JCB

Optogenetic activators of apoptosis, necroptosis and pyroptosis

Kateryna Shkarina, Eva Hasel de Carvalho, Jose Santos, Saray Ramos, Maria Leptin, and Petr Broz

Corresponding Author(s): Petr Broz, University de Lausanne

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November 4, 2021

Re: JCB manuscript #202109038

Prof. Petr Broz
University de Lausanne
Biochemistry
Chemin du Polny 25
Epalinges, Vaud 1066
Switzerland

Dear Prof. Broz,

Thank you for submitting your manuscript entitled "Optogenetic activators of apoptosis, necroptosis and pyroptosis". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that reviewers are enthusiastic about the toolset, which they think will be useful for the field of cell death. All of them recognize the experiments are thoroughly controlled and the data support the conclusions. They also acknowledge the study offers novel insights into the elimination of dead cells from epithelia. Nonetheless, reviewers #1 and #2 find more insights into the role of S1P signaling in the extrusion of apoptotic cells are needed, thus we hope you can address their points with new experimental data -in particular those raised by reviewer #1 (p3-5)- and extend the Discussion section accordingly and along the lines suggested by reviewer #2. We hope that you will be able to address each of reviewers' other points, though.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Toolss may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Toolss may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.
Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the

implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu.

Sincerely,

Anna Huttenlocher
Monitoring Editor
Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Cell death and elimination from the tissue are essential for homeostatic maintenance and repair, with misregulation of these key processes leading to pathogenesis. Here Shkarina et al. describes a new class of tools, optogenetically-controlled cell death effectors (optoCDEs), that enables light-mediated induction of three distinct types of programmed cell death - apoptosis, necroptosis and pyroptosis. The authors nicely show these tools are functional in multiple different model systems, such as mammalian cultured cells and developing zebrafish embryos. Importantly, the authors demonstrate these new tools can be used to control the degree of caspase activation to drive sub-lytic pyroptosis or ablation of single cells. Further, functional analysis with these tools show that cells are eliminated from the tissue in different manners depending if they are dying by apoptosis or programmed necrosis. Overall, this is an interesting study that introduces a novel toolset for studies of cell death and provides insight into mechanisms utilized to clear dying cells from the tissue. The authors' conclusions are largely supported by the data and the experiments are thoroughly controlled throughout. Below are suggestions for a few points of clarification and cases where the resulting conclusions require further justification.

Major Points:

On Pg10 (referring to Figure 4f), the authors state "a directed migration of the viable neighboring cells toward the lesion"- this is difficult to observe with the current images and level of resolution of provided. The use of a marker to label cell-cell junctions, such as CellMask used later in the manuscript, would add clarity to help support this claim.

Figure 8D and Supp. Figure 9E differ in how the extruding cell and partially extruding cells are defined, making it difficult for the reader to make direct comparisons with these data. It would be useful for the authors to use similar metrics or clarify why specific percentages were used in the analysis.

Given the key role that the authors have uncovered for S1P signaling in efferocytosis and necropoptotic extrusion, it seems important to provide evidence for the amount of S1P produced by the different forms of cell death using these new tools. This also brings up the question of whether the amount of S1P produced by either caspase 1, 8, or MLKL dictates the mode of clearance.

To further demonstrate specificity for this observation, it would be useful show if zVAD treatment after optogenetic activation of caspase 1 or 8 disrupts S1P production and/or the observed efferocytosis and necropoptotic extrusion.

Have the authors observed this role for S1P signaling in efferocytosis and necropoptotic extrusion in other cell lines (besides Caco2), and or in their new optoCDE-zebrafish model?

Minor Points:

In Fig 8b, providing an orthogonal view would be useful to the reader for distinguishing between extrusion events from those which apoptotic bodies are generated.

Page 7, the m or h designation should be used in front of optocaspase to distinguish between the mouse and human versions used

Likewise, on pgs 13-14, opto-zfCaspase8 should be consistently used throughout this section to denote the organismal version used.

References 32 and 65, and 59 and 61, respectively, refer the same publications.

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, the authors generate and describe a set of Cryptochrome-2-based optogenetic tools for light-mediated induction of different forms of cell death, including apoptosis, necroptosis and pyroptosis. The authors name these tools optoCDE (optogenetically-controlled cell death effectors). The tools are tested across different systems, including human, mouse cells and in vivo in Zebrafish. The use of optoCDE allows to authors to gain insight into how different types of cell death can change the mode and dynamics of cell elimination from epithelia. Finally, the authors use optoCDE to investigate the previously proposed role of shingosine-1-phosphate in modulating efferocytosis.

The work from Shkarina K and colleagues is sound and provides several novelties. It includes very convincing live imaging movies and a remarkable number of experiments across different experimental systems. The paper, albeit rather long and extremely data-dense, is overall easy to understand and flows logically. In a final form, it would require some additional controls and a deeper contextualization in the panorama of the published literature.

Major comments:

1. In Figure 1D (or in a supplementary figure), please show the frequency of cell death for cells that were imaged with the same settings and for the same time but not illuminated with the 488 laser (one can score cell death based on morphological features rather than CellTox incorporation). While the control presented by the authors (Cry2olig expressing controls) established the lack of toxicity of Cry2 alone and excludes photo-toxicity, it does not inform on the basal activity of their construct in the absence of illumination (or when illuminated with a non-activating wavelength). This additional control will show the ability of the tool to respond specifically to the correct wavelength (and eventually whether it has nonspecific activity). The authors do present the results of this type of control for a timespan of 10min (Figure 1F), but a longer stimulation (comparable to the one presented in Figure 1E) would be more appropriate
2. Supplementary Figure 3D: why is the illuminated HEK293T WT cell expressing optoCaspase-1 dying? Isn't GSDMD required for engagement of pyroptosis?
3. Can the authors comment on whether different levels of expression of (A) optoCaspase-1 or (B) GSDMD might be responsible for the difference in lytic/sublytic fate choices in their cell population? Unless they generate monoclonal cell lines, I would not expect them to be able to distinguish between fate choices linked to differential levels of expression versus differential activation of their optogenetic construct. Also, sublytic is not quite the right word. Maybe sublethal would be a better word.
4. While the effects of JTE-013 and SKI-II on apoptotic cell fragmentation and elimination are quite remarkable, non-specific effects of these inhibitors might confound result interpretation, as they might be due to off-target effects (especially at concentration as high as 30 micromolar). To further substantiate their finding, the authors could carry out a knock-down of SphK2 and/or S1PR2.
5. Finally, in the discussion the authors refer obliquely to an issue that must be discussed more fully. They acknowledge that their findings contradict many published papers that report extrusion of apoptotic cells and especially multiple papers from the Rosenblatt lab showing the role of S1P signaling in extrusion of apoptotic cells. The authors brush this aside with a cursory comment. At a minimum, a more thoughtful discussion is required. Even better, some understanding of the reason(s) for the differences would prevent the current work from creating confusion in the field.

Minor comments:
1. The authors should provide reference for the second part of their statement "Another more controlled approach is to force-oligomerize cell death executors using fusions with a chemically-dimerizable domain (DmrB/FKBP) (known as "clean" death system)9 but this is limited by poor reversibility and the inability to target and selectively kill individual cells in vitro or in vivo". What is the evidence for poor reversibility? In addition, according to the strategy used to deliver the dimerizable construct and the type of promoter employed, the system can likely be used to target and kill specific cells.
2. Figure 1: please include in the figure legend the definitions of all acronyms used
3. Do the HEK293T cells endogenously express caspase-1, -4 and -5?
4. The authors claim that CARD-deficient forms of Caspase-1 and -5 are more efficient at inducing cell death than the full-length variants. However, for Caspase-1 this is not a dramatic difference, and I would suggest rephrasing.

5. Can the authors show/specify what is the status of VC3AI in GSDMDtg HEK293T cells (related to Supplementary Figure 2E)?
6. Please specify the GSDMD status and caspase-1, -4, -5 status for each of the cell lines used in Figure 1I (transgenic expression or endogenous expression). Also specify endogenous caspase-8 and -9 levels for the cell lines used in Figure 5I
7. Can the authors discuss more about the necessity of using a doxycycline-inducible promoter to prevent cell death of U937 cells during differentiation? If the constructs are indeed inactive in the dark, this should not be necessary.
8. Please provide appropriate references when introducing and describing well established mechanisms of cell death
9. As currently shown, none of the data in Figure 5D-F and Supplementary Figure 6B support the activation of the genetically encoded caspase-3/-7 activity reporter before the acquisition of Annexin V staining. Please modify the figures or the text accordingly.
10. As reported in the discussion and the reference list, previous works focused on the generation of tools for light-inducible apoptosis. I do believe that especially the work of Smart and colleagues should be acknowledged and contextualized earlier in the manuscript, not only in the discussion. Mentioning this work will allow the reader to better understand what prompted the authors to develop different tools, and how their tool provides an advantage
11. Please explain the rationale behind using RHIM deficient RIP3
12. Please define "PMR"
13. Please verify the figures mentioned in "Optogenetic activation of zebrafish caspases allows spatial and temporal controlled induction of pyroptosis and apoptosis in vivo". There are incongruencies between the text and what shown
14. The authors say: "While the effect of opto-caspase induction in skin cells is visible within minutes the effect of their activation in muscle cells only becomes apparent after several hours". However, signs of apoptosis do not appear until ~40' after illumination. Please rephrase.
15. Please review Figure 7H, the insets do not look accurate (there is a confounding gray signal)
16. While Figure 9F shows the absence of fragmentation of apoptotic cells without neighbors, it would be advisable to include in the same panel a picture in which apoptotic bodies formation is clearly visible
17. Please review the sentence "(Fig. 8F-H and Supplementary Fig. B)" and the sentence "(Fig. 7E-G and Fig. 8I-LK)"
18. While indirect evidence points toward the reversibility of optoCDE (recovery and survival of cells after delivery of limited amount of illumination), it is unclear what are the actual kinetics and mode of inactivation. Is inactivation caused by feedback mechanisms, or is it linked to the conversion of optoCDE into an inactive conformation? Is survival actually linked to inactivation, or is it due to the inability of reaching a threshold of activity required to complete commitment to cell death? I believe this should be discussed. I would also suggest caution when claiming "optoCDE not only excel in their rapid kinetics of activation and reversibility (inactivate within minutes of ceasing illumination)..."
19. The term anastasis first appeared in PMID 22535522. It might be more appropriate to use this reference rather than PMID 30839720
20. In the method, please specify the concentration of Annexin V used (1 microgram/?)

