Targeted cell ablation in zebrafish using optogenetic transcriptional control

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MS TITLE: Targeted cell ablation in zebrafish using optogenetic transcriptional control

AUTHORS: Karen Mruk, Paulina Ciepla, Patrick Piza, Mohammad Alnaqib, and James K Chen

Many apologies for the delay in assessing your study, I'm afraid this got caught in the general slow down over the summer and then one of the referees was late in returning their report. However, I have now received all the referees' reports, 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. The referees all request clarification and additional information to help the reader on specific points and indicate that more precision is required in some places to describe and to critically evaluate the results. In addition, a clearer assessment and description of the death of surrounding cells is needed and inclusion of non-transgenic embryos to assess damage caused by leaky expression. I would also encourage you to follow Referee 3's suggestion to ensure crucial information is in the main text (not supplement).

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.

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.

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Reviewer 1

Advance summary and potential significance to field

Mruk et al. report the implementation of methods for optogenetic gene activation and inducible cell ablation.
In particular, the use of M2H37A and GAVPO will be of interest to fish researchers. The methods are far from perfect but there is information that will be useful for the field once the authors have addressed the following points.

Comments for the author

1. The authors are too generous about the potential use of these tools. For example, there are many overlapping data points in control and treated animals, making this tool much less robust than advertised. Please add a more measured and critical assessment of these technologies so that colleagues using these methods are prepared for potential limitations and frustration.

2. M2H37A-mediated killing seems to be non-apoptotic in expressing cells but apoptotic in surrounding cells. This feature undermines its use in many studies, with the exception of broader lesions. This effect is documented in the pilot experiments but not in the rest of the paper. In particular, readers need to know what happens cell autonomously and non-autonomously in the experiments in Figure 4.

3. Several experiments address the effect of M2H37A-mediated killing in comparison to drug-treated or non-illuminated transgenic embryos. But it is as important to compare to non-transgenic wild-type embryos to assess the damage caused by leaky expression and activity (e.g. Figure 4).

4. Figure 2: The authors write "The resulting embryos exhibited deformities of the notochord, head, and presumptive hatching gland…." Hatching gland defects are not documented. And why do the cells not die? A much more severe defect is expected based on previous shield ablation studies. And what were the non-autonomous defects in structures such as floor plate and ventral forebrain?

5. Figure S3A I cannot see evidence that "TUNEL-positive cells were limited to the CNS at 48 hpf in NTR-expressing embryos treated with metronidazole, but they were observed outside the CNS in M2H37A-expressing embryos cultured in the absence of rimantadine. And why is 32hpf TUNEL staining in DMSO-treated animals so strong?

6. The abstract is very vague and generic. Please provide details about the systems implemented here.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Mruk et al. describes an optogenetic cell ablation technique for zebrafish. Their method uses a light-sensitive transcription factor (GAVPO) to drive expression of either a cytotoxic ion channel (M2H37A) or pro-drug activating enzyme (nitroreductase, NTR). Using tissue-specific expression of GAVPO, they are are able demonstrate neural-specific cell ablation. This is the first demonstration that M2H37A is functional as a cytotoxic gene in zebrafish and in fact works better than NTR. This manuscript is also a further demonstration of the previously published GAVPO system. As such, this manuscript is certainly of interest to the zebrafish community and likely to the broader readership of Development as well.

Comments for the author

Suggestions for the authors:
1. In several places, the authors suggest that GAVPO-driven M2 or NTR expression levels scale with GAVPO concentration, light intensity, and duration of illumination. However, these conclusions

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appear to be drawn from qualitative assessments of fluorescence intensity. Their conclusions would be strengthened with some sort of quantitative assessment, either qPCR of transcript levels or quantifying fluorescence intensity.
2. Image acquisition and image processing methods for Fig. 1-3 are not described. In particular, what were the "short" and "long" exposure times used for Fig. 3A?
3. In Fig. 1, the defects are difficult for non-neurobiologists to appreciate. Could arrows be added to indicate specific defects (e.g., missing midbrain/hindbrain boundary)? Could the authors add a brief description of how the authors scored "neural ablation" for Table S1?
4. Fluorescence micrographs would be easier to see if presented in grayscale rather than red.
5. Fig. 3D is labeled as "global irradiation" however it appears to be similar to the "head irradiation" presented in Fig. 3B-C. "Global" implies that the entire embryo was irradiated.Perhaps authors could re-label as "whole head" or "whole field of view"
6. Arrowheads in Fig. 4A are not defined in the figure legend.
7. For Fig. 4B, arrows or brackets indicating axonal tracts would be helpful.

