extent similar to that of P_{ced-9m}::GFP in the wild-type. Moreover, the *blmp-1(s71)* mutation did not further enhance the expression of P_{ced-9m}::GFP.

We added the data to Figure 4I-M and P and inserted sentences to describe the results on page 7.

On Page 7, 2nd paragraph, line 13, the inserted sentences read: "Importantly, a *ced-9* promoter::GFP reporter transgene harboring the mutant sequence (P_{ced-9m}::GFP) is no longer down-regulated and shows a similar expression level when compared to P_{ced-9}::GFP in the *blmp-1(s71)* mutant (Figure 4I-L and P). Moreover, the *blmp-1(s71)* mutation did not further enhance the expression of P_{ced-9m}::GFP (Figure 4 M and P). These results suggest that in the tail-spike cell, BLMP-1 directly binds the TTTCAATTT sequence upstream of the *ced-9* ATG, blocking *ced-9* gene expression."

Second decision letter

MS ID#: DEVELOP/2020/193995

MS TITLE: BLMP-1 promotes developmental cell death in C. elegans by timely repression of ced-9/bcl-2 transcription

AUTHORS: Hang-Shiang Jiang, Piya Ghose, Hsiao-Fen Han, Yun-Zhe Wu, Ya-Yin Tsai, Huang-Chin Lin, Wei-Chin Tseng, Jui-Ching Wu, Shai Shaham, and YI-CHUN WU
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 2

Advance summary and potential significance to field

The authors have addressed most of the comments the editor and reviewers had and the revised manuscript is much stronger.

Comments for the author

I have no additional suggestions.

Reviewer 3

Advance summary and potential significance to field

This work shows that blmp-1 regulates tail spike removal through transcriptional regulation of ced-9 as part of a fine tuend temporal mechanism underlying this process.

Comments for the author

The authors have done a good job to address my previous concerns. I suggest to accept the paper.