Reviewer #3 (Comments to the Authors (Required)):

This study by Shkarina and colleagues describes the development and application of a suite of optogenetic approaches (centred on caspase or RIPK3/MLKL) to induce different forms of regulated cell death (apoptosis, necroptosis, pyroptosis) at the single cell level. The authors extensively validate their approach in pyroptosis before progressing to apoptosis and necroptosis. Beyond 2D analysis, they demonstrate effectiveness in 3D culture and in vivo (in zebrafish) Finally, they demonstrate differences in removal/expulsion of dying cells from an epithelial monolayer dependent on cell death modality, finding that necroptotic and pyroptotic cells are extruded, whereas apoptotic cells disintegrate and are absorbed by neighbouring cells.

In my opinion, the toolset (and generalisable optogenetic approach) the authors have developed is novel, powerful and as they demonstrate towards the end of the study will undoubtedly yield new biological insights. Their findings/methodology clearly surpasses any optogenetic approaches that have been developed to trigger cell death in the past, and one of the attractive points of this toolset is the apparently facile comparison of different cell death modalities using a similar experimental setup. Data fully support the authors' conclusions - I have a few minor comments.

- reference to optocaspase activity to trigger pyroptosis in commonly used cell lines (bottom page 2) are these cell lines listed here all pyroptosis (GSDMD) proficient ?

- with respect to the resistance of some cell lines to optoC8 or C9 (page 11), the authors speculate that this may be due to additional inhibitory mechanisms (such as XIAP upregulation, as their SMAC-mimetic expt. shows in some cell lines), really interesting that their are additional potential regulatory mechanisms, but could another reason be that optoC8 or C9 don't work as well in some cell types for trivial reasons (e.g misfolding) ? - I appreciate this is difficult to experimentally demonstrate, but a line stating this possible caveat would suffice.

- RIP3, should be changed to RIPK3

Response to reviewer comments

Note to all reviewers

We thank all reviewers for the detailed critique and their valuable suggestions, which we believe allowed us to improve the manuscript and make it more accessible to a broad audience. In this response letter, all original comments are quoted in black; our responses are shown in **blue**.

Reviewer #1 (Comments to the Authors (Required)):

Cell death and elimination from the tissue are essential for homeostatic maintenance and repair, with misregulation of these key processes leading to pathogenesis. Here Shkarina et al. describes a new class of tools, optogenetically-controlled cell death effectors (optoCDEs), that enables light-mediated induction of three distinct types of programmed cell death - apoptosis, necroptosis and pyroptosis. The authors nicely show these tools are functional in multiple different model systems, such as mammalian cultured cells and developing zebrafish embryos. Importantly, the authors demonstrate these new tools can be used to control the degree of caspase activation to drive sub-lytic pyroptosis or ablation of single cells. Further, functional analysis with these tools show that cells are eliminated from the tissue in different manners depending if they are dying by apoptosis or programmed necrosis. Overall, this is an interesting study that introduces a novel toolset for studies of cell death and provides insight into mechanisms utilized to clear dying cells from the tissue. The authors' conclusions are largely supported by the data and the experiments are thoroughly controlled throughout. Below are suggestions for a few points of clarification and cases where the resulting conclusions require further justification.

We thank the review for endorsing the novelty of our toolset and highlighting the broad functional characterization of the toolset in different model systems. We address all suggestions point-by-point below:

Major Points:

On Pg10 (referring to Figure 4f), the authors state "a directed migration of the viable neighboring cells toward the lesion"- this is difficult to observe with the current images and level of resolution of provided. The use of a marker to label cell-cell junctions, such as CellMask used later in the manuscript, would add clarity to help support this claim.

To allow the readers to better to discern the viable cells, we have added the images at higher resolution as shown below as Fig. 4F of the revised manuscript.

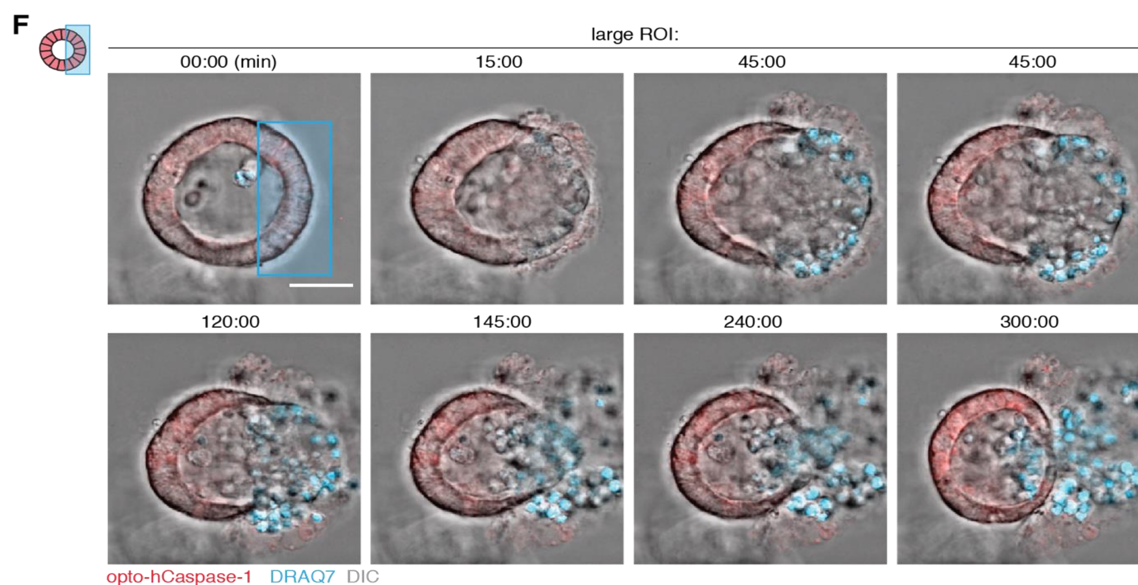


Figure 4F of the revised manuscript.

Figure 8D and Supp. Figure 9E differ in how the extruding cell and partially extruding cells are defined, making it difficult for the reader to make direct comparisons with these data. It would be useful for the authors to use similar metrics or clarify why specific percentages were used in the analysis.

The fully extruded, partially extruded and retained cells in Fig. 8D and Fig S9E, were quantified using the same criteria. However as pointed out by the reviewer, the colors (blue/orange) were switched between the figures. This has been corrected in the revised version of the manuscript (of note, S9E is now S4J).

Given the key role that the authors have uncovered for S1P signaling in efferocytosis and necropoptotic extrusion, it seems important to provide evidence for the amount of S1P produced by the different forms of cell death using these new tools. This also brings up the question of whether the amount of S1P produced by either caspase 1, 8, or MLKL dictates the mode of clearance.

The reviewer raises in important point. We have used an S1P ELISA to measure the amount of S1P that is released by the different forms of cell death.

Interestingly pyroptosis results in the highest level of S1P release, while necroptosis releases less S1P and apoptosis very little S1P at all. Since S1P signaling does not play a role in the extrusion of pyroptotic cells, even though pyroptosis releases more S1P than other forms of death, we conclude that the level of S1P production alone cannot be the deciding factor whether a cell is extruded or efferocytosed.
The S1P measurement has been included as Fig. 10D in the revised manuscript.

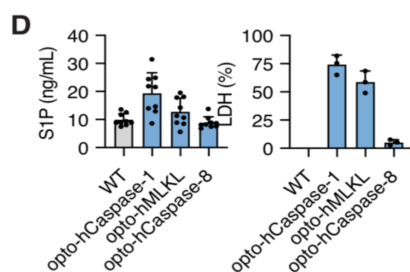


Fig. 10D of the revised manuscript

To further demonstrate specificity for this observation, it would be useful show if zVAD treatment after optogenetic activation of caspase 1 or 8 disrupts S1P production and/or the observed efferocytosis and necropoptotic extrusion.

The referee probably refers to the possibility that our optoCDE constructs would induce other signaling pathways that could drive S1P release independently of induction of cell death (scaffolding function of caspase-1-, 8 or RIPK3, MLKL). We are confident that this is not the case, as our optoCDE lack the respective domains (CARD, DED, RHIM etc..). Furthermore, we show in figures 1, 5 and 6 that mutating catalytic residues or chemical inhibition blocks the cell death induced by our optoCDE constructs. As a result the optoCDE expressing cells remain viable, proliferate and do not get extruded by their neighbors.

We have tried to perform the S1P ELISA as suggested, but unfortunately the presence of the inhibitors or the vehicle interfered with the assay (data not shown).

Have the authors observed this role for S1P signaling in efferocytosis and necropoptotic extrusion in other cell lines (besides Caco2), and or in their new optoCDE-zebrafish model?

In order to answer the referee's question, we have examined a role for S1P signaling in MCF10A and MDCK cell, as previous work by the Rosenblatt group and by Gagliardi et al. has shown that apoptotic MDCK or MCF10A cells are extruded (PMID: 21555463, PMID: 29162624).

Interestingly and in agreement with the published results, we found that both apoptotic and necrotic cell are extruded from MCF10A and MDCK monolayers, which is in contrast to our in Caco-2 cells and zebrafish keratinocytes.

We have also tested if inhibition of S1P signaling has an impact on the extrusion of apoptotic and necrotic MCF10A cells, and found that inhibition indeed slows down extrusion. We have included the new data in the revised manuscript as Fig. 10I and J (see below). The same data were obtained for MDCK cells but were not included due to space limitations (data not shown).