Not strictly necessary, but the appeal of the manuscript might be broadened if the authors could demonstrate M2-induced ablation in non-neuronal tissues.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Mruk et al describes a new tool for targeted cell ablation in zebrafish using optogenetics and the influenza M2 protein. In comparison to the nitroreductase system, the authors demonstrate that M2 had a more rapid effect on cells, although killing went beyond the cells expressing M2. M2 activity can be modulated by rimantadine adding additional control of the system. Furthermore in conjunction with tissue specific promoters, the authors use a light induced transcription factor to drive M2 expression in a more defined locale. Overall the use of both the light-induced GAVPO transcription factor and M2 as an alternative for cell ablation has high value for the zebrafish community.

Comments for the author

In general the work is convincing and I am in favor of publication. However the manuscript is difficult to read for someone not involved in the zebrafish field and some experiments and controls are missing. Overall, there is important data in the Supplemental material that isn’t strictly supplemental and should be in the main text of the manuscript.

Major points to address:
1 – How extensive is the cell death in surrounding tissues from M2 expression?
One assumes the expressing cells are killed, but how much more? This would be important for designing experiments to use with this system.

2 – How sensitive is GAVPO to standard room light? How careful will someone have to be with room light exposure if utilizing GAVPO for their experiments?
Controls for the GAVPO experiments showing room light compared to dark would be useful.

Main Text:
Page 6 – the promoters used are not familiar to someone outside the zebrafish field. It is important to explain that tuba1a is neuronal specific and myl7 is heart specific, or this part of the work is confusing.

Page 9 – a reference to Table S2 for numbers would be helpful in the final paragraph.

Page 11 – likely due to (rimantadine’s) protective effects – it would be helpful to be more explicit here. Ie the neurons expressing M2 don’t die, so there are more of them.

Figures:

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Figure 1A – how do you know these embryos are displaying neuronal defects as opposed to general morphological defects, especially given that M2 causes deformation in general from Figure S1.

Figure 1A – the bar graph here might be better in the main manuscript but if space is an issue, there are other supplemental data that should be included first – see below.

Figure 2 – Where is the experiment showing dose dependent manner of mCherry induction referred to in the text on page 8?

Figure 2 – The data in the supplemental info in S4 is as important as seeing fluorescent protein expression. I think this shouldn't be supplemental! Surely the bar graphs could be included in the report itself.

Figure 4B – what am I supposed to see in these images?

Supplemental data:
Table S1 – Why is there neuronal ablation in animals labeled as having only Tg(tuba1a:Gal4VP16; myl7:gfp)? Shouldn't these be controls?

Table S1 – the table is fine, but a simple bar chart for this data in the main text would be informative.

Table S2 part D - 14% of embryos show defects with GAVPO + the M2 line whether in 470nM light or not, or with rimantadine or not. Is the UAS line leaky? Or is this a drawback to this particular tool. Given this is introducing the tool, this is information experimenters need to know.

Table S2 – Where is Tg(UAS:NTR-mCherry uninjected and exposed to 470nm light?)

Fig S2 – there should be an overlap image provided to properly gauge the extent of co-expression. Is it known that elav and tuba1a don't overlap in expression? This should be mentioned.

Fig S3 – Why is the TUNEL stain so high in 32hpf DMSO embryos in this figure?

Fig S3 - How widespread is the ablation around cells expressing M2?

Fig S4 – I think both bar charts should be in the main manuscript.

Fig S6 – why do you see any M2 expression at all in the control at 12h?
Shouldn't these neurons be dead?

Materials and Methods:
1 - what is a "Bleeding Heart" construct?

2 - Why are embryos mounted on a vortex Genie for irradiation? Is there mixing taking place?

First revision

Author response to reviewers' comments

Responses to Reviewers' Comments
"Targeted cell ablation in zebrafish using optogenetic transcriptional control"
(DEVELOP/2019/183640)

Reviewer 1

Concern 1: The authors are too generous about the potential use of these tools. For example, there are many overlapping data points in control and treated animals, making this tool much less robust

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than advertised. Please add a more measured and critical assessment of these technologies so that colleagues using these methods are prepared for potential limitations and frustration.

Response: We appreciate the reviewer's concern about the robustness of the GAVPO system and its utility as a tool for biological research. To address this question, we have quantified light- dependent GAVPO activity using the UAS:NTR-mCherry reporter (Fig. 2C and Fig. S4C). Our findings corroborate the efficacy of the GAVPO system, particularly when stably integrated into transgenic animals. Transient GAVPO expression leads to a broader range of activity, likely due to the variability associated with mRNA microinjections. We have also modeled the sensitivity of GAVPO to ambient light by irradiating GAVPO-expressing embryos with a white LED (Fig. S4B-C). These results demonstrate that blue-light irradiation is required to achieve high levels of GAVPO activity.

Concern 2: M2H37A-mediated killing seems to be non-apoptotic in expressing cells but apoptotic in surrounding cells. This feature undermines its use in many studies, with the exception of broader lesions. This effect is documented in the pilot experiments but not in the rest of the paper. In particular, readers need to know what happens cell autonomously and non-autonomously in the experiments in Figure 4.