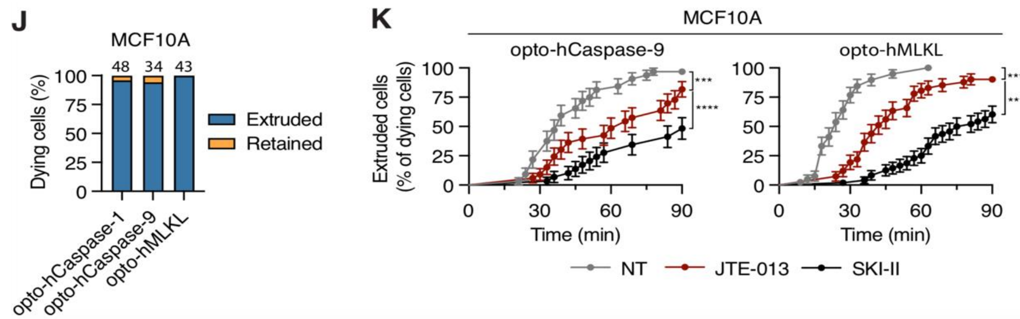


Fig. 10 J-K of the revised manuscript.

Minor Points:

In Fig 8b, providing an orthogonal view would be useful to the reader for distinguishing between extrusion events from those which apoptotic bodies are generated.

An 3D view and an orthogonal view of apoptotic Caco-2 cells is already provided in Fig. 8C and also in Fig. 9. Adding it to Fig. 8B would be partially redundant, and is not yet important at this point in the text as we discuss the actin cytoskeleton later.

Page 7, the m or h designation should be used in front of optocaspase to distinguish between the mouse and human versions used

The reviewer has raised an important point. To make the manuscript more accessible, we have decided on a common nomenclature for all constructs, using h, m and zf designations, such as:
-hOpto-Casp9
-mOpto-Casp9
-zfOpto-Casp-9

Likewise, on pgs 13-14, opto-zfCaspase8 should be consistently used throughout this section to denote the organismal version used.

This has been corrected accordingly

References 32 and 65, and 59 and 61, respectively, refer the same publications.

The references have been corrected

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, the authors generate and describe a set of Cryptochrome-2-based optogenetic tools for light-mediated induction of different forms of cell death, including apoptosis, necroptosis and pyroptosis. The authors name these tools optoCDE (optogenetically-controlled cell death effectors). The tools are tested across different systems, including human, mouse cells and in vivo in Zebrafish. The use of optoCDE allows to authors to gain insight into how different types of cell death can change the mode and dynamics of cell elimination from epithelia. Finally, the authors use optoCDE to investigate the previously proposed role of shingosine-1-phosphate in modulating efferocytosis.

The work from Shkarina K and colleagues is sound and provides several novelties. It includes very convincing live imaging movies and a remarkable number of experiments across different experimental systems. The paper, albeit rather long and extremely data-dense, is overall easy to understand and flows logically. In a final form, it would require some additional controls and a deeper contextualization in the panorama of the published literature.

We thank the reviewer for acknowledging the novelty of our toolset and broad validation of the tools in different model systems. We have added additional controls and improved to text as suggested. For detailed responses, see below:

Major comments:

1. In Figure 1D (or in a supplementary figure), please show the frequency of cell death for cells that were imaged with the same settings and for the same time but not illuminated with the 488 laser (one can score cell death based on morphological features rather than CellTox incorporation). While the control presented by the authors (Cry2olig expressing controls) established the lack of toxicity of Cry2 alone and excludes photo-toxicity, it does not inform on the basal activity of their construct in the absence of illumination (or when illuminated with a non-activating wavelength). This additional control will show the ability of the tool to respond specifically to the correct wavelength (and eventually whether it has nonspecific activity). The authors do present the results of this type of control for a timespan of 10min (Figure 1F), but a longer stimulation (comparable to the one presented in Figure 1E) would be more appropriate

The reviewer raises an important point, as basal activity of the constructs upon initiation of overexpression could be an issue. To address the comment, we have performed the experiment as suggested and tracked the viability of non-illuminated optoCasp-1/-4 and -5 expressing HEK293T cells over the same timeframe. The new data have been included in Fig 1D of the revised manuscript and show negligible toxicity of optoCasp-1/-4 and -5 constructs in non-illuminated cells.

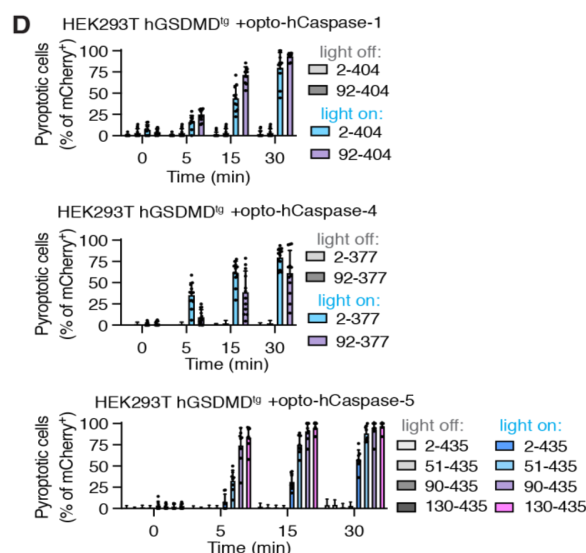


Fig. 1D of the revised manuscript.

3. Can the authors comment on whether different levels of expression of (A) optoCaspase-1 or (B) GSDMD might be responsible for the difference in lytic/sublytic fate choices in their cell population? Unless they generate

monoclonal cell lines, I would not expect them to be able to distinguish between fate choices linked to differential levels of expression versus differential activation of their optogenetic construct. Also, sublytic is not quite the right word. Maybe sublethal would be a better word.

We can only speculate to as what could be the determinant of the lethal/sub-lethal fates of individual cells. In response to this comment, we have re-analyzed our data and found that indeed cell with high optoCaspase-1 expression levels are more likely to die after the first illumination dose. The data are now included as Fig. 3I-J of the revised manuscript.

Other factors that could contribute is also the expression levels of endogenous GSDMD or cell death regulators, and it will be interesting to use to optoCDE system for future studies aimed at understanding how cells survive cell death induction.

We agree with the reviewers comment that sublytic is not necessarily the right word, and thus use 'sublethal' instead in the revised manuscript.

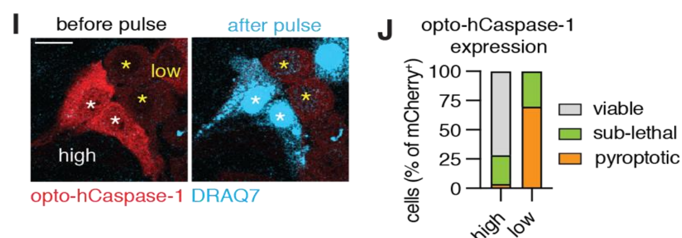


Fig. 3I-J of the revised manuscript.

4. While the effects of JTE-013 and SKI-II on apoptotic cell fragmentation and elimination are quite remarkable, non-specific effects of these inhibitors might confound result interpretation, as they might be due to off-target effects (especially at concentration as high as 30 micromolar). To further substantiate their finding, the authors could carry out a knock-down of SphK2 and/or S1PR2.

As suggested, we have knocked-down S1PR2 in 'receiver' cells and quantified apoptotic and necroptotic cell extrusion as before. The knock-down was able to recapitulate the inhibitor data, suggesting that the inhibitors specifically blocked S1P signaling. We included these data as Fig. 10H-I of the revised manuscript.

Furthermore, we have also analysed efferocytosis and extrusion of MCF10A and MDCK cells, and were able to recapitulate published data (PMID: 21555463) using JTE013 and SKI-II. (see Fig. 10J-K) of the revised manuscript). This further confirms that the inhibitors are specific.

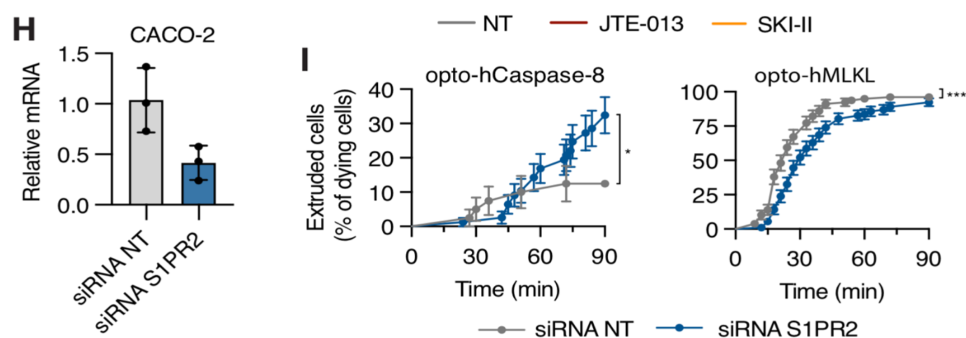


Fig. 10H-I of the revised manuscript.

5. Finally, in the discussion the authors refer obliquely to an issue that must be discussed more fully. They acknowledge that their findings contradict many published papers that report extrusion of apoptotic cells and especially multiple papers from the Rosenblatt lab showing the role of S1P signaling in extrusion of apoptotic cells. The authors brush this aside with a cursory comment. At a minimum, a more thoughtful discussion is

required. Even better, some understanding of the reason(s) for the differences would prevent the current work from creating confusion in the field.

We addressed the discrepancy between ours and published reports (such as from the Rosenblatt lab) by generating MCF10A and MDCK cell lines expressing optoCDEs and testing these for cell efferocytosis and extrusion (these are cell lines used in previous studies). Our findings show that apoptotic MCF10A and MDCK cells are not efferocytosed but extruded, in line with previous work. We thus conclude that the decision to efferocytose or extruded apoptotic cells is highly cell line specific. Nevertheless, S1P signaling plays an important role in both processes. As these results resolve the discrepancy and further highlight the versatility of the optoCDE approach, we have included them in the revised manuscript as Fig. 10J-K.

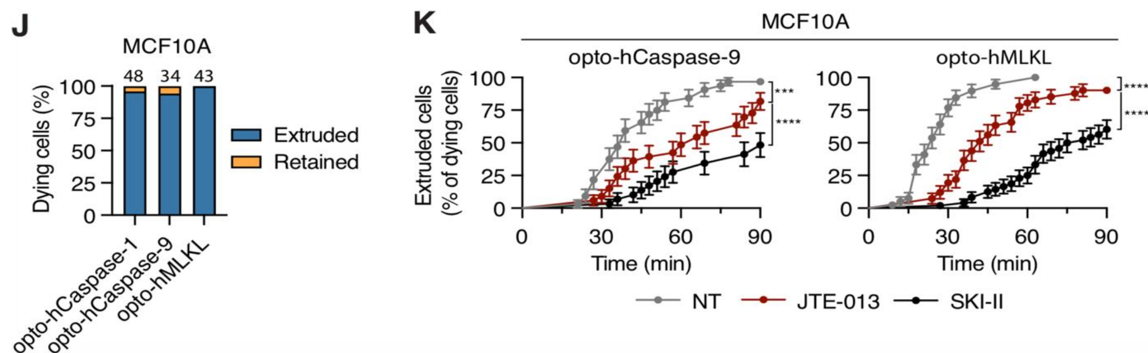


Fig. 10J-K of the revised manuscript.

Minor comments:

1. The authors should provide reference for the second part of their statement "Another more controlled approach is to force-oligomerize cell death executors using fusions with a chemically-dimerizable domain (DmrB/FKBP) (known as "clean" death system)9 but this is limited by poor reversibility and the inability to target and selectively kill individual cells in vitro or in vivo". What is the evidence for poor reversibility? In addition, according to the strategy used to deliver the dimerizable construct and the type of promoter employed, the system can likely be used to target and kill specific cells.

Homodimerization of DmrB domains can be reverted by using excess concentrations (1uM) of a washout ligand, and according to the manufacturer (Clontech) dissociation can be observed after ~30 min, which is slower than the dissociation of Cry2.
It is correct that chemical dimerization can be used to kill individual cells depending on the strategy used to deliver the constructs, but it does not allow to specifically kill a certain cell of choice like optogenetic can. This our emphasis on 'selectively kill individual cells'

To correctly describe the chemical-dimerization approach, we have rephrased this section in the revised manuscript.

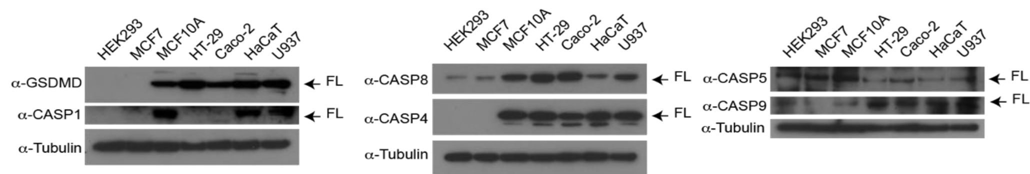
2. Figure 1: please include in the figure legend the definitions of all acronyms used

This has been corrected.

3. Do the HEK293T cells endogenously express caspase-1, -4 and -5?

HEK293T cell do not express inflammatory caspases (e.g. caspase-1, -4 and -5), ASC or the commonly studied inflammasome-forming receptors (such as NLRP3, AIM2, etc..). Due to this lack of endogenous components, they are thus often used as a model system for inflammasome reconstitution by overexpression (see for example PMID: 27221493).

In response to the comment, we immunoblotted for caspases and GSDMD in different cell lines used in our study, and provide the blots for the referee's convenience below.



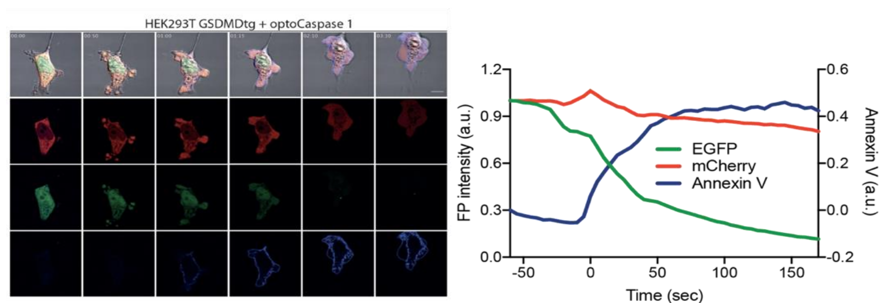
Rebuttal Figure 1: expression levels of Caspases and GSDMD in different cell lines.

4. The authors claim that CARD-deficient forms of Caspase-1 and -5 are more efficient at inducing cell death than the full-length variants. However, for Caspase-1 this is not a dramatic difference, and I would suggest rephrasing.

We have rephrased this statement.

5. Can the authors show/specify what is the status of VC3AI in GSDMDtg HEK293T cells (related to Supplementary Figure 2E)?

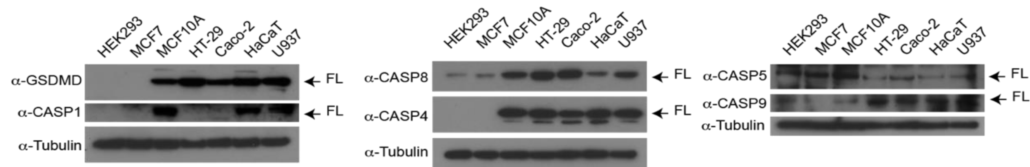
This would be an interesting experiment to be done, as previous work has shown that caspase-7 and caspase-3 is activated in pyroptotic macrophages (PMID: 32726624). Unfortunately, the experiment cannot be done with the VC3AI reporter in the GSDMDtg cells, since activation of GSDMD results in a loss of soluble cytosolic proteins, as shown below for GFP (Fig. R3), and thus we would lose the VC3AI reporter as soon as the cells lyse – making it impossible to measure its activation.



Rebuttal Figure 2: Loss of exogenously expressed GFP from HEK293T cells during pyroptosis. GSDMDtg HEK293T cells expressing optoCasp-1 were illuminated at t=0, and eGFP and Annexin-V signals recorded over time.

6. Please specify the GSDMD status and caspase-1, -4, -5 status for each of the cell lines used in Figure 1I (transgenic expression or endogenous expression). Also specify endogenous caspase-8 and -9 levels for the cell lines used in Figure 5I

In response to the comment, we immunoblotted for caspases and GSDMD in different cell lines used in our study, and provide the blots for the referee's convenience below.


HEK293
MCF7
MCF10A
HT-29
Caco-2
HaCaT
U937
α-GSDMD
α-CASP1
α-Tubulin
FL
FL
α-CASP8
α-CASP4
α-Tubulin
FL
FL
α-CASP5
α-CASP9
α-Tubulin
FL
FL

Rebuttal Figure 1: expression levels of Caspases and GSDMD in different cell lines.

7. Can the authors discuss more about the necessity of using a doxycycline-inducible promoter to prevent cell

death of U937 cells during differentiation? If the constructs are indeed inactive in the dark, this should not be necessary.

Apologies, we have not properly described what we mean here. We used a doxycycline-inducible promoter exactly for the reason that it allowed to culture the cells under the normal illumination conditions, and also to avoid toxicity by prolonged expression of the optoCDE constructs. We had observed that long constitutive expression (days) of certain constructs resulted in toxicity even without illumination.

We have also corrected the text in the revised manuscript to make that clearer.

8. Please provide appropriate references when introducing and describing well established mechanisms of cell death

We have added new references to the introduction.

9. As currently shown, none of the data in Figure 5D-F and Supplementary Figure 6B support the activation of the genetically encoded caspase-3/-7 activity reporter before the acquisition of Annexin V staining. Please modify the figures or the text accordingly.

This is correct. We have re-phrased the text accordingly.

10. As reported in the discussion and the reference list, previous works focused on the generation of tools for light-inducible apoptosis. I do believe that especially the work of Smart and colleagues should be acknowledged and contextualized earlier in the manuscript, not only in the discussion. Mentioning this work will allow the reader to better understand what prompted the authors to develop different tools, and how their tool provides an advantage

We discuss the different approaches for optogenetic activation of cell death, among the Smart et al, in the introduction.

11. Please explain the rationale behind using RHIM deficient RIP3

As shown in Fig. 6C, the RHIM-deficient construct 'opto-hRIPK3 RHIM NT' is more efficient as other RipK3 constructs.

12. Please define "PMR"

We have now defined MPR as plasma membrane rupture in the revised manuscript.

13. Please verify the figures mentioned in "Optogenetic activation of zebrafish caspases allows spatial and temporal controlled induction of pyroptosis and apoptosis in vivo". There are incongruencies between the text and what shown

The incongruencies have been corrected.

14. The authors say: "While the effect of opto-caspase induction in skin cells is visible within minutes the effect of their activation in muscle cells only becomes apparent after several hours". However, signs of apoptosis do not appear until ~40' after illumination. Please rephrase.

The text has been modified accordingly.

15. Please review Figure 7H, the insets do not look accurate (there is a confounding gray signal)

We have corrected this figure. The boxes outline the illuminated regions.
The grey was meant to show asc-gfp in these boxes. This has now been changes to green to make that clearer.

16. While Figure 9F shows the absence of fragmentation of apoptotic cells without neighbors, it would be advisable to include in the same panel a picture in which apoptotic bodies formation is clearly visible

We believe that the reviewer is referring to Supplementary Figure 9F, where we show the absence of apoptotic body formation in apoptotic Caco-2 cells. Indeed, it is difficult to judge whether apoptotic bodies are present or not from these images. We have thus included close-ups of these same cells, and in addition provide images of cells forming apoptotic bodies for comparison in Fig. S5B of the revised manuscript.

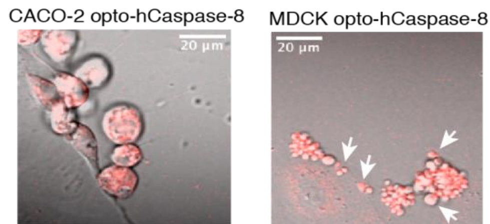


Fig. S5B of the revised manuscript.