Response: Our studies indicate that $M2^{H37A}$-expressing cells are killed through necrosis, a process that leads to the release of cytosolic contents into the surrounding tissue. This cellular debris can then cause inflammation that results in apoptotic cell death. While this feature may limit the use of $M2^{H37A}$ for some studies (e.g. cell fate mapping), $M2^{H37A}$-mediated killing recapitulates the cell death mechanisms that result from severe and acute injuries, making it a valuable tool for studying regenerative biology. Moreover, since inflammation is known to contribute to degenerative disease, the $M2^{H37A}$ system could prove to be useful for modeling these processes.

It is important to note that the non-autonomous cell death observed in our study was associated with pan-CNS $M2^{H37A}$ expression driven by the strong Gal4VP16 transactivator. In contrast, we observed localized notochord defects when we shield-irradiated *Tg(UAS:M2*^{H37A}*;myl7:mCherry)* embryos injected with *GAVPO* mRNA; the overlying floor plate remained intact (Fig. 3D). We have also now examined whether $M2^{H37A}$-mediated cell death in *Tg(elavl3:GAVPO;UAS:M2*^{H37A}*; myl7:mCherry)* embryos causes apoptosis by immunostaining the irradiated embryos for activated caspase-3 (Fig. S5B). The light-dependent loss of neurons in these embryos (Fig. 5) did not coincide with increased apoptosis, providing further evidence that $M2^{H37A}$ can be used to target specific cell populations.

Concern 3: Several experiments address the effect of M2H37A-mediated killing in comparison to drug-treated or non-illuminated transgenic embryos. But it is as important to compare to non-transgenic wild-type embryos to assess the damage caused by leaky expression and activity (e.g. Figure 4).

Response: We appreciate the reviewer's concern about leaky $M2^{H37A}$ expression in the *Tg(elavl3:GAVPO;UAS:M2*^{H37A}*;myl7:mCherry)* zebrafish. Since we cannot detect $M2^{H37A}$ protein in the non-irradiated embryos by immunostaining and that the animals develop normally, it is unlikely that there is significant $M2^{H37A}$-mediated cell death in the absence of blue-light illumination.

Concern 4: Figure 2: The authors write "The resulting embryos exhibited deformities of the notochord, head, and presumptive hatching gland…." Hatching gland defects are not documented. And why do the cells not die? A much more severe defect is expected based on previous shield ablation studies. And what were the non-autonomous defects in structures such as floor plate and ventral forebrain?

Response: The reviewer is correct that previous shield-ablation studies have reported more severe defects (e.g., Shin and Fraser, *Development* 122: 1313-122, 1996 and Saúde *et al.*, *Development* 127: 3407-17, 2000), likely because these surgical manipulations removed the entire shield at 6 hpf. In comparison, we irradiated a 100-µm anterior region within the shields of 6-hpf *Tg(UAS:M2*^{H37A}*;myl7:mCherry)* embryos that had previously been injected with *GAVPO* mRNA (now shown in Fig. 3D). Given the localized activation of GAVPO, the limited tissue penetration of blue light, and the temporal delay associated with gene transcription and protein translation, it is

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reasonable to expect that the developmental defects in our studies will be less severe than those reported in the previous studies. As discussed in our response to Concern 2, we did not observe significant non-autonomous defects in the floor plate, nor did the embryos appear to have ventral forebrain abnormalities. Since we have not characterized the hatching gland defects in detail, we have removed that point from our phenotypic description.

Concern 5: Figure S3A I cannot see evidence that "TUNEL-positive cells were limited to the CNS at 48 hpf in NTR-expressing embryos treated with metronidazole, but they were observed outside the CNS in M2H37A-expressing embryos cultured in the absence of rimantadine. And why is 32hpf TUNEL staining in DMSO-treated animals so strong?

Our DMSO-treated embryos have a high background since we intentionally overstained the samples to see the TUNEL-positive cells, which appear as small puncta. To corroborate our findings for the $M2^{H37A}$-expressing zebrafish, we have now also immunostained these samples for activated caspase-3 (Fig. S2B). Consistent with our TUNEL staining results (now shown in Fig. S2A), we observe widespread caspase-3 activation in the head by 48 hpf.

Concern 6: The abstract is very vague and generic. Please provide details about the systems implemented here.

Response: We appreciate this feedback, and we have revised the abstract to provide specific details about the systems implemented in our study.

Reviewer 2

Concern 1: In several places, the authors suggest that GAVPO-driven M2 or NTR expression levels scale with GAVPO concentration, light intensity, and duration of illumination. However, these conclusions appear to be drawn from qualitative assessments of fluorescence intensity. Their conclusions would be strengthened with some sort of quantitative assessment, either qPCR of transcript levels or quantifying fluorescence intensity.