17. Please review the sentence "(Fig. 8F-H and Supplementary Fig. B)" and the sentence "(Fig. 7E-G and Fig. 8I-LK)"

This has been corrected.

18. While indirect evidence points toward the reversibility of optoCDE (recovery and survival of cells after delivery of limited amount of illumination), it is unclear what are the actual kinetics and mode of inactivation. Is inactivation caused by feedback mechanisms, or is it linked to the conversion of optoCDE into an inactive conformation? Is survival actually linked to inactivation, or is it due to the inability of reaching a threshold of activity required to complete commitment to cell death? I believe this should be discussed. I would also suggest caution when claiming "optoCDE not only excel in their rapid kinetics of activation and reversibility (inactivate within minutes of ceasing illumination)..."

We have changed the text, as indeed we have not properly evaluated the reversibility of the toolset.

19. The term anastasis first appeared in PMID 22535522. It might be more appropriate to use this reference rather than PMID 30839720
We have added the new reference

20. In the method, please specify the concentration of Annexin V used (1 microgram/?)
It is 1ug/ml. We have corrected the material and methods section accordingly.

Reviewer #3 (Comments to the Authors (Required)):

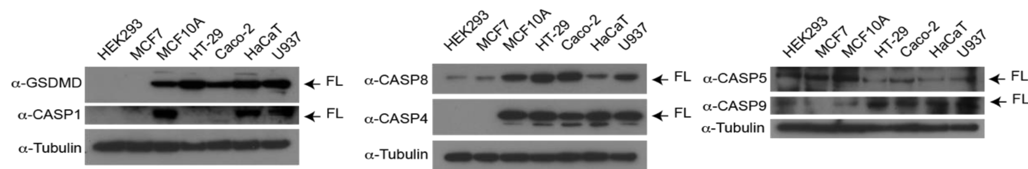
This study by Shkarina and colleagues describes the development and application of a suite of optogenetic approaches (centred on caspase or RIPK3/MLKL) to induce different forms of regulated cell death (apoptosis, necroptosis, pyroptosis) at the single cell level. The authors extensively validate their approach in pyroptosis before progressing to apoptosis and necroptosis. Beyond 2D analysis, they demonstrate effectiveness in 3D culture and in vivo (in zebrafish) Finally, they demonstrate differences in removal/expulsion of dying cells from an epithelial monolayer dependent on cell death modality, finding that necroptotic and pyroptotic cells are extruded, whereas apoptotic cells disintegrate and are absorbed by neighbouring cells.

In my opinion, the toolset (and generalisable optogenetic approach) the authors have developed is novel, powerful and as they demonstrate towards the end of the study will undoubtedly yield new biological insights. Their findings/methodology clearly surpasses any optogenetic approaches that have been developed to trigger cell death in the past, and one of the attractive points of this toolset is the apparently facile comparison of different cell death modalities using a similar experimental setup. Data fully support the authors' conclusions - I have a few minor comments.

We thank the reviewer for the positive feedback on our manuscript and for highlighting the novelty of the toolset.

- reference to optocaspase activity to trigger pyroptosis in commonly used cell lines (bottom page 2) are these cell lines listed here all pyroptosis (GSDMD) proficient ?

In response to referee #1 and #2's request we have analyzed the expression levels of caspases and GSDMD in the different cell lines. All except MCF7 and HEK293 express GSDMD and thus are theoretically pyroptosis proficient. Pyroptosis induction was achieved by overexpressing hGSDMD in these lines.



Rebuttal Figure 1: expression levels of Caspases and GSDMD in different cell lines.

- with respect to the resistance of some cell lines to optoC8 or C9 (page 11), the authors speculate that this may be due to additional inhibitory mechanisms (such as XIAP upregulation, as their SMAC-mimetic expt. shows in some cell lines), really interesting that their are additional potential regulatory mechanisms, but could another reason be that optoC8 or C9 don't work as well in some cell types for trivial reasons (e.g misfolding) ? - I appreciate this is difficult to experimentally demonstrate, but a line stating this possible caveat would suffice.

We cannot completely exclude that misfolding of the construct could be a reason. However, since misfolded proteins are usually degraded and we can observe strong expression and functionality of the constructs (based on the presence of the mCherry signal and its clustering upon illumination), we believe that this is probably not the reason for the failure to induce apoptosis in these lines. We have nevertheless, rephrased the text as it cannot be 100% excluded.

- RIP3, should be changed to RIPK3

We have changed the text accordingly.

March 2, 2022

RE: JCB Manuscript #202109038R

Prof. Petr Broz
University de Lausanne
Biochemistry
Chemin du Polny 25
Epalinges, Vaud 1066
Switzerland

Dear Prof. Broz:

Thank you for submitting your revised manuscript entitled "Optogenetic activators of apoptosis, necroptosis and pyroptosis". We have now assessed your revised manuscript and we would be happy to publish your paper in JCB pending revisions to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised.
Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figures limits: Articles and Tools may have up to 10 main text figures.

*** Please provide main figures as individual files.

3) Figure formatting:
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

*** Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to main Fig 3E (inset magnifications), 4A (inset magnifications), 4E (inset magnifications), 8C, 8I, 9B, 9D, 9E, and supplemental Fig 1B (inset magnification), 1F, 2H, 5H.

*** Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. Please ensure that the particular red and green hues used in main Fig 1E, 2A, 5D-E, 6D-E, 6J, 7C, 7E-H, 8B-C, 8I and supplemental Fig 1C, 1E-H, 2D, 2F-G, 3C, 3H, 4B, 4E, 4G are distinctive with any of the colorblind types. If not, please modify colors accordingly or present separate micrographs for each channel.

4) Statistical analysis:
*** Error bars on graphic representations of numerical data must be clearly described in the figure legend.

*** The number of independent data points (n) represented in a graph must be indicated in the legend. Please indicate whether n refers to technical replicates or independent samples.

Statistical methods should be explained in full in the materials and methods.

For figures presenting pooled data the statistical measure should be defined in the figure legends.

Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.).

*** As you used parametric tests in your study (i.e. t-tests), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described." Also, the materials and methods should be included with the main manuscript text and not in the supplementary materials.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods.

You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

8) Microscope image acquisition:
The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials:
There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. There is no limit for supplemental tables.

*** Please note that supplemental figures and tables should be provided as individual, editable files.

*** A summary of all supplemental material should appear at the end of the Materials and Methods section. We would need that you include more details in this section - please see any recent JCB paper for an example of this summary.

11) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

*** It should begin with "First author name(s) et al..." to match our preferred style.

12) Conflict of interest statement:
*** JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) A separate author contribution section is required following the Acknowledgments in all research manuscripts.

*** All authors should be mentioned and designated by their first and middle initials and full surnames and the CRediT nomenclature should be used (https://casrai.org/credit/).

14) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

15) Materials and data sharing: As a condition of publication, authors must make protocols and unique materials (including, but not limited to, cloned DNAs; antibodies; bacterial, animal, or plant cells; and viruses) described in our published articles freely available upon request by researchers, who may use them in their own laboratory only. All materials must be made available on request and without undue delay.

All datasets included in the manuscript must be available from the date of online publication, and the source code for all custom computational methods, apart from commercial software programs, must be made available either in a publicly available database or as supplemental materials hosted on the journal website. Numerous resources exist for data storage and sharing (see Data Deposition: https://rupress.org/jcb/pages/data-deposition), and you should choose the most appropriate venue based on your data type and/or community standard. If no appropriate specific database exists, please deposit your data to an appropriate publicly available database.

16) Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

*** Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.

*** Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Additionally, JCB encourages authors to submit a short video summary of their work. These videos are intended to convey the main messages of the study to a non-specialist, scientific audience. Think of them as an extended version of your abstract, or a short poster presentation. We encourage first authors to present the results to increase their visibility. The videos will be shared on social media to promote your work. For more detailed guidelines and tips on preparing your video, please visit https://rupress.org/jcb/pages/submission-guidelines#videoSummaries.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Anna Huttenlocher
Monitoring Editor
Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

Response to reviewer comments

Note to all reviewers

We thank all reviewers for the detailed critique and their valuable suggestions, which we believe allowed us to improve the manuscript and make it more accessible to a broad audience. In this response letter, all original comments are quoted in black; our responses are shown in **blue**.

Reviewer #1 (Comments to the Authors (Required)):

Cell death and elimination from the tissue are essential for homeostatic maintenance and repair, with misregulation of these key processes leading to pathogenesis. Here Shkarina et al. describes a new class of tools, optogenetically-controlled cell death effectors (optoCDEs), that enables light-mediated induction of three distinct types of programmed cell death - apoptosis, necroptosis and pyroptosis. The authors nicely show these tools are functional in multiple different model systems, such as mammalian cultured cells and developing zebrafish embryos. Importantly, the authors demonstrate these new tools can be used to control the degree of caspase activation to drive sub-lytic pyroptosis or ablation of single cells. Further, functional analysis with these tools show that cells are eliminated from the tissue in different manners depending if they are dying by apoptosis or programmed necrosis. Overall, this is an interesting study that introduces a novel toolset for studies of cell death and provides insight into mechanisms utilized to clear dying cells from the tissue. The authors' conclusions are largely supported by the data and the experiments are thoroughly controlled throughout. Below are suggestions for a few points of clarification and cases where the resulting conclusions require further justification.

We thank the review for endorsing the novelty of our toolset and highlighting the broad functional characterization of the toolset in different model systems. We address all suggestions point-by-point below:

Major Points:

On Pg10 (referring to Figure 4f), the authors state "a directed migration of the viable neighboring cells toward the lesion"- this is difficult to observe with the current images and level of resolution of provided. The use of a marker to label cell-cell junctions, such as CellMask used later in the manuscript, would add clarity to help support this claim.

To allow the readers to better to discern the viable cells, we have added the images at higher resolution as shown below as Fig. 4F of the revised manuscript.