Response: We have now quantified light-dependent activation of the UAS:NTR-mCherry reporter, mediated by both transient and stable GAVPO expression (Fig. 2C, Fig. 4, and Fig. S4C, respectively).

Concern 2: Image acquisition and image processing methods for Fig. 1-3 are not described. In particular, what were the "short" and "long" exposure times used for Fig. 3A?

Response: Due to space constraints, the image acquisition and imaging processing protocols for our study are described in our methods section. We have also added exposure times to Fig. 4A (formerly Fig. 3A) and Fig. S4A-B.

Concern 3: In Fig. 1, the defects are difficult for non-neurobiologists to appreciate. Could arrows be added to indicate specific defects (e.g., missing midbrain/hindbrain boundary)? Could the authors add a brief description of how the authors scored "neural ablation" for Table S1?

Response: We have added arrowheads to demarcate the mindbrain/hindbrain boundary in Fig. 1C. We have also added a description for how "neural ablation" was scored to Table S1.

Concern 4: Fluorescence micrographs would be easier to see if presented in grayscale rather than red.

Response: We have converted fluorescent micrographs throughout the manuscript to grayscale images. Since several of the original images were acquired with a color camera, this conversion is associated with some information loss. If the editor would prefer the color images for certain data

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sets, we can revert to those micrographs.

Concern 5: Fig. 3D is labeled as "global irradiation" however it appears to be similar to the "head irradiation" presented in Fig. 3B-C. "Global" implies that the entire embryo was irradiated. Perhaps authors could re-label as "whole head" or "whole field of view"

Response: The images shown in Fig. 4D and 4F (formerly Fig. 3D and 3F) are of globally and spot-irradiated fish, respectively. To more clearly demonstrate the difference between these two irradiation protocols, we now include images of trunk regions within the same animals.

Concern 6: Arrowheads in Fig. 4A are not defined in the figure legend.

Response: We now define the arrowheads in the legend for Fig. 5A (formerly Fig. 4A).

Concern 7: For Fig. 4B, arrows or brackets indicating axonal tracts would be helpful.

Response: We now use dashed lines in Fig. 5B (formerly Fig. 4B) to indicate axonal tract widths for selected regions.

Concern 8: Not strictly necessary, but the appeal of the manuscript might be broadened if the authors could demonstrate M2-induced ablation in non-neuronal tissues.

Response: Our shield-irradiation studies demonstrate that $M2^{H37A}$ can be used to ablate mesoderm tissues (Fig. 3D). We have also recently generated *Tg(ClaudinK:GAVPO;UAS-NTR- mCherry)* embryos that express in the photoactivatable transactivator in oligodendrocytes and Schwann cells and exhibit light-dependent mCherry expression in these cells (Figure R1). Although the latter results are encouraging and provide further evidence for the general utility of the GAVPO system, we will need significantly more time to thoroughly characterize this line for publication.

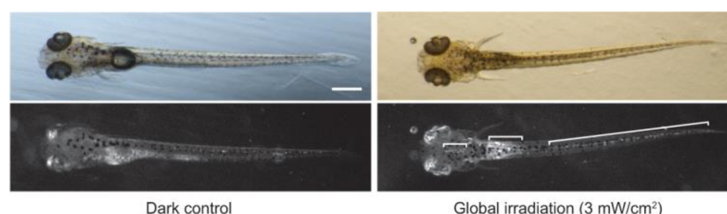

Figure R1. Light-inducible, myelin specific expression using the GAVPO system. *Tg(ClaudinK:GAVPO; UAS:NTR-mCherry)* embryos were globally irradiated with blue light at 5 dpf for 2 h and imaged 8 h later. Areas with mCherry-positive cells are indicated by the brackets. Embryo orienation: dorsal view, anterior left. Scale bar: 300 µm.

Reviewer 3

Concern 1: How extensive is the cell death in surrounding tissues from M2 expression? One assumes the expressing cells are killed, but how much more? This would be important for designing experiments to use with this system.

Response: As described in our response to Reviewer 1 (Concern 2), the non-autonomous cell death observed in our study was associated with pan-CNS $M2^{H37A}$ expression driven by the strong Gal4VP16 transactivator. In contrast, we observed localized notochord defects when we shield- irradiated *Tg(UAS:M2^{H37A};myl7:mCherry)* embryos injected with *GAVPO* mRNA; the overlying floor plate remained intact (Fig. 3D). We also have now examined whether $M2^{H37A}$-mediated cell death in *Tg(elavl3:GAVPO;UAS:M2^{H37A};myl7:mCherry)* embryos causes apoptosis by immunostaining the irradiated embryos for activated caspase-3 (Fig. S5B). The light-dependent loss of neurons in these

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embryos (Fig. 5) did not coincide with increased apoptosis, providing further evidence that M2^{H37A} can be used to target specific cell populations.

Concern 2: How sensitive is GAVPO to standard room light? How careful will someone have to be with room light exposure if utilizing GAVPO for their experiments? Controls for the GAVPO experiments showing room light compared to dark would be useful.

Response: Since fluorescent and LED lights are also sources of blue light, we irradiated *Tg(elavl3:GAVPO;UAS:NTR-mCherry)* embryos with white LEDs (intensity at 470 nm = 1.8 mW/cm^2) for 2 hours. The resulting mCherry fluorescence was significantly lower than that observed in transgenic embryos irradiated with blue light (Fig. S4B-C). This white LED condition represents a worst-case scenario; we have determined that the ambient light in our laboratory has a 470-nm intensity of 0.93 mW/cm^2, and we take steps to minimize light exposure when handling the embryos. As described in our methods section, we also use a Wratten filter to attenuate the intensity of our microscope light sources.

Concern 3: Page 6 – the promoters used are not familiar to someone outside the zebrafish field. It is important to explain that tuba1a is neuronal specific and myl7 is heart specific, or this part of the work is confusing.

Response: We've added these details to the manuscript text.

Concern 4: Page 9 – a reference to Table S2 for numbers would be helpful in the final paragraph.

Response: To maintain consistency with our other figures and supplementary tables, we would prefer to cite Table S2 in the legend for Fig. 2.

Concern 5: Page 11 – likely due to (rimantadine's) protective effects – it would be helpful to be more explicit here. i.e. the neurons expressing M2 don't die, so there are more of them.

Response: We have added this explanation to the manuscript text.

Concern 6: Figure 1A – how do you know these embryos are displaying neuronal defects as opposed to general morphological defects, especially given that M2 causes deformation in general from Figure S1.

Response: The *tuba1a* promoter used to control Gal4VP16 expression and consequently M2^{H37A}-mediated killing is a well-characterized, CNS-specific driver. Moreover, M2^{H37A} is known to act cell autonomously to cause cell lysis. As we discuss in our manuscript, we also observed apoptotic cell death at later developmental stages, as ascertained by TUNEL staining and caspase-3 activation (Fig. S2A-B). Thus, M2^{H37A} induces not only neuronal necrosis in these transgenic animals, but also damage to cells that are in close proximity to the CNS.

Concern 7: Figure 1A – the bar graph here might be better in the main manuscript but if space is an issue, there are other supplemental data that should be included first – see below.

Response: We presume that the reviewer is referring to the graph in Fig. S1C of our original manuscript? We have added this data and the corresponding micrographs to Fig. 1.

Concern 8: Figure 2 – Where is the experiment showing dose dependent manner of mCherry induction referred to in the text on page 8?

Response: Fig. 2B shows representative micrographs for *Tg(UAS:NTR-mCherry)* embryos injected with 100 pg or 200 pg of *GAVPO* mRNA and then globally irradiated with blue-light. In addition to these

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images, we now include a graph of the quantified GAVPO- and light-dependent mCherry fluorescence for this experiment (Fig. 2C).

Concern 9: Figure 2 – The data in the supplemental info in S4 is as important as seeing fluorescent protein expression. I think this shouldn't be supplemental! Surely the bar graphs could be included in the report itself.

Response: We agree with this comment, and we have moved these data to the Fig. 2D and Fig. 3B.

Concern 10: Figure 4B – what am I supposed to see in these images?

Response: This figure panel (now Fig. 5B) shows confocal images of spinal axonal tracts of *Tg(elavl3:GAVPO;UAS:M2^{H37A};myl7:mCherry)* embryos that have been injected with an *elavl3:mCherry-CAAX* construct. The embryos exhibit mosaic neuronal mCherry expression, allowing individual tracts to be discerned by fluorescence microscopy. Irradiation of the embryos with blue light leads a reduction in the number of axonal tracts and those that remain are disorganized. We have also added dashed lines to indicate the regions that were used for quantifying axon tract widths.

Concern 11: Table S1 – Why is there neuronal ablation in animals labeled as having only Tg(tuba1a:Gal4VP16; myl7:gfp)? Shouldn't these be controls?

Response: The reviewer is correct that these are controls. We apologize for this error.

Concern 12: Table S1 – the table is fine, but a simple bar chart for this data in the main text would be informative.

Response: Tables S1-S4 provide the statistical data associated with Figs. 1-4. To avoid cluttering these figures with additional bar graphs, we would prefer to refer the readers to these tables.

Concern 13: Table S2 part D - 14% of embryos show defects with GAVPO + the M2 line whether in 470nM light or not, or with rimantadine or not. Is the UAS line leaky? Or is this a drawback to this particular tool. Given this is introducing the tool, this is information experimenters need to know.