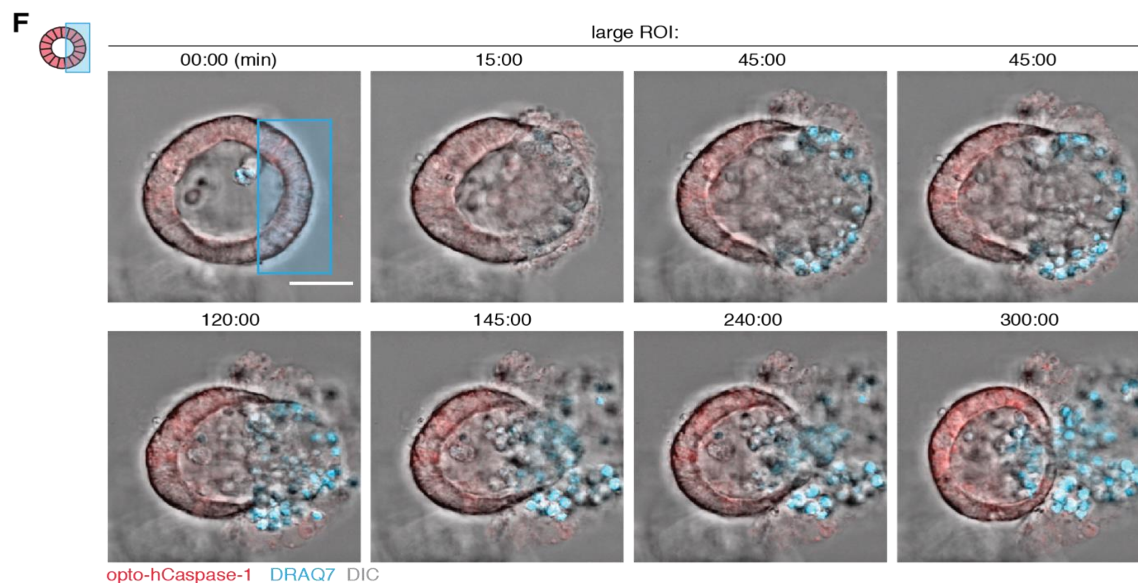


Figure 4F of the revised manuscript.

Figure 8D and Supp. Figure 9E differ in how the extruding cell and partially extruding cells are defined, making it difficult for the reader to make direct comparisons with these data. It would be useful for the authors to use similar metrics or clarify why specific percentages were used in the analysis.

The fully extruded, partially extruded and retained cells in Fig. 8D and Fig S9E, were quantified using the same criteria. However as pointed out by the reviewer, the colors (blue/orange) were switched between the figures. This has been corrected in the revised version of the manuscript (of note, S9E is now S4J).

Given the key role that the authors have uncovered for S1P signaling in efferocytosis and necropoptotic extrusion, it seems important to provide evidence for the amount of S1P produced by the different forms of cell death using these new tools. This also brings up the question of whether the amount of S1P produced by either caspase 1, 8, or MLKL dictates the mode of clearance.

The reviewer raises in important point. We have used an S1P ELISA to measure the amount of S1P that is released by the different forms of cell death.

Interestingly pyroptosis results in the highest level of S1P release, while necroptosis releases less S1P and apoptosis very little S1P at all. Since S1P signaling does not play a role in the extrusion of pyroptotic cells, even though pyroptosis releases more S1P than other forms of death, we conclude that the level of S1P production alone cannot be the deciding factor whether a cell is extruded or efferocytosed.
The S1P measurement has been included as Fig. 10D in the revised manuscript.

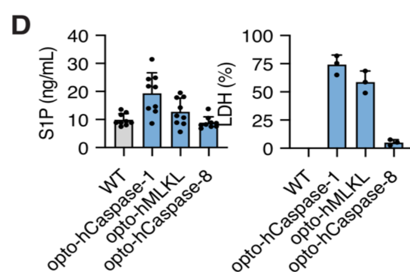


Fig. 10D of the revised manuscript

To further demonstrate specificity for this observation, it would be useful show if zVAD treatment after optogenetic activation of caspase 1 or 8 disrupts S1P production and/or the observed efferocytosis and necropoptotic extrusion.

The referee probably refers to the possibility that our optoCDE constructs would induce other signaling pathways that could drive S1P release independently of induction of cell death (scaffolding function of caspase-1-, 8 or RIPK3, MLKL). We are confident that this is not the case, as our optoCDE lack the respective domains (CARD, DED, RHIM etc..). Furthermore, we show in figures 1, 5 and 6 that mutating catalytic residues or chemical inhibition blocks the cell death induced by our optoCDE constructs. As a result the optoCDE expressing cells remain viable, proliferate and do not get extruded by their neighbors.

We have tried to perform the S1P ELISA as suggested, but unfortunately the presence of the inhibitors or the vehicle interfered with the assay (data not shown).

Have the authors observed this role for S1P signaling in efferocytosis and necropoptotic extrusion in other cell lines (besides Caco2), and or in their new optoCDE-zebrafish model?

In order to answer the referee's question, we have examined a role for S1P signaling in MCF10A and MDCK cell, as previous work by the Rosenblatt group and by Gagliardi et al. has shown that apoptotic MDCK or MCF10A cells are extruded (PMID: 21555463, PMID: 29162624).

Interestingly and in agreement with the published results, we found that both apoptotic and necrotic cell are extruded from MCF10A and MDCK monolayers, which is in contrast to our in Caco-2 cells and zebrafish keratinocytes.

We have also tested if inhibition of S1P signaling has an impact on the extrusion of apoptotic and necrotic MCF10A cells, and found that inhibition indeed slows down extrusion. We have included the new data in the revised manuscript as Fig. 10I and J (see below). The same data were obtained for MDCK cells but were not included due to space limitations (data not shown).

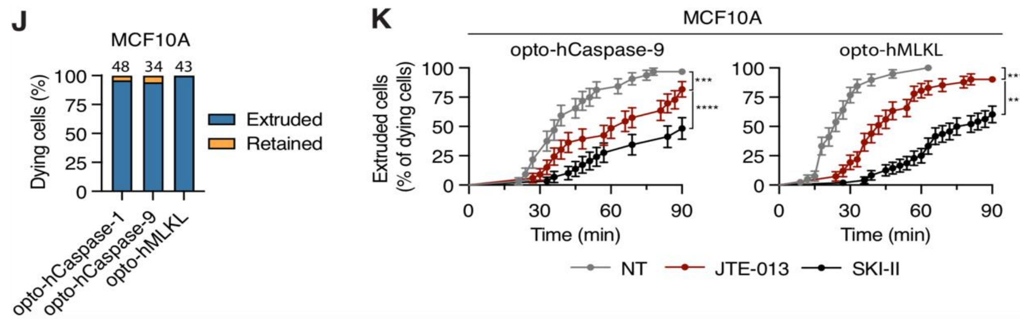


Fig. 10 J-K of the revised manuscript.

Minor Points:

In Fig 8b, providing an orthogonal view would be useful to the reader for distinguishing between extrusion events from those which apoptotic bodies are generated.

An 3D view and an orthogonal view of apoptotic Caco-2 cells is already provided in Fig. 8C and also in Fig. 9. Adding it to Fig. 8B would be partially redundant, and is not yet important at this point in the text as we discuss the actin cytoskeleton later.

Page 7, the m or h designation should be used in front of optocaspase to distinguish between the mouse and human versions used

The reviewer has raised an important point. To make the manuscript more accessible, we have decided on a common nomenclature for all constructs, using h, m and zf designations, such as:
-hOpto-Casp9
-mOpto-Casp9
-zfOpto-Casp-9

Likewise, on pgs 13-14, opto-zfCaspase8 should be consistently used throughout this section to denote the organismal version used.

This has been corrected accordingly

References 32 and 65, and 59 and 61, respectively, refer the same publications.

The references have been corrected

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, the authors generate and describe a set of Cryptochrome-2-based optogenetic tools for light-mediated induction of different forms of cell death, including apoptosis, necroptosis and pyroptosis. The authors name these tools optoCDE (optogenetically-controlled cell death effectors). The tools are tested across different systems, including human, mouse cells and in vivo in Zebrafish. The use of optoCDE allows to authors to gain insight into how different types of cell death can change the mode and dynamics of cell elimination from epithelia. Finally, the authors use optoCDE to investigate the previously proposed role of shingosine-1-phosphate in modulating efferocytosis.

The work from Shkarina K and colleagues is sound and provides several novelties. It includes very convincing live imaging movies and a remarkable number of experiments across different experimental systems. The paper, albeit rather long and extremely data-dense, is overall easy to understand and flows logically. In a final form, it would require some additional controls and a deeper contextualization in the panorama of the published literature.

We thank the reviewer for acknowledging the novelty of our toolset and broad validation of the tools in different model systems. We have added additional controls and improved to text as suggested. For detailed responses, see below:

Major comments:

1. In Figure 1D (or in a supplementary figure), please show the frequency of cell death for cells that were imaged with the same settings and for the same time but not illuminated with the 488 laser (one can score cell death based on morphological features rather than CellTox incorporation). While the control presented by the authors (Cry2olig expressing controls) established the lack of toxicity of Cry2 alone and excludes photo-toxicity, it does not inform on the basal activity of their construct in the absence of illumination (or when illuminated with a non-activating wavelength). This additional control will show the ability of the tool to respond specifically to the correct wavelength (and eventually whether it has nonspecific activity). The authors do present the results of this type of control for a timespan of 10min (Figure 1F), but a longer stimulation (comparable to the one presented in Figure 1E) would be more appropriate

The reviewer raises an important point, as basal activity of the constructs upon initiation of overexpression could be an issue. To address the comment, we have performed the experiment as suggested and tracked the viability of non-illuminated optoCasp-1/-4 and -5 expressing HEK293T cells over the same timeframe. The new data have been included in Fig 1D of the revised manuscript and show negligible toxicity of optoCasp-1/-4 and -5 constructs in non-illuminated cells.

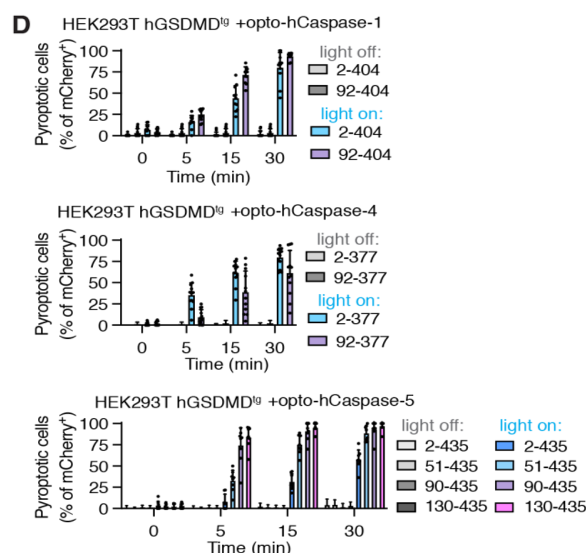


Fig. 1D of the revised manuscript.

3. Can the authors comment on whether different levels of expression of (A) optoCaspase-1 or (B) GSDMD might be responsible for the difference in lytic/sublytic fate choices in their cell population? Unless they generate

monoclonal cell lines, I would not expect them to be able to distinguish between fate choices linked to differential levels of expression versus differential activation of their optogenetic construct. Also, sublytic is not quite the right word. Maybe sublethal would be a better word.

We can only speculate to as what could be the determinant of the lethal/sub-lethal fates of individual cells. In response to this comment, we have re-analyzed our data and found that indeed cell with high optoCaspase-1 expression levels are more likely to die after the first illumination dose. The data are now included as Fig. 3I-J of the revised manuscript.

Other factors that could contribute is also the expression levels of endogenous GSDMD or cell death regulators, and it will be interesting to use to optoCDE system for future studies aimed at understanding how cells survive cell death induction.

We agree with the reviewers comment that sublytic is not necessarily the right word, and thus use 'sublethal' instead in the revised manuscript.

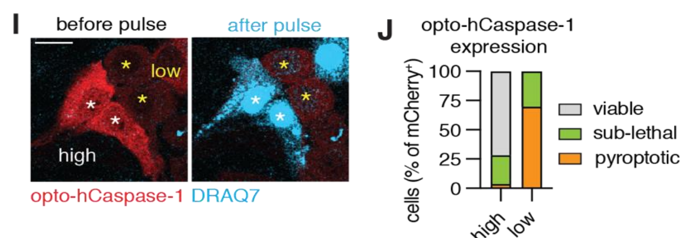


Fig. 3I-J of the revised manuscript.

4. While the effects of JTE-013 and SKI-II on apoptotic cell fragmentation and elimination are quite remarkable, non-specific effects of these inhibitors might confound result interpretation, as they might be due to off-target effects (especially at concentration as high as 30 micromolar). To further substantiate their finding, the authors could carry out a knock-down of SphK2 and/or S1PR2.

As suggested, we have knocked-down S1PR2 in 'receiver' cells and quantified apoptotic and necroptotic cell extrusion as before. The knock-down was able to recapitulate the inhibitor data, suggesting that the inhibitors specifically blocked S1P signaling. We included these data as Fig. 10H-I of the revised manuscript.

Furthermore, we have also analysed efferocytosis and extrusion of MCF10A and MDCK cells, and were able to recapitulate published data (PMID: 21555463) using JTE013 and SKI-II. (see Fig. 10J-K) of the revised manuscript). This further confirms that the inhibitors are specific.

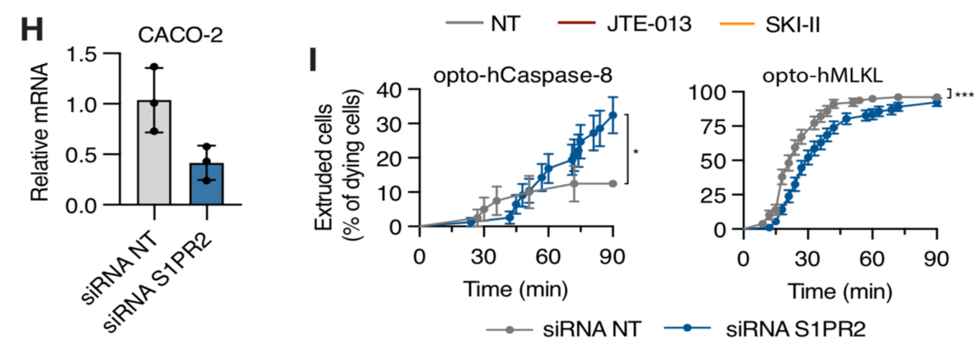


Fig. 10H-I of the revised manuscript.

5. Finally, in the discussion the authors refer obliquely to an issue that must be discussed more fully. They acknowledge that their findings contradict many published papers that report extrusion of apoptotic cells and especially multiple papers from the Rosenblatt lab showing the role of S1P signaling in extrusion of apoptotic cells. The authors brush this aside with a cursory comment. At a minimum, a more thoughtful discussion is

required. Even better, some understanding of the reason(s) for the differences would prevent the current work from creating confusion in the field.

We addressed the discrepancy between ours and published reports (such as from the Rosenblatt lab) by generating MCF10A and MDCK cell lines expressing optoCDEs and testing these for cell efferocytosis and extrusion (these are cell lines used in previous studies). Our findings show that apoptotic MCF10A and MDCK cells are not efferocytosed but extruded, in line with previous work. We thus conclude that the decision to efferocytose or extruded apoptotic cells is highly cell line specific. Nevertheless, S1P signaling plays an important role in both processes. As these results resolve the discrepancy and further highlight the versatility of the optoCDE approach, we have included them in the revised manuscript as Fig. 10J-K.

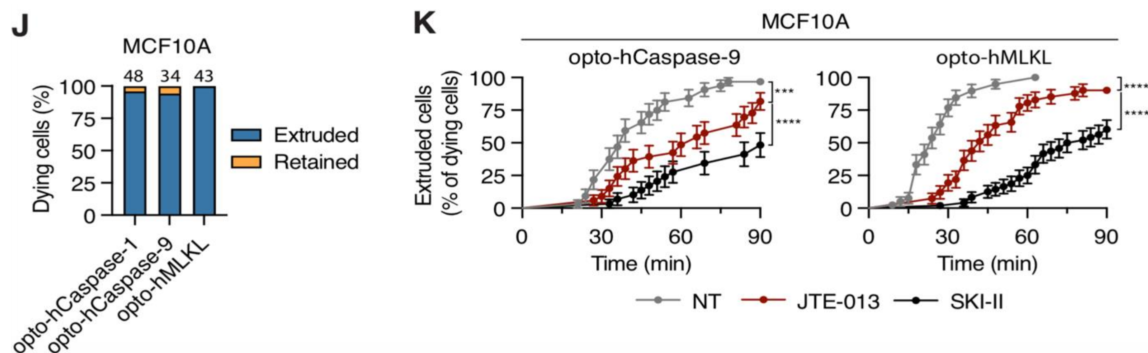


Fig. 10J-K of the revised manuscript.

Minor comments:

1. The authors should provide reference for the second part of their statement "Another more controlled approach is to force-oligomerize cell death executors using fusions with a chemically-dimerizable domain (DmrB/FKBP) (known as "clean" death system)9 but this is limited by poor reversibility and the inability to target and selectively kill individual cells in vitro or in vivo". What is the evidence for poor reversibility? In addition, according to the strategy used to deliver the dimerizable construct and the type of promoter employed, the system can likely be used to target and kill specific cells.

Homodimerization of DmrB domains can be reverted by using excess concentrations (1uM) of a washout ligand, and according to the manufacturer (Clontech) dissociation can be observed after ~30 min, which is slower than the dissociation of Cry2.
It is correct that chemical dimerization can be used to kill individual cells depending on the strategy used to deliver the constructs, but it does not allow to specifically kill a certain cell of choice like optogenetic can. This our emphasis on 'selectively kill individual cells'

To correctly describe the chemical-dimerization approach, we have rephrased this section in the revised manuscript.

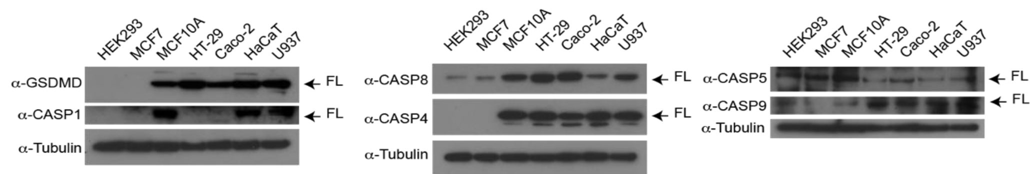
2. Figure 1: please include in the figure legend the definitions of all acronyms used

This has been corrected.

3. Do the HEK293T cells endogenously express caspase-1, -4 and -5?

HEK293T cell do not express inflammatory caspases (e.g. caspase-1, -4 and -5), ASC or the commonly studied inflammasome-forming receptors (such as NLRP3, AIM2, etc..). Due to this lack of endogenous components, they are thus often used as a model system for inflammasome reconstitution by overexpression (see for example PMID: 27221493).

In response to the comment, we immunoblotted for caspases and GSDMD in different cell lines used in our study, and provide the blots for the referee's convenience below.



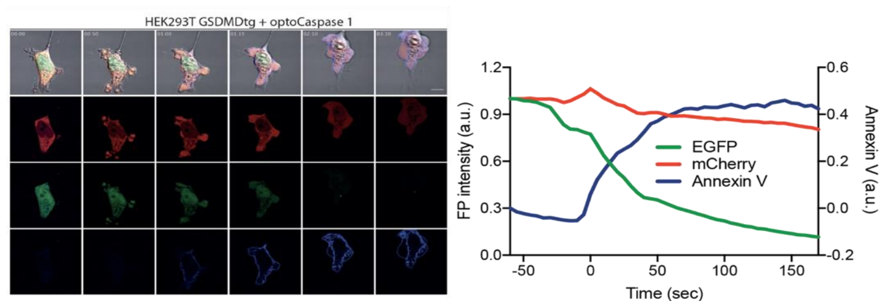
Rebuttal Figure 1: expression levels of Caspases and GSDMD in different cell lines.