Response: We now include RT-PCR data that confirms that higher doses of *GAVPO* mRNA can promote light-independent M2^{H37A} expression (Fig. 3C), which can lead to developmental defects. This leakiness appears to be less of a concern when GAVPO is stably expressed through a tissue- specific promoter, as demonstrated by the lack of detectable M2^{H37A} expression in *Tg(elavl3:GAVPO;UAS:M2^{H37A};myl7:mCherry)* embryos prior to irradiation (Fig. S5A).

Concern 14: Table S2 – Where is Tg(UAS:NTR-mCherry uninjected and exposed to 470nm light?)

Response: We apologize for the oversight, and we have added these data to the Table S2.

Concern 15: Fig S2 – there should be an overlap image provided to properly gauge the extent of co-expression. Is it known that elav and tuba1a don't overlap in expression? This should be mentioned.

Response: We have included the merged image to help the readers (now Fig. S1). Previous studies have shown that *elavl3* is a post-mitotic early neuronal marker (Kim *et al.*, *Neurosci. Lett.* 216: 109-112, 1996) and *tuba1a* is expressed throughout the developing CNS (Hieber *et al.*, *J. Neurobiol.* 37:429-420, 1998; Goldman *et al.*, *Transgenic Res.* 10: 21-33, 2001). We now describe this difference between the two promoters in the manuscript text.

Concern 16: Fig S3 – Why is the TUNEL stain so high in 32hpf DMSO embryos in this figure?

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Response: As described in our response to Reviewer 1 (Concern 5), our DMSO-treated embryos have a high background since we intentionally overstained the samples to see the TUNEL- positive cells, which appear as small puncta. To corroborate our findings for the M2^{H37A}- expressing zebrafish, we have now also immunostained these samples for activated caspase-3 (Fig. S2B). Consistent with our TUNEL staining results, we observe widespread caspase-3 activation in the head by 48 hpf.

Concern 17: Fig S3 - How widespread is the ablation around cells expressing M2?

Response: We assume that the reviewer is referring to Fig. 4B? Fig. S3 in our original manuscript used *Tg(UAS:NTR-mCherry)* zebrafish injected with *GAVPO* mRNA. As described in our response to Reviewer 1 (Concern 2), the non-autonomous cell death observed in our study was associated with pan-CNS M2^{H37A} expression driven by the strong Gal4VP16 transactivator. In contrast, we observed localized notochord defects when we shield-irradiated *Tg(UAS:M2^{H37A};myl7:mCherry)* embryos injected with *GAVPO* mRNA; the overlying floor plate remained intact (Fig. 3D). We also have now examined whether M2^{H37A}-mediated cell death in *Tg(elavl3:GAVPO;UAS:M2^{H37A};myl7:mCherry)* embryos causes apoptosis by immunostaining the irradiated embryos for activated caspase-3 (Fig. S5B). The light-dependent loss of neurons in these embryos (Fig. 5) did not coincide with increased apoptosis, providing further evidence that M2^{H37A} can be used to target specific cell populations.

Concern 18: Fig S4 – I think both bar charts should be in the main manuscript.

Response: We agree with this comment, and we have moved these data to the Fig. 2D and Fig. 3B.

Concern 19: Fig S6 – why do you see any M2 expression at all in the control at 12h? Shouldn't these neurons be dead?

Response: The M2^{H37A} levels required for cell death and the precise rate at which this channel kills individual cells is not known. We believe that the M2^{H37A} expression observed in these embryos (now Fig. S5A) corresponds to cells that have not yet died in response to this cytotoxic channel.

Concern 20: What is a "Bleeding Heart" construct?

Response: We apologize for using this colloquial term, and we now refer to this construct by its standard *myl7* nomenclature.

Concern 21: Why are embryos mounted on a vortex Genie for irradiation? Is there mixing taking place?

Response: For global irradiations, the embryo cultures were vortexed to ensure uniform irradiation from all sides (the embryos can tumble freely in the culture medium). We now include this explanation in the methods section.

Second decision letter

MS ID#: DEVELOP/2019/183640

MS TITLE: Targeted cell ablation in zebrafish using optogenetic transcriptional control

AUTHORS: Karen Mruk, Paulina Ciepla, Patrick Piza, Mohammad Alnaqib, and James K Chen

I have now received 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.

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The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. In particular, Referee 2 has some remaining points that need clarification and addressing these will help readers. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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.

Reviewer 1

Advance summary and potential significance to field

The study reports two important advances for zebrafish researchers: a light inducible transcriptional activator and a necrosis-inducing channel. The authors have improved the manuscript but they still oversell the power of their system with respect to specificity and background activity.

Comments for the author

Please use a more measured tone in discussing the impact of this technology.