4. The authors claim that CARD-deficient forms of Caspase-1 and -5 are more efficient at inducing cell death than the full-length variants. However, for Caspase-1 this is not a dramatic difference, and I would suggest rephrasing.

We have rephrased this statement.

5. Can the authors show/specify what is the status of VC3AI in GSDMDtg HEK293T cells (related to Supplementary Figure 2E)?

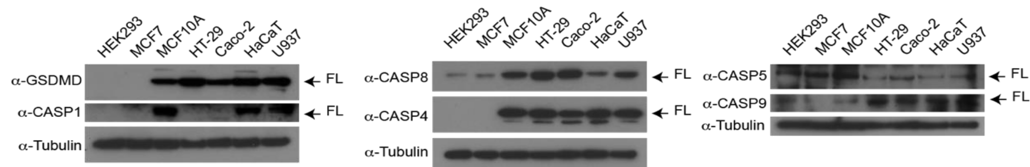
This would be an interesting experiment to be done, as previous work has shown that caspase-7 and caspase-3 is activated in pyroptotic macrophages (PMID: 32726624). Unfortunately, the experiment cannot be done with the VC3AI reporter in the GSDMDtg cells, since activation of GSDMD results in a loss of soluble cytosolic proteins, as shown below for GFP (Fig. R3), and thus we would lose the VC3AI reporter as soon as the cells lyse – making it impossible to measure its activation.



Rebuttal Figure 2: Loss of exogenously expressed GFP from HEK293T cells during pyroptosis. GSDMDtg HEK293T cells expressing optoCasp-1 were illuminated at t=0, and eGFP and Annexin-V signals recorded over time.

6. Please specify the GSDMD status and caspase-1, -4, -5 status for each of the cell lines used in Figure 1I (transgenic expression or endogenous expression). Also specify endogenous caspase-8 and -9 levels for the cell lines used in Figure 5I

In response to the comment, we immunoblotted for caspases and GSDMD in different cell lines used in our study, and provide the blots for the referee's convenience below.



Rebuttal Figure 1: expression levels of Caspases and GSDMD in different cell lines.

7. Can the authors discuss more about the necessity of using a doxycycline-inducible promoter to prevent cell

death of U937 cells during differentiation? If the constructs are indeed inactive in the dark, this should not be necessary.

Apologies, we have not properly described what we mean here. We used a doxycycline-inducible promoter exactly for the reason that it allowed to culture the cells under the normal illumination conditions, and also to avoid toxicity by prolonged expression of the optoCDE constructs. We had observed that long constitutive expression (days) of certain constructs resulted in toxicity even without illumination.

We have also corrected the text in the revised manuscript to make that clearer.

8. Please provide appropriate references when introducing and describing well established mechanisms of cell death

We have added new references to the introduction.

9. As currently shown, none of the data in Figure 5D-F and Supplementary Figure 6B support the activation of the genetically encoded caspase-3/-7 activity reporter before the acquisition of Annexin V staining. Please modify the figures or the text accordingly.

This is correct. We have re-phrased the text accordingly.

10. As reported in the discussion and the reference list, previous works focused on the generation of tools for light-inducible apoptosis. I do believe that especially the work of Smart and colleagues should be acknowledged and contextualized earlier in the manuscript, not only in the discussion. Mentioning this work will allow the reader to better understand what prompted the authors to develop different tools, and how their tool provides an advantage

We discuss the different approaches for optogenetic activation of cell death, among the Smart et al, in the introduction.

11. Please explain the rationale behind using RHIM deficient RIP3

As shown in Fig. 6C, the RHIM-deficient construct 'opto-hRIPK3 RHIM NT' is more efficient as other RipK3 constructs.

12. Please define "PMR"

We have now defined MPR as plasma membrane rupture in the revised manuscript.

13. Please verify the figures mentioned in "Optogenetic activation of zebrafish caspases allows spatial and temporal controlled induction of pyroptosis and apoptosis in vivo". There are incongruencies between the text and what shown

The incongruencies have been corrected.

14. The authors say: "While the effect of opto-caspase induction in skin cells is visible within minutes the effect of their activation in muscle cells only becomes apparent after several hours". However, signs of apoptosis do not appear until ~40' after illumination. Please rephrase.

The text has been modified accordingly.

15. Please review Figure 7H, the insets do not look accurate (there is a confounding gray signal)

We have corrected this figure. The boxes outline the illuminated regions.
The grey was meant to show asc-gfp in these boxes. This has now been changes to green to make that clearer.

16. While Figure 9F shows the absence of fragmentation of apoptotic cells without neighbors, it would be advisable to include in the same panel a picture in which apoptotic bodies formation is clearly visible

We believe that the reviewer is referring to Supplementary Figure 9F, where we show the absence of apoptotic body formation in apoptotic Caco-2 cells. Indeed, it is difficult to judge whether apoptotic bodies are present or not from these images. We have thus included close-ups of these same cells, and in addition provide images of cells forming apoptotic bodies for comparison in Fig. S5B of the revised manuscript.

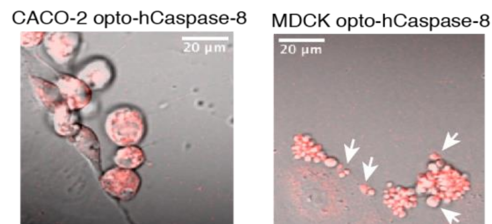


Fig. S5B of the revised manuscript.

17. Please review the sentence "(Fig. 8F-H and Supplementary Fig. B)" and the sentence "(Fig. 7E-G and Fig. 8I-LK)"

This has been corrected.

18. While indirect evidence points toward the reversibility of optoCDE (recovery and survival of cells after delivery of limited amount of illumination), it is unclear what are the actual kinetics and mode of inactivation. Is inactivation caused by feedback mechanisms, or is it linked to the conversion of optoCDE into an inactive conformation? Is survival actually linked to inactivation, or is it due to the inability of reaching a threshold of activity required to complete commitment to cell death? I believe this should be discussed. I would also suggest caution when claiming "optoCDE not only excel in their rapid kinetics of activation and reversibility (inactivate within minutes of ceasing illumination)..."

We have changed the text, as indeed we have not properly evaluated the reversibility of the toolset.

19. The term anastasis first appeared in PMID 22535522. It might be more appropriate to use this reference rather than PMID 30839720
We have added the new reference

20. In the method, please specify the concentration of Annexin V used (1 microgram/?)
It is 1ug/ml. We have corrected the material and methods section accordingly.

Reviewer #3 (Comments to the Authors (Required)):

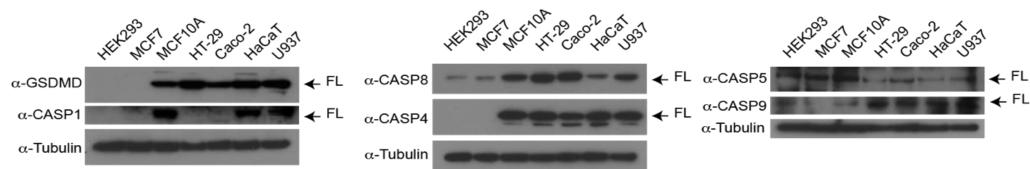
This study by Shkarina and colleagues describes the development and application of a suite of optogenetic approaches (centred on caspase or RIPK3/MLKL) to induce different forms of regulated cell death (apoptosis, necroptosis, pyroptosis) at the single cell level. The authors extensively validate their approach in pyroptosis before progressing to apoptosis and necroptosis. Beyond 2D analysis, they demonstrate effectiveness in 3D culture and in vivo (in zebrafish) Finally, they demonstrate differences in removal/expulsion of dying cells from an epithelial monolayer dependent on cell death modality, finding that necroptotic and pyroptotic cells are extruded, whereas apoptotic cells disintegrate and are absorbed by neighbouring cells.

In my opinion, the toolset (and generalisable optogenetic approach) the authors have developed is novel, powerful and as they demonstrate towards the end of the study will undoubtedly yield new biological insights. Their findings/methodology clearly surpasses any optogenetic approaches that have been developed to trigger cell death in the past, and one of the attractive points of this toolset is the apparently facile comparison of different cell death modalities using a similar experimental setup. Data fully support the authors' conclusions - I have a few minor comments.

We thank the reviewer for the positive feedback on our manuscript and for highlighting the novelty of the toolset.

- reference to optocaspase activity to trigger pyroptosis in commonly used cell lines (bottom page 2) are these cell lines listed here all pyroptosis (GSDMD) proficient ?

In response to referee #1 and #2's request we have analyzed the expression levels of caspases and GSDMD in the different cell lines. All except MCF7 and HEK293 express GSDMD and thus are theoretically pyroptosis proficient. Pyroptosis induction was achieved by overexpressing hGSDMD in these lines.



Rebuttal Figure 1: expression levels of Caspases and GSDMD in different cell lines.

- with respect to the resistance of some cell lines to optoC8 or C9 (page 11), the authors speculate that this may be due to additional inhibitory mechanisms (such as XIAP upregulation, as their SMAC-mimetic expt. shows in some cell lines), really interesting that their are additional potential regulatory mechanisms, but could another reason be that optoC8 or C9 don't work as well in some cell types for trivial reasons (e.g misfolding) ? - I appreciate this is difficult to experimentally demonstrate, but a line stating this possible caveat would suffice.

We cannot completely exclude that misfolding of the construct could be a reason. However, since misfolded proteins are usually degraded and we can observe strong expression and functionality of the constructs (based on the presence of the mCherry signal and its clustering upon illumination), we believe that this is probably not the reason for the failure to induce apoptosis in these lines. We have nevertheless, rephrased the text as it cannot be 100% excluded.

- RIP3, should be changed to RIPK3

We have changed the text accordingly.