Reviewer 2

Advance summary and potential significance to field

The revised manuscript by Mruk et al. describes an optogenetic cell ablation technique for zebrafish. Their method uses a light-sensitive transcription factor (GAVPO) to drive expression of either a cytotoxic ion channel (M2H37A) or pro-drug activating enzyme (nitroreductase, NTR). Using tissue-specific expression of GAVPO, they are are able demonstrate neural-specific cell ablation. This is the first demonstration that M2H37A is functional as a cytotoxic gene in zebrafish and in fact works better than NTR. This manuscript is also a further demonstration of the previously published GAVPO system. As such, this manuscript is certainly of interest to the zebrafish community and likely to the broader readership of Development as well. This revised manuscript includes additional characterization and analysis of the GAVPO system that improves upon the original submission. The addition of quantitative assessments in some figures is especially commendable.

Comments for the author

1. The authors demonstrate that M2 expression induces necrosis in target cells, and indirectly leads to apoptosis of surrounding cells. The authors point out that this combination of necrosis + apoptosis mimics what happens in “real-life” injury, suggesting this could be a good model of tissue injury and repair. However, as pointed out by the other reviewers, this secondary, non-cell autonomous cell death precludes the use of the M2 system in other applications like lineage tracing. I think a slightly expanded discussion of these advantages and limitations of the M2 system would be helpful and interesting to readers.

2. In Fig. 2D, was this experiment performed in the absence of metronidazole? If so, can the authors comment on why are there so many more deformed/dead embryos with GAVPO/Gal4-VP16 expression compared to uninjected embryos (not mentioned in text)?

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3. Is there similar quantification (normal/deformed/dead) for embryos injected with 50 pg GAVPO mRNA? This is the concentration used elsewhere in the manuscript (e.g., Fig. 3)

4. In Fig. 3A, it would be helpful for non-zebrafish people if the mesodermal structures (head mesoderm, hatching gland, notochord) were pointed out, e.g. with arrows.

5. In Fig 4B-C, It is unclear whether the dashed lines represented irradiated area or the region of interest used for pixel intensity measurements as in 4A. If the dashed lines represent areas used for measurement, authors should additionally note irradiated area (i.e., define "head").
This would help readers assess the level of off-target activation or activation due to light scattering.

6. Fig. 4D-F. An explanation of the differences in trunk fluorescence between 4D and 4F would be helpful (i.e., that lack of trunk fluorescence in 4F demonstrates restricted GAVPO activation).

7. In 4E, spot illumination is shown on a dorsal-view embryo but resulting expression is shown in lateral view. For readers unfamiliar with zebrafish embryo anatomy, it would be helpful if the spot illumination was also denoted in the lateral-view panel (an approximation might be sufficient)

8. In Fig. 5, was this experiment performed using global irradiation?

9. In Fig. S5, why is the anti-M2 staining apparently so much brighter in rimantadine-treated embryo 2 hours post-irradiation? Is this due to autofluorescence from rimantadine?

Second revision

Author response to reviewers' comments

Responses to Reviewers' Comments

"Targeted cell ablation in zebrafish using optogenetic transcriptional control"
(DEVELOP/2019/183640)

Reviewer 1

Concern 1: Please use a more measured tone in discussing the impact of this technology.

Response: We appreciate the reviewer's feedback, and we have modified the text accordingly.

Reviewer 2

Concern 1: The authors demonstrate that M2 expression induces necrosis in target cells, and indirectly leads to apoptosis of surrounding cells. The authors point out that this combination of necrosis + apoptosis mimics what happens in "real-life" injury, suggesting this could be a good model of tissue injury and repair. However, as pointed out by the other reviewers, this secondary, non-cell autonomous cell death precludes the use of the M2 system in other applications like lineage tracing. I think a slightly expanded discussion of these advantages and limitations of the M2 system would be helpful and interesting to readers.

Response: We have expanded the discussion to describe the advantages and limitations of M2-mediated cell ablation in more detail.

Concern 2: In Fig. 2D, was this experiment performed in the absence of metronidazole? If so, can the authors comment on why are there so many more deformed/dead embryos with GAVPO/Gal4-VP16 expression compared to uninjected embryos (not mentioned in text)?

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Response: The experiment shown in Fig. 2D was conducted in the absence of metronidazole. The defects observed in some embryos injected with 200 pg *GAVPO* mRNA is likely due to toxicity from the *in vitro* transcription product. A previous study also reported embryonic toxicity at this *GAVPO* mRNA dose [Reade et al. (2017) *Development* 144: 345-355]. In comparison, *Tg(elavl3:GAVPO)* zebrafish develop normally, indicating the GAVPO protein is well-tolerated. To minimize the toxicity of *in vitro* transcribed GAVPO mRNA, we conducted our transient expression experiments with 50 pg/embryo of the transcript, which was sufficient to achieve robust GAVPO activity. The greater toxicity observed with *Gal4VP16* mRNA is consistent with the findings of earlier zebrafish studies [Köster et al. (2001) *Dev. Biol.* 233: 329-346; Scott et al. (2007) *Nat. Methods* 4: 323-326]. We have added these points and their corresponding references to our manuscript.

Concern 3: Is there similar quantification (normal/deformed/dead) for embryos injected with 50 pg *GAVPO* mRNA? This is the concentration used elsewhere in the manuscript (e.g., Fig. 3).

Response: We did not include the 50 pg *GAVPO* mRNA condition in the study shown in Fig. 2D because we had previously found that 100 pg was well-tolerated by zebrafish embryos. However, we did quantify the toxicity of embryos injected with 50 pg *GAVPO* mRNA in a separate experiment (Figure R1). As expected, this transcript dose leads to even fewer deformed/dead embryos.

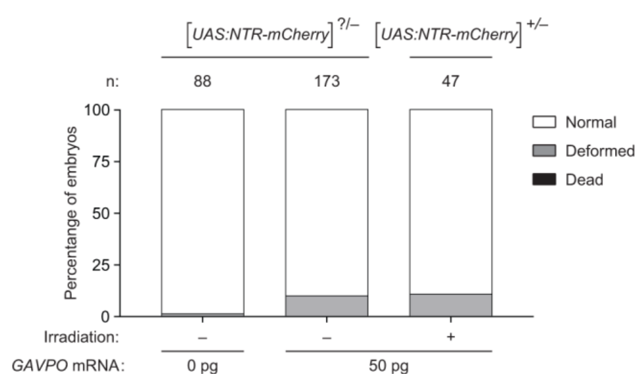

Figure R1. Effects of 50 pg *GAVPO* mRNA on zebrafish development. Zygotes obtained by crossing *Tg(UAS: NTR-mCherry)* heterozygotes and wild-type zebrafish were each injected with 50 pg of *GAVPO* mRNA, and the embryos were either cultured in the dark or globally exposed to blue LED light (3 mW/cm^2) from 6 to 8 hpf. Developmental phenotypes were then scored at 24 hpf, using the indicated number of embryos (n) per condition.

Concern 4: In Fig. 3A, it would be helpful for non-zebrafish people if the mesodermal structures (head mesoderm, hatching gland, notochord) were pointed out, e.g. with arrows.

Response: We have added arrowheads to the figure.

Concern 5: In Fig 4B-C, It is unclear whether the dashed lines represented irradiated area or the region of interest used for pixel intensity measurements as in 4A. If the dashed lines represent areas used for measurement, authors should additionally note irradiated area (i.e., define "head"). This would help readers assess the level of off-target activation or activation due to light scattering.

Response: We mistakenly omitted the description in the figure legend for panels B and C, and we apologize for this oversight. All dashed lines represent the area used to quantify pixel intensities. We have also added a description of the head-irradiation procedure to the methods section.

Concern 6: Fig. 4D-F. An explanation of the differences in trunk fluorescence between 4D and 4F would be helpful (i.e., that lack of trunk fluorescence in 4F demonstrates restricted GAVPO activation).

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Response: We appreciate this suggestion, and we have added an explanation of these differences to the manuscript.

Concern 7: In 4E, spot illumination is shown on a dorsal-view embryo but resulting expression is shown in lateral view. For readers unfamiliar with zebrafish embryo anatomy, it would be helpful if the spot illumination was also denoted in the lateral-view panel (an approximation might be sufficient)

Response: We understand the reviewer's concern; however, we are hesitant to delineate the illuminated region in lateral view since we have not directly observed it from this perspective. In principle, the light path through the embryo will depend on the focal point of illumination and the transmission efficiency of the embryo tissue. Unfortunately, any approximation we could provide would be speculative.

Concern 8: In Fig. 5, was this experiment performed using global irradiation?

Response: This experiment utilized global irradiation, and we have clarified this point in the figure legend.

Concern 9: In Fig. S5, why is the anti-M2 staining apparently so much brighter in rimantadine-treated embryo 2 hours post-irradiation? Is this due to autofluorescence from rimantadine?

Response: We do not entirely know why the anti-M2 staining is brighter for this experimental condition. The chemical structure of rimantadine indicates that it is not intrinsically autofluorescent, and its reaction products with paraformaldehyde are not likely to be either. One possibility is that adamantine skeleton in this channel blocker can increase the hydrophobicity of the treated embryos, thereby promoting non-specific antibody binding. If the amount of anti-M2 antibody is limiting during whole-mount immunostaining, the level of this non-specific staining would decrease as more $M2^{H37A}$ is expressed. This could explain why the anti-M2 staining of *Tg(elavl3:GAVPO;UAS:M2*^{H37A}*;myl7:mCherry)* embryos is brighter at the earliest time point after irradiation.

Third decision letter

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ARTICLE TYPE: Techniques and Resources Article

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

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